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# Abnormalities of High Density Lipoproteins in Abetalipoproteinemia *

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**Summary. Detailed studies of the high density lipoproteins from three patients with abetalipoproteinemia have revealed the following principal abnormalities: 1) High density lipoprotein 3 (HDL3) is reduced in both absolute and relative concentration, although HDL2 is present in normal amounts. 2) The phospholipid distribution of both HDL fractions is abnormal, with low concentrations of lecithin and an increased percentage (though normal absolute quantity) of sphingomyelin. 3) In both HDL fractions, lecithin contains less linoleate and more oleate than normal. The cholesteryl esters are also low in linoleic acid, and the sphingomyelin is high in nervonic acid. Dietary intake influences the linoleic acid concentration within 2 weeks, and perhaps sooner, but the elevated sphingomyelin nervonic acid is little affected by up to 6 months of corn oil supplementation. Qualitatively similar changes in fatty acid composition, but not phospholipid distribution, are also found in other malabsorption states.**

**The available evidence suggests that the abnormally low levels of HDL3 and the deranged phospholipid distribution are more specific for abetalipoproteinemia than the fatty acid abnormalities. However, the absence of these abnormalities in obligate heterozygous subjects makes their relationship to the primary defect of abetalipoproteinemia difficult to assess.**

## Introduction

Abetalipoproteinemia is a rare hereditary disease characterized by a degenerative neurological process, malabsorption, retinal degeneration, thorny red cells (acanthocytosis), and absent beta lipoprotein. In all but two (3, 4) of the patients reported in the literature, the plasma cholesterol and phospholipid levels were lower than expected from the absence of beta lipoprotein alone, suggesting an accompanying deficiency in alpha-1 or high density lipoproteins; this has been confirmed by electrophoretic and ultracentrifugal analyses in several patients (4–8). In addition to the low plasma cholesterol, triglyceride, and lipid phosphorus values resulting from these lipoprotein deficiences, several investigators have found plasma phospholipid distribution to be abnormal (3, 4, 9), with a relative increase in sphingomyelin and a decrease in lecithin.

In the present study, we have examined the chemical composition of the high density lipoproteins in abetalipoproteinemia in detail to define better the high density lipoprotein abnormalities and their relationship to the underlying molecular defect in this disease.

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Portions of this work have been previously reported (1, 2).

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## Methods

*Subjects.* The following three patients with abetalipoproteinemia were studied: M.S. (age 8–10), J.D. (age 7), and J. G. (age 7). Erythrocyte (4, 9, 10) and gastrointestinal (11) studies on M.S. have been previously reported. Fresh plasma from J.D.[1] was sent from New York City by air express at 4° C, and analysis was begun 12 hours after venesection. The clinical history of J.G.[2] has been reported previously (11).

In all three patients, the diagnosis of abetalipoproteinemia was established on the basis of clinical history, acanthocytosis, absence of beta lipoprotein by immunoelectrophoresis, and characteristic duodenal biopsy. Although patients J.D. and M.S. were on unrestricted diets at the time of the study, both limited fat intake voluntarily. The diet of M.S. was supplemented with variable amounts of safflower oil. J. G., except as otherwise noted, was also consuming a free choice diet, which averaged 0.88 g of fat per kg body weight each day.

Plasma lipoproteins were analyzed from seven control subjects, two women and five men (aged 25 to 35), whose total plasma lipid phosphorus and cholesterol values were within the generally accepted normal range. In addition, pooled plasma from three children 7 to 9 years old was examined. These children had asymptomatic hay fever or bronchial asthma, for which they were being desensitized in the outpatient clinic. No attempt was made to estimate or control fat consumption in the control subjects.

*Separation and extraction of plasma lipoproteins.* Blood obtained after an overnight fast was anticoagulated with EDTA (1 mg per ml blood), and the plasma and cells were separated by centrifugation at 4° C for 20 minutes. The plasma was aspirated and then recentrifuged for another 20 minutes to sediment any remaining formed elements. The lipoproteins were isolated in a Spinco model L ultracentrifuge with a type 50 rotor at 150,000 *g*. All density adjustments were made with a solution of potassium bromide and sodium chloride (12) or by the addition of solid potassium bromide alone.

The initial centrifugation was at a density of 1.063 for 15 hours. The tubes were sliced approximately 1 cm below the visible top layer, and the $D < 1.063$ fraction (low density lipoproteins, LDL) was removed. The infranatant fractions from all tubes were combined, adjusted to a density of 1.110, and centrifuged for 31 hours to isolate HDL2 in the new supernatant fraction. The infranatant fraction was adjusted to a density of 1.21 and centrifuged for 31 hours to separate HDL3 from the $D > 1.21$ residue. In some instances, when a type 40 rotor was used, the material was centrifuged longer to compensate for the lower centrifugal force. The lipoprotein fractions were transferred to cellulose tubing and dialyzed against 3 to 4 L of 0.15 M sodium chloride with 0.001 M EDTA. The LDL and HDL2 fractions were dialyzed for 24 hours with three changes of the dialysis solution; HDL3 and $D > 1.21$ fractions were dialyzed for 48 hours with four changes of solution. Portions of whole plasma and the lipoprotein fractions were extracted with methanol and chloroform as previously described (13).

