

## The Nature of Pre-beta (Very Low Density) Lipoproteins \*

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The plasma lipoproteins and "particles"<sup>1</sup> fall into four major groups. As defined by paper electrophoresis (3) these are the beta, alpha, and pre-beta lipoproteins, and chylomicrons. The latter two are rich in glyceride and very low in density ( $< 1.006$ ,  $S_f > 20$ ). It is now common to reserve the term chylomicrons for the relatively large particles that arise in the gut from exogenous or ingested fat and that usually have  $S_f$  values  $> 10^5$  (2). The endogenous glycerides are found primarily in the pre-beta lipoproteins and particles; in the analytical ultracentrifuge these form concentration maximums distinct from those of the beta-migrating low density lipoproteins of  $S_f$  0 to 20, and when isolated as a group in the preparative ultracentrifuge they are often referred to as very low density lipoproteins (VLDL) (3, 4).

The VLDL have sometimes been considered to represent beta lipoproteins in association with variable amounts of glycerides added by the liver, a viewpoint encouraged by the prevailing immunochemical data. The latter are not all in agreement (5, 6), but most of the evidence, including that presented in the most recent review of the subject (6), suggests that VLDL are antigenically homogenous and identical to the beta lipoproteins. There exist certain other pieces of information, however, which are not in accord with this assumption.

Some of the properties of VLDL or pre-beta lipoproteins suggest that polypeptides in addition

to those in the beta lipoproteins are present. A greater negative charge is needed to explain the pre-beta mobility on paper (3) or  $\alpha_2$  mobility on starch (7) of VLDL. Also, if triglyceride were simply added to beta lipoproteins, the ratio of cholesterol to phospholipid would be expected to remain unchanged; instead it falls sharply as the flotation rate of lipoproteins exceeds  $S_f$  20 (8).

Furthermore, in contrast to beta lipoproteins (5, 9-11), glutamic acid has not been the sole amino-terminal acid in proteins isolated from VLDL (10-12). Included have been significant amounts of aspartic acid, known to be amino-terminal in the polypeptide characteristic of alpha lipoprotein (5, 9-11), as well as serine and threonine. Pertinent to this last finding are innumerable observations that the plasma concentrations of alpha lipoproteins bear a reciprocal relationship to the concentrations of VLDL and the establishment that patients with severe deficiencies of alpha lipoproteins have VLDL of quite unusual lipid composition (13).

The present studies have been designed to resolve the paradox created by the suggestion that alpha lipoproteins have some relationship to the formation and metabolism of VLDL despite immunochemical evidence that allows for the presence of only beta lipoproteins. Pre-beta lipoproteins have been studied by several techniques, including immunoelectrophoresis before and after lipid extractions. The relationship between alpha lipoproteins and endogenous glyceride transport has also been examined experimentally in normal subjects and patients with either hyperlipoproteinemia or alpha lipoprotein deficiency. Proof is presented that alpha lipoproteins are normally part of the VLDL and account for the pre-beta mobility of these lipoprotein complexes.

### Methods

Blood samples were collected in EDTA, 1 mg per ml, and usually after an overnight fast. In addition to fre-

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<sup>1</sup> The term "particle" is used in the context suggested by Dole and Hamlin (2) to signify any lipoprotein complex of sufficient size to be seen in a dark field microscope.

quent small samples, 150 ml of blood was obtained from some normal subjects after at least 1 week of a high carbohydrate diet containing <5 g of fat. The large samples were used for large-scale preparations of pre-beta lipoproteins or other fractions. Plasma was stored at 4° C, and most of the analyses or fractionations were begun on the day of collection. The major donors included 11 normal male and female volunteers 18 to 25 years of age and the following sets of patients: five considered to be homozygous for familial high density lipoprotein deficiency (Tangier disease), two sets of parents who presumably had a single gene for Tangier disease, two patients with severe liver disease, and a number of patients with various types of familial hyperlipoproteinemia (14).