*Chromatographic separation of major lipid classes.* Neutral lipids were separated from total phospholipid on silicic acid columns by using 2 g of silicic acid per mg lipid phosphorus. Neutral lipids were eluted with chloroform (60 ml per g silicic acid). Subsequently, chloroform : methanol 1 : 1 (15 ml per g silicic acid), chloroform : methanol 1 : 9 (30 ml per g), and 3% water in methanol were used as eluents and then combined. (All solvent ratios are expressed as volume : volume.) This stepwise elution of the phospholipids resulted in 97 to 100% recovery of the lipid phosphorus (14). Neutral lipids were then rechromatographed to separate cholesteryl esters from free cholesterol, triglycerides, and free fatty acids. One gram of silicic acid stored at 110° C was slurried in chloroform and packed in columns made from 10-ml serological pipettes. The column was washed with 25 ml chloroform, then 20 ml of benzene : hexane 1 : 3. The sample (up to 5 mg of total cholesterol) was transferred to the column in benzene : hexane 1 : 3, with three separate 2-ml transfers. The cholesteryl esters were eluted with an additional 14 ml of benzene : hexane 1 : 3. The remaining neutral lipids were eluted in bulk with 25 ml of chloroform. This technique usually gave complete separation of cholesteryl esters and triglyceride, but the purity of the fractions was always checked by thin layer chromatography. If separation was incomplete, the sample was rechromatographed. Phospholipid distribution was determined by elution and measurement of phosphorus after chromatography of total lipid or total phospholipid on paper impregnated with silicic acid (13, 15).

*Isolation and preparation of lipids for fatty acid analysis.* The combined phospholipids from the silicic acid columns were further separated by thin layer chromatography. Silica gel G[3] was washed twice with each of the following solvents: chloroform, chloroform : methanol 1 : 1, chloroform : methanol 1 : 3, and methanol. After drying the silica gel overnight in a vacuum oven, we prepared thin layer (0.25 mm) plates and activated them at 110° C for at least 2 hours. Up to 0.3 mg of lipid phosporus was applied as a streak across a 20-cm plate and developed in chloroform : methanol : water 75 : 35 : 4. The phospholipids were detected by spraying guide lanes with 1% iodine in methanol or with a specific phosphorus spray (16). The corresponding unstained areas were scraped from the plates with glass slides. The phospholipids were eluted by stirring the silica gel in 5 ml of chloroform : methanol 1 : 1 and, after 20 minutes, pouring the slurry into a small column. The lipid was then eluted with three 5-ml portions of chloroform : methanol 1 : 1, 10 ml chloroform : methanol 1 : 3, and 20 ml 3% water in methanol. A blank area was also eluted to monitor con-

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[2] Referred to us by Drs. Richard Deemer and Lloyd Brandborg of San Francisco, Calif.

[3] E. Merck, A. G., Darmstadt, Germany.

tamination. This elution technique was found to be quantitative by Masoro, Rowell, and McDonald (17) for muscle phospholipid phosphorus. In one determination, we recovered 102% of the lipid phosphorus from an extract of whole plasma. With chromatographically pure plasma lecithin, fatty acid ester recovery was 103%, as determined by infrared analysis (4).

After elution, approximately 5% of each fraction was rechromatographed on a silica gel plate to determine its purity. The sphingomyelin and lysolecithin samples showed single spots with the same $R_f$ as the originally eluted material. The lecithin, however, consistently showed three spots, the major one with the $R_f$ of lecithin, a smaller spot in the lysolecithin region, and a nonphosphorus-containing spot at the solvent front. Apparently, some lecithin was hydrolyzed during the elution procedure. Despite this, the fatty acid ester content of the eluted lecithin was not significantly diminished (*vide supra*), so the degree of deacylation was minor. These alterations in the lecithin were not accompanied by changes in fatty acid composition. As shown in Table I, the fatty acid composition of purified lecithin chromatographed and eluted from the thin layer plate was virtually identical to that of a sample transesterified directly.

Methyl esters were prepared from the phospholipid fractions separated by thin layer chromatography and from the cholesteryl esters and triglycerides isolated from silicic acid columns by transesterification in sulfuric acid and methanol (4). Their identification and quantification by gas-liquid chromatography were done as previously described (4, 14). Fatty acid composition was expressed as moles per 100 moles of fatty acid.

*Other analytical procedures.* Protein determinations were done on the intact HDL2 and HDL3 fractions by the method of Lowry, Rosebrough, Farr, and Randall (18), with crystalline human albumin as reference standard. Lipid phosphorus was measured by established methods (14, 19). Glyceride glycerol was quantified by a periodate oxidation method (20), using the neutral lipid fraction obtained by silicic acid column chromatography. Total cholesterol (0.08 to 0.5 mg) was quantified by the following modification of the Liebermann-Burchard reaction. After drying under nitrogen, the lipid sample was dissolved in 2 ml of dichloroethane: acetic acid 1:1. After the addition of 1 ml of acetic anhydride and 0.1 ml of sulfuric acid, the contents were well mixed and placed in the dark for exactly 30 minutes. The optical density at 615 mμ was then determined against a reagent blank. Standards were run concurrently. Free and ester cholesterol were determined by the same technique after their separation on silicic acid columns. The Liebermann-Burchard reaction gives a higher molar extinction coefficient for esterified cholesterol than for free cholesterol. Since the values for total cholesterol reported in Table II were not corrected for this effect, they are 10 to 12% too high (21). When we analyzed the column fractions, we used a standard of cholesteryl palmitate to measure accurately the cholesterol content of the ester fraction.

TABLE I

*The fatty acid composition of plasma lecithin directly transesterified compared with that eluted after thin layer chromatography and then transesterified*

| Fatty acid | Transesterified before chromatography | Transesterified after thin layer chromatography |
|---|---|---|
| | *moles/100 moles fatty acid* | |
| 14:0 | 0.2 | 0.4 |
| 16:0 | 28.9 | 29.8 |
| 16:1 | 0.9 | 1.6 |
| 18:0 | 13.7 | 13.7 |
| 18:1 | 10.8 | 11.0 |
| 18:2 | 26.4 | 25.2 |
| 20:3 | 2.5 | 2.5 |
| 20:4 | 10.7 | 10.3 |
| 22:5 | 0.9 | 1.0 |
| 22:6 | 2.7 | 2.5 |

## Results

*Lipid and protein concentrations.* The lipid and protein composition of HDL2 and HDL3 is given in Table II. In abetalipoproteinemia, the HDL2 lipid phosphorus, cholesterol, and protein values were within the normal range, but in HDL3 all were reduced to about one-half to one-third of normal. All three patients had lower percentages of cholesteryl ester in HDL2 and of triglyceride in HDL3 than any of the normal adults or children. The $D > 1.21$ fraction was lower in lipid phosphorus content, but the cholesterol levels were similar to those of normal subjects.

To determine whether the decrease in HDL3 resulted from deletion of some subfraction within this lipoprotein class, we centrifuged samples of whole plasma from two patients and one normal subject at densities of 1.110, 1.123, 1.160, and 1.21. The derived lipid phosphorus and cholesterol values in Figure 1 show that the major quantitative deficiency in the abnormal plasma was in the 1.123 to 1.160 range, which was the main HDL fraction in the normal subject.

*Phospholipid distribution.* The phospholipid distributions in whole plasma and in the individual lipoproteins are summarized in Table III. The whole plasma data from normal subjects was similar to those previously described (13, 22–24). When the separated normal lipoproteins were compared, increasing lipoprotein density was accompanied by a decrease in the relative amount of sphingomyelin and an increase in lecithin. These differences in phospholipid composition between HDL2 and HDL3, although small, were consistent

TABLE II

*Composition of plasma and various lipoprotein fractions in abetalipoproteinemia**

| | | Normal adults | | Normal children | Abetalipoproteinemia | | | Family of JG | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Males (5) | Females (2) | | JD | MS | JG | Father | Mother | Sister |
| Lipid phosphorus (*mg/100 ml plasma*) | WP† | 7.58–9.08 | 8.63–10.2 | 9.05 | | | | 7.68 | 9.65 | 10.1 |
| | LDL | 3.18–4.28 | 2.86–3.89 | 2.99 | | | 0.11 | 4.06 | 3.84 | |
| | HDL2 | 0.53–1.08 | 0.76–2.40 | 1.05 | 0.94 | 1.10 | 0.75 | 0.21 | 1.77 | |
| | HDL3 | 2.02–2.54 | 2.25–3.11 | 3.08 | 0.69 | 0.79 | 0.90 | 2.43 | 2.70 | |
| | >1.21 | 0.52–0.78‡ | 0.59–0.70 | 1.01 | 0.16 | 0.19 | 0.24 | 0.86 | 0.59 | |
| Total cholesterol (*mg/100 ml plasma*) | WP | 174–262 | 220–230 | 216 | | | | 219 | 211 | 230 |
| | LDL | 102–176 | 112–176 | 121 | | | 3 | 167 | 146 | |
| | HDL2 | 10–21 | 16–34 | 23 | 22 | 24 | 18 | 6 | 28 | |
| | HDL3 | 32–39 | 33–42 | 45 | 18 | 17 | 13 | 30 | 38 | |
| | >1.21 | 4–7‡ | 3–6 | 10 | 4 | 2 | 3 | 6 | 3 | |
| Cholesteryl ester (%) | WP | | | | | | | 74 | | 73 |
| | LDL | | | | | | | 71 | 67 | |
| | HDL2 | 82, 83§ | 72‖ | 70 | 57 | 53 | 54 | 67 | 67 | |
| | HDL3 | 87, 87§ | 87‖ | 72 | 70 | 70 | 63 | 79 | 75 | |
| Triglyceride (*mg/100 ml plasma*) | WP | 108 | 37 | 73.9 | | | | 118 | | |
| | LDL | 42, 83§ | | 48.1 | | | | 93 | 69 | |
| | HDL2 | 2, 2§ | 3 | 4.3 | 1.9 | 1.2 | 0.9 | 2 | 6 | |
| | HDL3 | 3, 7§ | 4 | 5.1 | 2.7 | 1.1 | 0.8 | 10 | 6 | |
| Protein (*mg/100 ml plasma*) | HDL2 | 18–40¶ | 66 | 30 | 40 | 33 | 20 | | 74 | |
| | HDL3 | 125–148¶ | 138 | 146 | 44 | 47 | 55 | | 218 | |

* J. G.'s plasma was centrifuged initially at D 1.063. The plasma from J. D. and M. S. was centrifuged first at D 1.110. The HDL2 data, therefore, include the small increment that might have been in the D 1.063 supernatant. In another experiment, the plasma of M. S. was also centrifuged at D 1.063. The D 1.063 supernatant, like that of J. D., contained small amounts of the total plasma lipid phosphorus and cholesterol.

† Abbreviations: WP = whole plasma; LDL = low density lipoprotein; HDL2 = high density lipoprotein 2; HDL3 = high density lipoprotein 3.

‡ n = 4.

§ n = 2.

‖ n = 1.

¶ n = 3.

in all normal subjects. Usually, lysolecithin could not be demonstrated on paper or thin layer chromatography of the HDL2 and HDL3 subfractions, but, as previously reported by Phillips (25), this

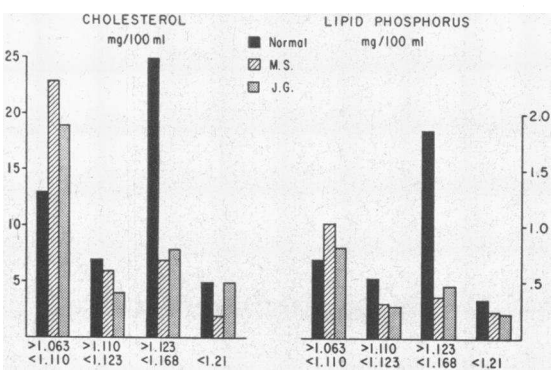


FIG. 1. CHOLESTEROL AND LIPID PHOSPHORUS VALUES IN HIGH DENSITY LIPOPROTEIN 2 (HDL2, 1.063 TO 1.110) AND THREE SUBFRACTIONS OF HDL3 FROM ONE NORMAL CONTROL SUBJECT AND PATIENTS M.S. AND J.G. Portions of native plasma were centrifuged simultaneously at densities of 1.110, 1.123, 1.168, and 1.21. (The normal sample was initially centrifuged at 1.063 to remove low density lipoprotein.) The values expressed for each density range were obtained by subtraction, i.e., the 1.110 to 1.123 values were calculated by subtracting the supernatant values obtained by centrifuging at D 1.110 from the supernatant values obtained by centrifuging at D 1.123.

phospholipid accounted for approximately 50% of the lipid phosphorus in the D > 1.21 fraction.