#### *Dietary studies*

The "carbohydrate induction" of hyperlipemia in normal subjects is described in detail elsewhere (15). Diets composed of ordinary foods were fed on a metabolic ward, where caloric intake and body weight were recorded daily. The content of carbohydrate was varied from <1 to >10 g per kg body weight. The diets were usually altered by interchanging fat and carbohydrate in isocaloric quantities while keeping the protein relatively constant. Occasionally more carbohydrate was added to a previously isocaloric diet. The effects on plasma lipoproteins under study were the same whether the diets were hypercaloric or isocaloric. The daily cholesterol intake was maintained between 300 and 700 mg in all diets; in a few subjects it was held at a constant level of approximately 300 mg.

#### *Isolation of lipoprotein fractions*

*Preparative ultracentrifugation.* Lipoprotein fractions were separated in the Spinco model L preparative ultracentrifuge as described elsewhere (16). For quantification of plasma high density lipoproteins a solution of NaCl and KBr was directly added to plasma to raise its density to 1.063. The plasma was then centrifuged at 40,000 rpm and 10° C in the 40.3 rotor for 16 hours. The concentrations of the constituents of the top and bottom fractions obtained by tube slicing were brought to the concentration of the starting plasma by adding 0.9% NaCl solution.

For preparation of lipoproteins on a larger scale, 25 to 50 ml plasma was centrifuged in the 40.3 rotor at 40,000 rpm for 30 minutes to remove the particles of  $S_r > 400$  (2). The top 1 ml in the tube was discarded, and the infranate was recentrifuged for 16 hours. The grossly turbid upper 2 ml was removed, layered under isotonic saline, and recentrifuged for an additional 16 hours. The turbid 1 to 2 ml supernate after this centrifugation represented the  $D < 1.006$  fraction further identified below.

The infranate of the initial 16-hour isolation of the  $D < 1.006$  fraction was brought to  $D 1.019$  by the addition of NaCl and KBr (17) and centrifuged for 16 hours at 40,000 rpm. The small amount of turbidity at the top of the tube was discarded. Fractions of  $D 1.019$  to  $1.063$

and  $D 1.063$  to  $1.21$  were then prepared from this infranate by serial centrifugations at appropriate densities (16, 17). These were each washed by one more centrifugation at their respective densities.

Thus, three lipoprotein fractions were separated sequentially:  $D < 1.006$ , or very low density lipoproteins (VLDL);  $D 1.019$  to  $1.063$ , or low density lipoproteins (LDL); and  $D 1.063$  to  $1.21$ , or high density lipoproteins (HDL). The fractions were dialyzed at 4° C against 40 vol of 0.15 M NaCl containing 0.001 M EDTA at pH 7. The dialysis fluid was changed three times during the 24-hour period. The freedom of the dialyzates from non-lipoprotein contaminants was established by paper electrophoresis (3) and immunoelectrophoresis (16).

#### *Delipidation of lipoproteins*

*Ether extraction.* The ultracentrifugal fractions were extracted with organic solvents using a slight modification of the method of Avigan (18). Partial delipidation with diethyl ether alone was carried out by layering 50 vol of peroxide-free ether, arbitrarily adjusted to pH 9 by equilibration with dilute  $\text{NH}_4\text{OH}$  (1 drop  $\text{NH}_4\text{OH}$  per L distilled  $\text{H}_2\text{O}$ ) over 1 vol of lipoproteins in saline in a bubble-free sealed round-bottom flask that was gently agitated continuously for 4 to 24 hours at 4° C. The ether phase was then removed by suction and any remaining solvent blown off by nitrogen. The ether-treated lipoproteins in the aqueous phase were used without delay for immunochemical studies and electrophoresis.

*Ethanol-ether extraction.* Lipoprotein solutions or suspensions (1 to 2 vol) were injected through a no. 25 needle into 50 vol of ethanol: ether (3:2, vol: vol), and the mixture was left standing at 4° C for 18 to 24 hours. The precipitate was collected by centrifugation in the cold for about 50 minutes, washed once in ether, and dissolved in 1 ml of 0.9% NaCl (pH 7) containing 0.001 M EDTA. This was used immediately for immunochemical and electrophoretic studies. Ethanol-ether is known to precipitate irreversibly the polypeptide of beta lipoprotein (18, 19), and some of the precipitate obtained after delipidation of VLDL and LDL was not soluble in the saline. When preparations of either VLDL or HDL were extracted by ether or ethanol-ether, the ether phase always contained a small amount of protein precipitated by antisera specific for alpha lipoproteins (HDL).

*Precipitation of low density lipoproteins.* To 3-ml aliquots of plasma was added 0.15 ml 0.1 M manganese chloride and 6 mg of sodium heparin (20). A precipitate was allowed to form for 15 minutes at 4° C, and the tube was then centrifuged for 15 minutes at 4° C. The supernate contained only alpha lipoproteins when screened by immunoelectrophoresis (16).

*Electrophoresis.* Plasma lipoproteins were separated by paper electrophoresis with buffer containing albumin (3). All plasma samples were evaluated for changes in lipoprotein content by this technique within 24 hours after the sample was obtained.

The ultracentrifugal fractions VLDL, LDL, and HDL, and their products after delipidation were subjected to electrophoresis in barbital buffer of 0.1 ionic strength

without added albumin. These strips were run for 16 hours at 120 v, dried at 100° C for 30 minutes, and stained for protein with bromophenol blue. In the absence of albumin the VLDL trailed to the origin, but its pre-beta mobility (3) was still clearly apparent. The beta mobility of the LDL and the alpha<sub>1</sub> mobility of HDL were unaltered by the addition of albumin to the buffer.

**Immunochemical methods.** Immunelectrophoresis and double diffusion in agar were performed with techniques and antisera described earlier (16). Immunelectrophoresis was usually carried out in 0.05 M barbital buffer at pH 8.2 by using constant current (58 ma) for exactly 40 minutes. For the soluble products of ethanol-ether delipidation, a current of 60 ma was employed for 50 minutes. The method of development and staining of the immunelectrophoresis plates has been previously described (16).

In some experiments, 1% agarose<sup>2</sup> was used in place of 2% Noble agar,<sup>3</sup> and electrophoresis was carried out for 40 minutes at a constant current of 44 ma. The beta lipoproteins move away from the origin in agarose and have the mobility of  $\beta$ -globulin (21), and the pre-beta lipoproteins have the mobility of  $\alpha_2$ -globulin. This makes it easier to distinguish beta lipoproteins from pre-beta lipoproteins and from the two forms of alpha

lipoprotein, designated elsewhere as  $\alpha$ LP<sub>A</sub> and  $\alpha$ LP<sub>B</sub> (16).

**Chemical analysis.** Plasma or lipoprotein fractions were extracted in chloroform methanol and the cholesterol and phospholipid determined (22, 23). Triglycerides were extracted and measured directly (24). FFA was determined by the method of Dole (25). The total protein content of VLDL was estimated directly on small portions of the lipoproteins by the method of Lowry, Rosebrough, Farr, and Randall (26).

**Nomenclature and quantification of lipoproteins.** The index terms used subsequently to describe the lipoproteins will be based on the bands (beta, alpha, and pre-beta) obtained by paper electrophoresis (3). This departure from the convention of identifying lipoproteins in operational terms (27) is required by the necessity for frequent cross-reference to fractions isolated by electrophoresis on several media, preparative ultracentrifugation, or precipitation. The correlations between lipoprotein bands and fractions obtained in normal subjects by different methods are shown in Figure 1. All have been substantiated experimentally (3, 4, 7).

**Concentrations of alpha lipoproteins.** The plasma concentrations of alpha lipoproteins were expressed in terms of the cholesterol content in mg per 100 ml of plasma. Initially these lipoproteins were isolated by ultracentrifugation as HDL (Figure 1). Later the precipitation technique was usually employed (20). In

<sup>2</sup> Marine Colloid, Springfield, N. J.

<sup>3</sup> Difco Laboratories, Detroit, Mich.