In abetalipoproteinemia, whole plasma showed a relative increase in sphingomyelin and decrease in lecithin similar to that previously reported (3, 4, 9). As in normal subjects, the HDL2 fraction contained more sphingomyelin and less lecithin than HDL3, but both fractions reflected the abnormal sphingomyelin: lecithin ratio seen in whole plasma. In plasma from M.S. and J.G., a small amount of lipid phosphorus was found in the fraction floating at a density of 1.063, despite the absence of beta lipoprotein by immunoelectrophoresis. In both instances, the sphingomyelin: lecithin ratio was even greater than in the HDL2 fraction.

Despite the reduction in total high density lipid phosphorus seen in the patients, the absolute sphingomyelin concentration in HDL was within the normal range, 18 to 26 μmoles per 100 ml in the patients compared to 16 to 30 in the normal subjects.[4]

[4] Since the paper chromatographic method did not separate sphingomyelin and phosphatidyl inositol (PI), combined silicic acid column and paper chromatography (14) were employed to determine if the increase in sphingomyelin was in part due to increased PI. Whole plasma phospholipid from patient J.G. contained 1.4%

TABLE III

*Percentage distribution of plasma lipoprotein phospholipids in patients with abetalipoproteinemia and control subjects**

| | | Normal adults† | | Normal children | Abetalipoproteinemia | | | Family of JG | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Range | | JD | MS | JG | Father | Mother | Sister |
| Lecithin | WP | 67 | 65–70 | 66 | 59 | 44 | 52 | 60 | | 69 |
| | LDL | 65 | 63–68 | 62 | | 39 | 45 | 68 | 63 | |
| | HDL2 | 71 | 65–72 | 73 | 54 | 47‡ | 54 | 67 | 69 | |
| | HDL3 | 74 | 70–77 | 78 | 59 | 56‡ | 65 | 74 | 81 | |
| | >1.21 | 33 | 28–38 | 43 | 35 | 31‡ | 42 | | 26 | |
| Sphingomyelin | WP | 21 | 20–23 | 20 | 30 | 48 | 31 | 22 | | 19 |
| | LDL | 27 | 26–32 | 30 | | 54 | 46 | 28 | 32 | |
| | HDL2 | 21 | 19–29 | 22 | 35 | 47‡ | 39 | 22 | 17 | |
| | HDL3 | 17 | 15–18 | 16 | 31 | 38‡ | 30 | 20 | 14 | |
| | >1.21 | 10 | 8–12 | 14 | 16 | 21‡ | 23 | | 11 | |
| Lysolecithin | WP | 7 | 4–9 | 9 | 5 | 7 | 8 | 10 | | 5 |
| | >1.21 | 48 | 40–58 | 32 | 34 | 34‡ | 32 | | 55 | |
| Phosphatidyl ethanolamine | WP | 4 | 3–5 | 4 | 3 | 1 | 6 | 3 | 2 | 2 |
| | LDL | 5 | 3–5 | 4 | | 3 | 3 | 2 | 1 | |
| | HDL2 | 5 | 3–8 | 3 | 0.2 | 0.9‡ | 1 | 4 | 3 | |
| | HDL3 | 5 | 3–10 | 5 | 3 | 2 | 1 | 3 | 2 | |
| | >1.21 | 5 | 3–8 | 6 | 6 | 8 | 0 | | | |

* All values are expressed as per cent of total phospholipid recovered from the chromatograms. Recovery ranged from 85 to 111%. In all analyses, 0 to 7% of the phosphorus recovered was in areas not corresponding to any of those shown (mostly at the origin).

† No apparent differences were noted among five normal male and two female values. Hence, they have been combined.

‡ These values are the averages of two determinations.

In addition to the more obvious changes in sphingomyelin and lecithin, there was an apparent relative and absolute decrease in the amount of phosphatidyl ethanolamine, especially in HDL2.[5] Lysolecithin was the predominant phospholipid in the D > 1.21 fraction, but it accounted for less than 50% of the phospholipid in that fraction.

*Fatty acid composition of lipid fractions.* The fatty acid compositions of lecithin, sphingomyelin, and phosphatidyl ethanolamine isolated from HDL2 and HDL3 are shown in Tables IV, V, and VI. In normal subjects, there was no significant difference between the lecithin fatty acids of HDL2 and HDL3. The lecithin fatty acids in abetalipoproteinemia differed from normal mainly in their lower linoleic acid contents (18:2) (in normal subjects 20 to 34%, in the patients 6.5 to 12%) and their comparably higher oleic acid (18:1) contents. Despite the lower level of 18:2, its biosynthetic product arachidonic acid (20:4) was present in normal concentration in all three patients, with the exception of the value of 3.3% in HDL2 of M.S. In J.G. and J.D., there was a small amount (< 1%) of a fatty acid tentatively identified by retention data as 5,8,11-eicosatrienoic acid. This fatty acid was not found in lecithin from normal subjects or from M.S., who had the highest percentage of linoleic acid and who was receiving a safflower oil supplement in his diet at the time of the study.

The average sphingomyelin fatty acid composition showed more variation between normal HDL2 and HDL3 than did the lecithin fractions, but when the data from each individual were examined, no consistent differences between fractions could be demonstrated (Table V). In contrast to the observations of Sweeley (27), significant amounts of 18:1, 18:2, and 20:4 were present. This was probably due to contamination of our sphingomyelin samples with phosphatidyl inositol.[6]

PI, compared to 2.0% in simultaneously analyzed normal adult plasma.

[5] The recent work of Dodge, Cohen, Kayden, and Phillips (26) indicates that the tissue lipids of patients with abetalipoproteinemia are more susceptible to peroxidation than those from normal individuals because of concomitant vitamin E deficiency. Since phosphatidyl ethanolamine contains a high concentration of polyunsaturated fatty acids, the low levels of plasma phosphatidyl ethanolamine in abetalipoproteinemia may be artifactual.

[6] The mobility of phosphatidyl inositol relative to that of sphingomyelin was variable in our thin layer system.

TABLE IV

*The fatty acid composition of high density lipoprotein lecithins in patients with abetalipoproteinemia and control subjects**

| Fatty acid | Normal adults† | | Children's pool‡ | | Abetalipoproteinemia‡ | | | | | | Family of JG§ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | M.S. | | J.G. | | J.D. | | Father | Mother | Sister |
| | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL | HDL | WP |
| | *moles/100 moles fatty acid* | | | | | | | | | | | | |
| 16:0 | 28.7±2.1 | 28.4±2.2 | 28.5 | 27.9 | 35.7 | 30.2 | 33.4 | 32.6 | 32.5 | | 27.9 | 30.7 | 27.3 |
| 16:1 | 1.1±0.9 | 1.1±0.9 | 1.2 | 1.1 | 2.4 | 1.6 | 2.9 | 3.3 | 2.3 | | 1.6 | 2.0 | 1.2 |
| 18:0 | 13.9±0.6 | 14.1±1.3 | 14.1 | 14.4 | 15.0 | 16.6 | 10.1 | 9.9 | 10.6 | | 14.6 | 12.6 | 16.2 |
| 18:1 | 12.1±1.3 | 12.1±1.1 | 12.3 | 11.5 | 24.4 | 17.8 | 25.1 | 25.7 | 25.4 | | 14.6 | 13.4 | 11.7 |
| 18:2 | 25.1±5.2 | 24.8±5.8 | 25.1 | 24.3 | 12.1 | 12.3 | 6.5 | 6.5 | 7.5 | | 26.6 | 25.8 | 25.2 |
| 20:3 | 2.7±0.6 | 2.6±0.4 | 2.4 | 2.7 | 1.3 | 2.2 | 3.8 | 4.2 | 3.5 | | 2.2 | 2.4 | 3.2 |
| 20:4 | 10.0±2.4 | 10.5±2.4 | 9.4 | 10.2 | 3.3 | 8.4 | 9.4 | 9.6 | 11.0 | | 6.0 | 8.2 | 8.7 |
| 22:4 | 0.3±0.2 | 0.4±0.4 | 0.4 | 0.4 | 0.7 | 1.0 | 1.5 | 1.1 | 1.4 | | 0.6 | 0.3 | 0.4 |
| 22:5 | 1.0±0.8 | 1.2±1.1 | 1.1 | 1.9 | 0.4 | 1.7 | 3.1 | 3.2 | 1.8 | | 1.8 | 0.8 | 1.4 |
| 22:6 | 2.3±2.0 | 2.4±2.4 | 3.2 | 3.4 | 1.4 | 5.2 | 1.9 | 2.1 | 2.4 | | 1.3 | 2.4 | 1.8 |

* Less than 1 mole per 100 moles of 14:0, 17:0, 18:3, 20:2, and 20:5 were also identified but are not shown.
† Mean ± standard deviation for six adults, two females and four males.
‡ Values for pooled children's samples; the patients represent single analyses.
§ Percentages given for the mother and father are the averages of HDL2 and HDL3, which were virtually identical. The sister's data are from analyses of whole plasma.

TABLE V

*The fatty acid composition of high density lipoprotein sphingomyelins in patients with abetalipoproteinemia and control subjects**

| Fatty acid | Normal adults (mean ± SD, n = 5) | | Normal children† (pooled sample) | | Abetalipoproteinemia | | | | | | Family of JG‡ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | M.S. | | J.G. | | J.D. | | Father | Mother | Sister |
| | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL2 | HDL3 | HDL | HDL | WP |
| | *moles/100 moles fatty acid* | | | | | | | | | | | | |
| 16:0 | 28.5±4.9 | 24.3±2.6 | 26.1 | 27.6 | 26.2 | 26.5 | 26.0 | 26.7 | 34.8 | | 32.1 | 35.6 | 34.0 |
| 18:0 | 13.7±5.5 | 14.7±5.2 | 12.2 | 11.3 | 10.6 | 9.0 | 11.6 | 12.5 | 10.7 | | 9.6 | 9.2 | 7.3 |
| 18:1 | 5.3±1.6 | 4.2±0.9 | 3.3 | 4.2 | 2.7 | 6.1 | 2.4 | 3.1 | 3.6 | | 5.5 | 3.1 | 1.5 |
| 18:2 | 2.7±1.3 | 2.7±0.3 | 1.1 | 1.8 | 0.6 | 2.6 | 0.3 | 1.0 | 0.8 | | 2.2 | 0.6 | 1.8 |
| 20:0 | 3.3±0.7 | 3.3±0.7 | 3.6 | 3.1 | 3.2 | 2.8 | 3.3 | 3.2 | 2.8 | | 3.3 | 4.0 | 3.3 |
| 20:4 | 4.2±4.0 | 5.9±4.4 | 4.7 | 3.8 | 0§ | 0§ | 0§ | 0§ | 0§ | | 0 | 0 | 0 |
| 22:0 | 9.3±2.4 | 11.0±2.8 | 12.7 | 11.1 | 4.4 | 3.7 | 6.1 | 7.8 | 3.7 | | 12.7 | 10.5 | 15.3 |
| 22:1 | T‖ T | T T | T | T | 3.2 | 2.7 | 1.6 | 2.8 | 3.6 | | 0.8 | 2.7 | 1.4 |
| 23:0 | 6.2±3.4 | 5.9±1.2 | 6.7 | 5.5 | T | T | 1.5 | 0.9 | 1.4 | | 1.8 | 4.8 | 5.7 |
| 24:0 | 7.4±2.2 | 8.1±2.5 | 10.8 | 8.8 | 3.7 | 3.3 | 4.6 | 4.2 | 3.6 | | 8.7 | 6.2 | 12.2 |
| 24:1 | 12.5±0.6 | 14.2+1.8 | 14.0 | 12.6 | 31.3 | 24.7 | 31.2 | 31.4 | 26.6 | | 12.3 | 15.8 | 12.4 |
| 24:2¶ | 0.9±0.9 | 1.2±1.1 | 1.5 | 1.2 | 8.3 | 8.5 | 4.1 | 1.8 | 2.4 | | 1.4 | 2.8 | 1.1 |
| 25:0¶ | | | | | | | | | | | | | |