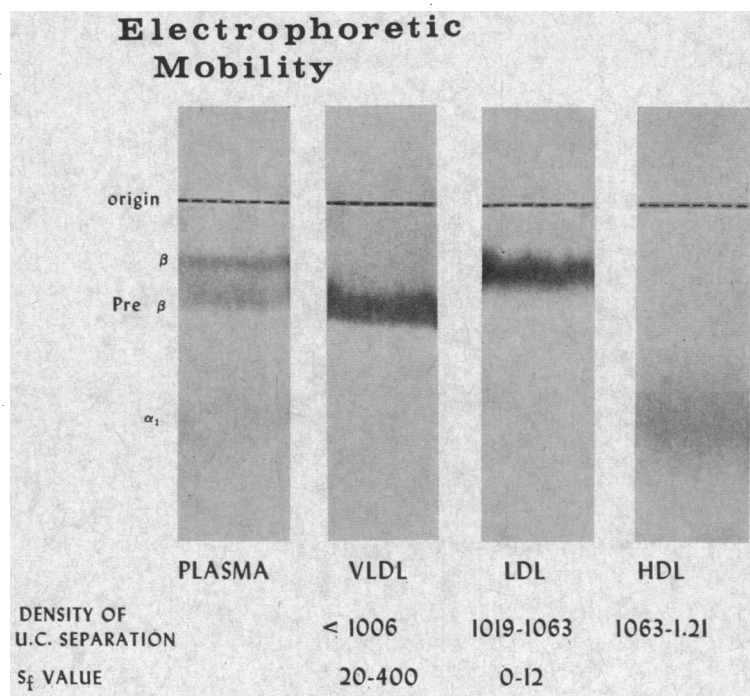


FIG. 1. THE CORRELATIONS BETWEEN LIPOPROTEIN BANDS OBTAINED BY ELECTROPHORESIS ON PAPER WITH THEIR ULTRACENTRIFUGAL (U.C.) COUNTERPARTS IN NORMAL SUBJECTS. Electrophoretic strips were stained with Oil Red O. VLDL = very low density, LDL = low density, and HDL = high density, lipoproteins.

TABLE I  
*Responses of plasma lipid and alpha lipoprotein concentrations to high carbohydrate feeding in normal subjects*

Subject	Age	Sex	At onset of high CHO diet*				At maximal hyperlipemia on diet			
			Plasma		α LP		Plasma		α LP	
			Chol.	Trig.	Chol.	PL†	Chol.	Trig.	Chol.	PL†
	<i>years</i>				<i>mg per 100 ml plasma</i>					
J.B.	18	M	122	61	48	101	110	309	30	66
A.G.	20	M	191‡		46	100	146		32	70
L.K.	21	F	180	80	64	112	170	284	37	74
G.L.	19	M	145	81	51	109	125	284	26	67
L.M.	18	M	120	50	49	102	110	240	33	63
S.M.	19	F	124	40	51	100	122	216	33	71
L.P.	18	M	104‡		38	82	88		28	64
N.K.	19	F	128	40	63	108	130	265	28	62
L.S.	19	F	120	35	39	86	142	149	32	74
B.W.	20	F	132	45	49	94	151	251	30	64
C.W.	18	F	140	59	46	98	148	327	29	68

\* Abbreviations: CHO = carbohydrate; chol. = cholesterol; trig. = triglyceride; LP = lipoprotein; PL = phospholipid.

† PL includes phospholipid of D > 1.21.

‡ Patients went directly from ad libitum diets to 500-g CHO low fat diets, omitting the cholesterol restricted preparative diets eaten by all other subjects.

study of each of the 11 normal subjects both methods were employed at least once during each diet period. The difference in the cholesterol content obtained by the two methods was only  $0.2 \pm 1.8$  mg per 100 ml (mean and standard deviation of 46 samples). Moreover, the changes in alpha lipoprotein cholesterol and phospholipid concentrations were similar in all samples drawn during diet study (Table I).