* Less than 1 mole per 100 moles of 15:0, 17:0, 20:1, 21:0, and 23:1 were also identified but are not shown. Larger quantities (but less than 4%) of 14:0 and 16:1 were present, but since they did not differ from the normal by more than 2 SD, they are not given.
† Values for normal children and the patients represent single analyses.
‡ The percentages given for the mother and father are the averages of HDL2 and HDL3, which were virtually identical in all respects. Data for the sister were obtained on whole plasma. All of the family samples were purified before transesterification by deacylation in mild alkali followed by silicic acid chromatography (see text and footnote 4), hence the absence of 20:4.
§ These samples were transesterified both before and after decylation in mild alkali. The values were virtually the same by both methods; in neither case was 20:4 present (see text and footnote 4).
‖ T = trace (<0.2 moles per 100 moles fatty acid).
¶ 24:2 and 25:0 did not separate on the chromatograms, so these peaks were calculated together. In the patients almost all of this peak was 24:2, as estimated by hydrogenation and rechromatography.

TABLE VI

*The fatty acid composition of high density lipoprotein phosphatidyl ethanolamines in abetalipoproteinemic patients and control subjects**

| Fatty acid | Normal adults | | Children's pool | | Patient M.S. | |
|---|---|---|---|---|---|---|
| | HDL2† | HDL3‡ | HDL2 | HDL3 | HDL2 | HDL3 |
| | *moles/100 moles fatty acid* | | | | | |
| 16:0 | 13.4 | 14.6±4.1 | 11.8 | 14.5 | 28.7 | 31.4 |
| 16:1 | 2.0 | 2.1+1.7 | 4.1 | 5.8 | 3.3 | 8.1 |
| 18:0 | 14.7 | 14.3±1.0 | 16.5 | 17.1 | 10.0 | 15.6 |
| 18:1 | 6.2 | 7.7±1.0 | 8.7 | 9.4 | 8.7 | 5.4 |
| 18:2 | 10.5 | 8.9±2.6 | 10.1 | 5.9 | 7.3 | |
| 20:4 | 27.7 | 23.2±2.5 | 23.0 | 22.2 | 3.8 | 4.4 |
| 22:6 | 13.4 | 13.5+2.8 | 12.2 | 13.3 | 3.8 | 6.2 |
| 24:0 | T | T | T | | 4.7 | 4.7 |
| 24:1 | T | T | T | | 16.7 | 9.9 |

* Less than 1 mole per 100 moles of 18:3 and 20:0 was present, so it is not included. From 0.5 to 2.3 moles per 100 moles of 20:3 and 22:4 were found in the normal samples but not in abetalipoproteinemia. 14:0, 20:5, and 22:5 were also present (never exceeding 5 moles per 100 moles), but no differences were noted between normal and abnormal. All of these have been omitted.
† Mean for two adults.
‡ Mean ± standard deviation for five adults.

The sphingomyelin fatty acids of three patients with abetalipoproteinemia differed significantly from those of the normal subjects, but with few exceptions HDL2 and HDL3 fatty acids were very similar. The greatest difference was the high nervonic acid (24:1) value, which was twice normal. Despite the low total HDL phospholipid value of about 50% of normal, the absolute quantity of HDL sphingomyelin nervonate was higher in all three patients. Accompanying this were both a relative and an absolute decrease in 22:0, 23:0, and 24:0. In M.S., 24:2 was markedly elevated, but this was not found in the other two patients. In contrast to findings on normal subjects, arachidonic acid (20:4) was not detectable in any of the patients' sphingomyelin fractions, and the linoleic acid content was lower than normal.

The phosphatidyl ethanolamine fatty acid data were limited because of the small sizes of the samples available for analysis. In normal subjects, the polyunsaturated fatty acids 20:4 and 22:6 predominated. Significant amounts of 16:0, 18:0, and 18:1 dimethylacetals derived from plasmalogens were present. In patient M.S., the phosphatidyl ethanolamine fractions contained much less 20:4 than normal.[5] The same dimethylacetals were found as in normal plasma.

The fatty acid composition of cholesteryl ester is listed in Table VII. Since no significant differences were found between HDL2 and HDL3, the average of these is given. Both J.G. and M.S. were receiving dietary supplements containing linoleic acid at the time of these analyses. Cholesteryl linoleate accounted for over 50% of the total cholesteryl esters in normal subjects. In the patients, linoleic acid was only 23 and 26% of the total, despite the supplementary linoleic acid. Palmitate, 16:1, and 18:1 were all relatively higher, and the 20:4 content was essentially that of the normal adult material.

Sufficient triglyceride from HDL was not obtained for valid fatty acid analyses.

*Effects of dietary alterations on plasma lipids.* In patient J.G., whole plasma lipids were analyzed while he was on diets that varied in fat content. The total phospholipid and cholesterol values are given in Figure 2. The initial studies were done while he was on his unrestricted home diet, which contained an average of 0.88 g of fat per kg body weight each day. During 34 days when he consumed only 0.3 g of total fat per day, both phospholipid and cholesterol levels declined. Subsequently, his diet was changed to an average daily fat level of 8.5 g; this was supplemented with 28 g of corn oil daily. Changes in lecithin and cholesteryl ester fatty acids during this time are also depicted in Figure 2. When the fat-free diet was instituted, lecithin linoleic acid and arachidonic acid decreased, with a reciprocal increase in oleic

TABLE VII

*The fatty acid composition of cholesteryl ester in abetalipoproteinemic patients and control subjects**

| Fatty acid | Normal adults (n = 3)† | Children's pool | Patients | |
|---|---|---|---|---|
| | | | M.S. | J.G. |
| | | *moles/100 moles fatty acid* | | |
| 16:0 | 12.3 (10.2–15.0) | 12.4 | 24.5 | 27.8 |
| 16:1 | 4.2 (3.2–5.0) | 3.6 | 10.0 | 6.3 |
| 18:0 | 1.1 (0.9–1.3) | 1.3 | 1.0 | 1.1 |
| 18:1 | 18.0 (14.4–20.5) | 18.8 | 31.1 | 31.9 |
| 18:2 | 57.4 (50.1–67.8) | 53.4 | 22.9 | 25.8 |
| 20:3 | T | T | T | 1.0 |
| 20:4 | 6.3 (2.6–8.4) | 7.5 | 5.3 | 4.9 |

* The values given represent the average of HDL2 and HDL3 except for J.G., in whom the data were derived from whole plasma.
† Mean and range.

One phosphatidyl inositol sample isolated from normal plasma by column chromatography contained 11% 18:1, 12% 18:2, and 23% 20:4. In subsequent studies, we have found that the unsaturated fatty acids 18:2 and 20:4 can be eliminated from the "sphingomyelin fraction" by mild alkaline hydrolysis, which removes the glycerol-bound fatty acid. The sphingomyelins are then isolated by silicic acid column chromatography before methanolysis. The studies on the family members were done with the latter technique (Table V).

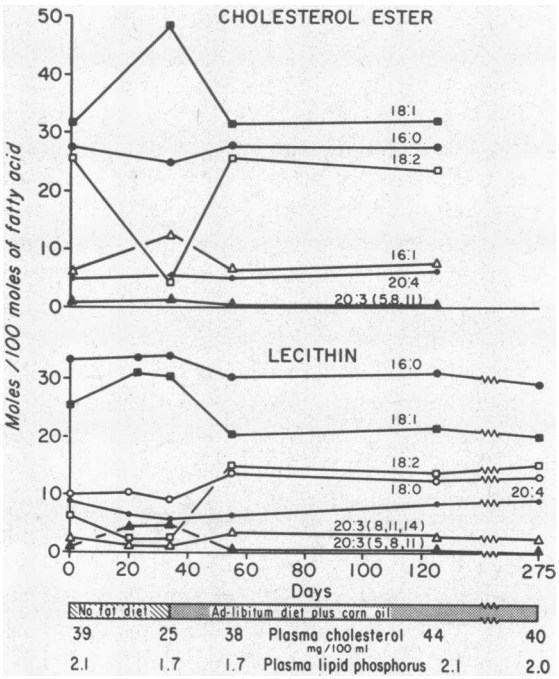


FIG. 2. FATTY ACID COMPOSITION OF PLASMA LECITHIN AND CHOLESTERYL ESTER FROM PATIENT J.G. ON VARIOUS LEVELS OF FAT INTAKE. The zero time samples were obtained before any dietary manipulation. The patient had been on an unrestricted diet calculated to contain 0.88 g fat per kg body weight per day. During the period of corn oil supplementation, the patient received 10 to 30 ml per day.

acid and 5,8,11-eicosatrienoic acid. When corn oil was returned to the diet, these changes were reversed. The lecithin arachidonic acid increased more slowly than linoleic acid, but the 5,8,11-eicosatrienoic acid level fell rapidly with a rise in the arachidonic acid precursor—8,11,14-eicosatrienoic acid. Even after 3 months of corn oil supplementation, however, a small amount of 5,8,11-eicosatrienoic acid was still detectable. While J.G. was on the fat-free diet, the cholesteryl esters contained 4.4% linoleic acid. This value increased to 25.8% when corn oil was added, with a reciprocal fall in oleic acid. A small amount of 5,8,11-eicosatrienoic acid was also present in this fraction during the fat-free diet.

*Family studies.* Detailed lipoprotein studies were done on the parents of patient J.G. in an attempt to demonstrate some partial expression of the lipid abnormalities found in homozygous subjects. In addition, whole plasma from his 5-year-old sister was examined. The lipoprotein data summarized in Tables II and III show that all values fell within the normal range, except for the lower level of HDL2 in the father. Lecithin and sphingomyelin fatty acid patterns are summarized in Tables IV and V. The only departure from normal was the value of 1.8% for sphingomyelin 23:0 in the father, compared with the average normal value of 6%.

## Discussion

Levy, Fredrickson, and Laster studied the immunologic characteristics, amino acid composition, and N-terminal amino acids of whole HDL, HDL2, and HDL3 isolated from patients with abetalipoproteinemia (28). When compared with similarly isolated normal material, no differences were apparent. These data suggest that an abnormality of the protein moiety of HDL in abetalipoproteinemia is unlikely and that the generally observed low level of HDL (4–8) must have another basis.

In this study we have shown that the low levels of total HDL are due only to a reduction within the ultracentrifugal fraction designated HDL3. However, both HDL2 and HDL3 manifest certain differences in lipid composition from their normal counterparts, *1*) a relative increase in sphingomyelin and decrease in lecithin; *2*) significant abnormalities in the fatty acid composition of lecithins, sphingomyelins, and cholesteryl esters; and *3*) a decrease in the percentage of esterified cholesterol. The presence of these abnormalities in family members would have supported the concept that they closely reflect the fundamental defect in abetalipoproteinemia. However, none of the defects were partially expressed in two obligate heterozygous subjects and a sibling of one propositus (Tables II and III). Thus, the degree to which the changes in HDL are specific to abetalipoproteinemia becomes the paramount consideration.