In over half of the plasma samples obtained from the normal subjects alpha lipoproteins were also crudely estimated by immunoelectrophoresis or double diffusion in agar. The density and location of the precipitation bands with reference to the antibody well changed in a mode consistent with the chemical measurements of the lipids in alpha lipoproteins. Further qualitative agreement with the chemical methods was provided by the intensity of the Oil Red O stain taken by the alpha lipoprotein band on paper electrophoresis.

## Results

*Dietary studies in normal subjects.* The plasma triglyceride concentrations of 11 normal subjects were elevated by high carbohydrate feeding. In every subject the plasma alpha lipoprotein concentrations varied inversely with those of triglyceride and pre-beta lipoprotein (Table I). Concentrations of alpha lipoproteins fell 20 to 60%, whereas those of the plasma triglycerides increased 2 to 3 times. In all the normal subjects on regular diets pre-beta lipoproteins were not in sufficient concentration to be detectable on paper electrophoresis; they appeared in all subjects as the glycerides

rose above about 150 mg per 100 ml. The changes in concentrations of alpha lipoproteins and triglycerides and the appearance and disappearance of pre-beta lipoproteins shown in Figures 2 and 3 are typical of the group. As is illustrated in both Figure 2 and Table I, the alterations in concentrations of alpha lipoprotein cholesterol bore no consistent relationship to any changes in plasma cholesterol concentrations produced by the diets. The plasma FFA concentrations were determined at least every 3 days throughout each study. In every subject they remained between 0.3 to 0.7  $\mu$ Eq FFA per ml. They were generally lower when the subjects were on high-carbohydrate diets.

*Decomposition of pre-beta lipoprotein.* The above observations suggested that alpha lipoproteins were incorporated into pre-beta lipoproteins as the latter appeared in plasma with rising glyceride concentrations. This possibility was tested in two ways. One was an attempt to dissociate alpha lipoproteins from pre-beta lipoproteins by promoting intravascular hydrolysis of glyceride. The second utilized partial extraction of pre-beta lipoproteins by lipid solvents and characterization of the decomposition products.

*In vivo lipolysis.* Sodium heparin, 10 U per kg, was administered intravenously to five subjects when their glyceride concentrations were maximally elevated by carbohydrate feeding.

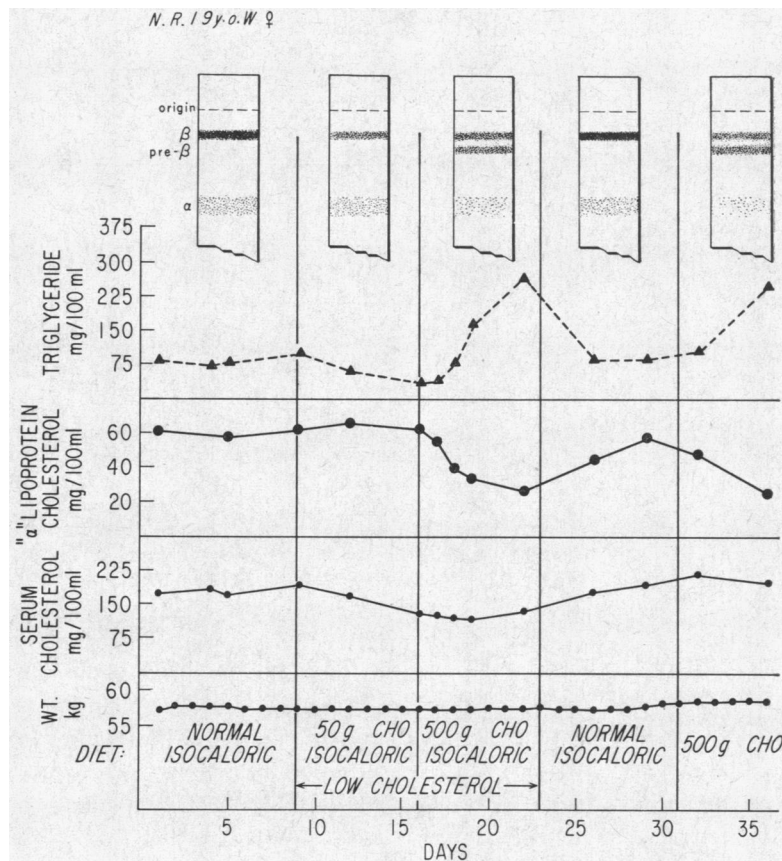


FIG. 2. CARBOHYDRATE INDUCTION IN A NORMAL SUBJECT. CHO = carbohydrate.