Total HDL is absent in Tangier disease (29), absent or reduced in chronic biliary obstruction (30, 31), and decreased in liver disease (32), in some patients with the nephrotic syndrome (30, 33, 34), and in certain hyperlipemias (30, 35). In most of these conditions, however, there are accompanying changes in the very low density ($D < 1.019$) and low density ($D$ 1.019 to 1.063) lipoproteins, with an apparent inverse relationship be-

tween very low density and high density lipoprotein concentrations (36, 37). Conversely, in abetalipoproteinemia, with almost complete absence of all low density lipoproteins, the total high density fraction is decreased rather than increased. The relationship between HDL2 and HDL3 observed in this study may also be unique. Whereas both physiologic and pathologic variations in the concentrations of HDL2 and HDL3 do occur (30, 36, 38), there have been no reports of normal HDL2 and low HDL3.[7]

The specificity of the altered distribution of HDL phospholipid found in abetalipoproteinemia is difficult to establish. A similar but less striking reduction in the lecithin : sphingomyelin ratio is found in fetal cord whole plasma, LDL, and HDL (13, 38). Changes equal to those in abetalipoproteinemia have been found in the whole plasma of patients with nephrotic syndrome by Nye and Waterhouse, but they were apparently confined to the low density lipoproteins (34). Since malabsorption is a prominent feature of abetalipoproteinemia, we have evaluated plasma phospholipid distribution in four patients with steatorrhea of another etiology. With one exception, they had approximately half the normal levels of total plasma cholesterol and phospholipid, but their whole plasma lecithin : sphingomyelin ratio was normal (38).

The fatty acid abnormalities found in abetalipoproteinemia appear to be at least partly nonspecific. Three subjects with steatorrhea of another etiology all had low levels of linoleic acid in their plasma lipids. In J.G., the low level of linoleic acid in lecithin and cholesteryl esters was raised by adding corn oil to the diet. During this supplementation, J.G. had a linoleic acid intake well above the 2.8% level of total calories sufficient to maintain normal plasma linoleic and arachidonic acid levels in adults (40). Nevertheless, his lecithin linoleic acid content did not reach a normal level even after 8 months of corn oil supplementation (10 to 15 ml per day). This suggests that factors other than diet were influencing fatty acid composition. Whether the sphingomyelin fatty acid data can be explained, even in part, by a deficiency of absorbed or dietary fat remains unclear. The striking increase in the level of 24:1 has not been previously reported for other diseases and was not diminished appreciably in J.G. by dietary change or supplementation. However, the plasma sphingomyelin fatty acids from one patient with malabsorption of another cause and one obese subject starved for 3 months revealed high levels of 24:1 (23.2 and 25.7 moles per 100 ml, respectively) (38). This suggests that the change in 24:1 may also be a function of the type or amount of fat absorbed, but perhaps slower to change than the lecithin and cholesteryl ester linoleate fractions.

To recapitulate, it appears that the low levels of HDL3 and the phospholipid abnormalities of the magnitude seen in the HDL of our patients may be specific to abetalipoproteinemia. The changes in fatty acid composition, on the other hand, are, at least in part, related to the malabsorption.

If the low HDL3 and the abnormalities of HDL phospholipid distribution are related to the primary defect in abetalipoproteinemia, how are they to be explained? One possibility is that such abnormalities are secondary to the absence of beta lipoprotein. Also, they might reflect a fundamental derangement of phospholipid metabolism that causes both absence of beta lipoprotein and abnormal alpha lipoprotein formation. If the first explanation is correct, one could reasonably suspect that the primary defect lies in the production of apolipoprotein. Some evidence exists that lipid-free beta lipoprotein can recognize antibodies to LDL; if so, the apobeta lipoprotein is not circulating in the plasma of these patients (28).[8]

[7] Levy and Fredrickson recently demonstrated that, during ultracentrifugation, the electrophoretically homogeneous alpha-1 lipoproteins from normal subjects (39) and from patients with abetalipoproteinemia (28) become heterogeneous. HDL2 retains the electrophoretic and immunological characteristics of the alpha-1 lipoprotein of fresh whole plasma, whereas HDL3 consists of two fractions, one identical to the original alpha-1 lipoprotein, and the other with different electrophoretic migration and immunologic reactivity. This altered fraction presumably contains less lipid relative to protein. These findings suggest that, to an unknown extent, the data reported in the present study on the concentration of HDL2 and HDL3 are artifactual. This does not, however, alter the significance of a reduction in total HDL and differences between the relative proportions of HDL2 and HDL3 in patients with abetalipoproteinemia compared to normal subjects.

[8] It seems likely that the protein common to most or all LDL is not present in the plasma, since antibodies used for detection of LDL apparently "recognize" the

On the other hand, if the phospholipid abnormalities are primary, one might expect similar changes in phospholipid distribution in other tissues. Reversals in the lecithin: sphingomyelin ratio of the red cell are well documented (3, 4), but these have been shown to be secondary to the plasma abnormalities (41). Limited data indicate that the gastrointestinal mucosa of patients with abetalipoproteinemia has a normal phospholipid distribution (11); in any event, the gross abnormalities seen in plasma are not present.

Although the nature of the fundamental defect remains unresolved by direct evidence, the available data build a circumstantial case against the presence of the apobeta lipoprotein in plasma. Whether it is not synthesized, is not released from its site of synthesis, or is destroyed before reaching the plasma compartment cannot be determined from the evidence on hand.

Finally, in retraction of a previous statement (4), we suggest that abetalipoproteinemia in the untreated state should now be provisionally regarded as an example of essential fatty acid deficiency in man. Earlier, less sensitive gas-liquid chromatographic studies failed to reveal the presence of 5,8,11-eicosatrienoic acid, long regarded as the chemical signature of essential fatty acid deficiency in experimental animals. This study and those of others (42) have now demonstrated the presence of this abnormal fatty acid on the basis of retention data on gas-liquid chromatography. Only fatty acid degradation studies remain to obtain final proof.

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