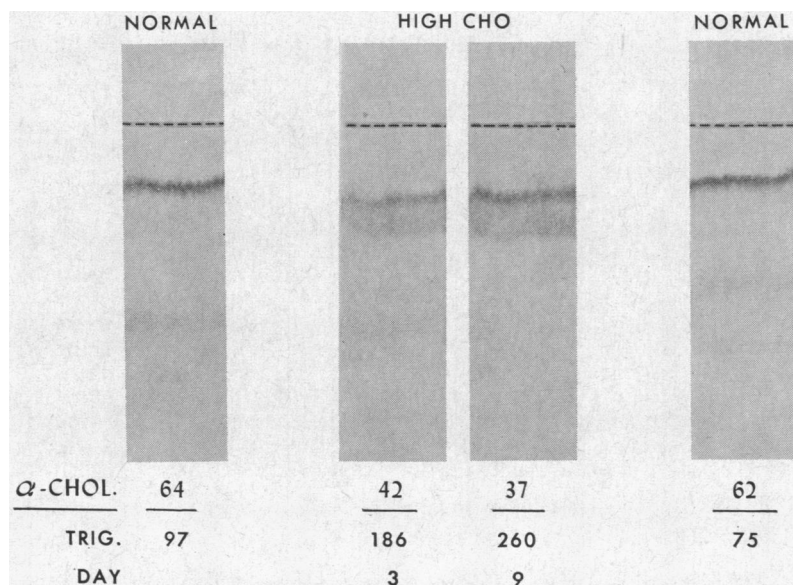


FIG. 3. CARBOHYDRATE INDUCTION OF HYPERLIPEMIA. The actual paper electrophoretic strips obtained on a normal subject during carbohydrate induction. Strips stained with Oil Red O. Trig. = triglycerides; chol. = cholesterol.

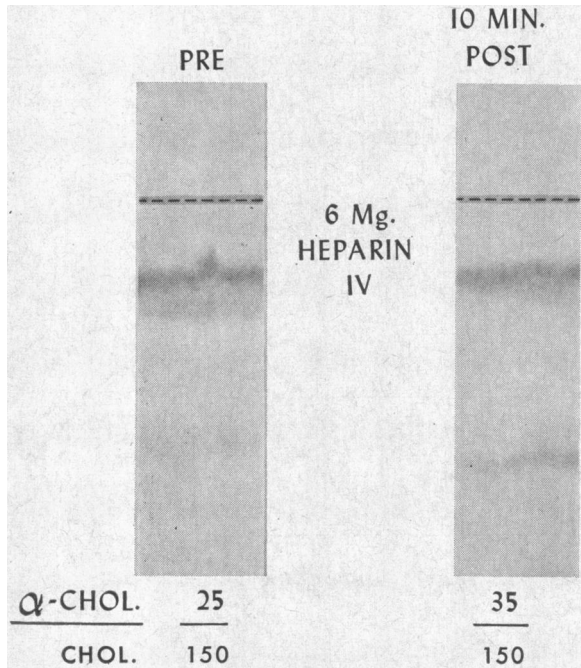


FIG. 4. THE EFFECTS OF HEPARIN ON THE LIPOPROTEIN PATTERN OF A NORMAL SUBJECT WITH ENDOGENOUS HYPERLIPEMIA.

Plasma was obtained 10 minutes later, separated at 4°, and kept at this temperature until analyzed. The changes in lipoprotein pattern observed after

TABLE II  
Compositions of representative isolates of pre-beta lipoproteins\*

Subject	Cholesterol	Phospholipid	Triglyceride	Protein†
<i>mg per 100 ml</i>				
1	43	65	183	42
2	48	63	198	43
3	54	82	265	50
4	51	77	230	46

\* Referred to concentrations in plasma.

† The protein content was determined directly on the pre-beta lipoprotein isolates after centrifugation and is therefore not strictly comparable to the lipid contents. The latter were calculated as the difference between the contents of cholesterol, phospholipid, and triglyceride, respectively, in the plasma and fraction of D > 1.006.

heparin were striking (Figure 4). In every instance the pre-beta lipoprotein was greatly decreased or disappeared, and alpha lipoprotein cholesterol increased 20 to 45% (mean 28%). The latter changes were supported by increases in alpha lipoproteins observable by paper electrophoresis (Figure 4) and semiquantitative immunochemical measurements by the method of Preer (16, 28). The plasma triglycerides decreased an average of 50% (40 to 65%) during this 10-minute period.

By contrast, repetition of the heparin experiments in the same five subjects when they were

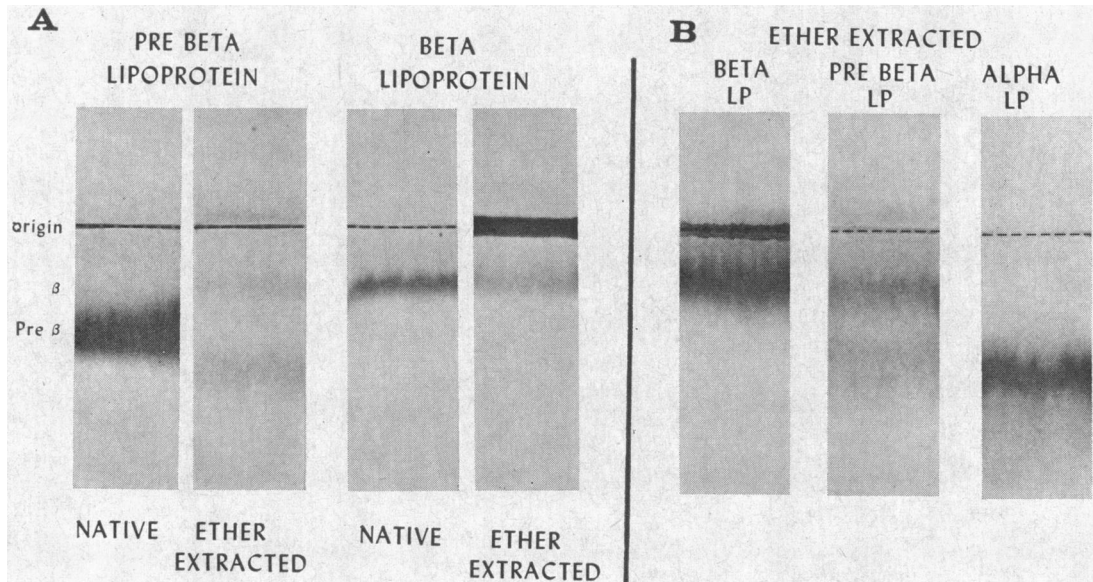


FIG. 5. A) PAPER ELECTROPHORETIC STRIPS OF BETA AND PRE-BETA LIPOPROTEINS BEFORE AND AFTER DELIPIDATION. B) PAPER ELECTROPHORESIS OF ALPHA, BETA, AND PRE-BETA LIPOPROTEINS AFTER ETHER DELIPIDATION. Section A was run in barbital-albumin buffer and stained with Oil Red O. Section B was run in plain barbital buffer and stained with bromophenol blue.



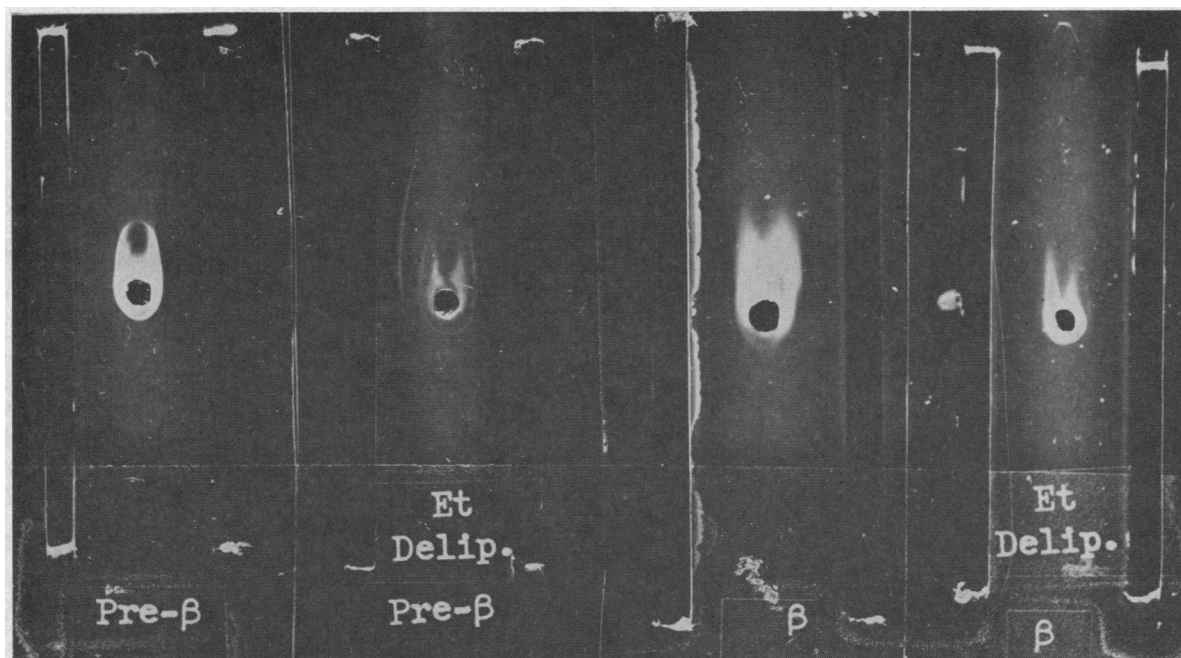


FIG. 6. IMMUNOELECTROPHORESIS ON AGAR OF PRE-BETA AND BETA LIPOPROTEINS BEFORE AND AFTER DELIPIDATION WITH ETHER (Et). Antiserum  $Sl_A$  (combined anti-alpha and beta lipoprotein sera) was employed in the left-hand troughs, and antiserum  $R_7$  (anti-beta lipoprotein serum) in the right-hand troughs. All precipitin bands have been subsequently stained with Oil Red O.

eating regular diets and had no pre-beta lipoproteins produced no alteration in their normal paper electrophoretic patterns and no significant change ( $< \pm 3\%$ ) in the concentration of alpha lipoproteins.

*Solvent extraction.* Pre-beta lipoproteins were isolated from eight different normal subjects and the gross chemical compositions determined. Four representative examples are given in Table II.

Each of the isolates migrated as a distinct and homogenous band on paper electrophoresis. Each of these bands was completely decomposed by ether extraction to two other bands having the mobilities of alpha and beta lipoproteins (Figure 5). The beta lipoproteins, by contrast, retained their mobility after identical treatment with ether (Figure 5).

Ethanol-ether extraction of beta lipoproteins yielded a denatured protein that remained at the origin on electrophoresis. When pre-beta lipoproteins were extracted with ethanol-ether, they were converted to an alpha lipoprotein band and a band remaining at the origin consistent with denatured beta lipoproteins.

*Immunoelectrophoresis and solvent extraction.* Pre-beta and beta lipoproteins migrated similarly on agar. Both types reacted with antiserum to beta lipoproteins, but neither gave a true precipitin line with anti-alpha lipoprotein serum (Figure 6). When examined by double diffusion in agar both types of lipoprotein formed single, apparently identical, precipitation lines with anti-beta serum.

However, after ether extraction of pre-beta lipoproteins, different reactions were observed. Two antigens were demonstrated by using combined antibodies to alpha and beta lipoproteins (16) (Figure 6). Both precipitation lines were stained by Oil Red O. One of these precipitation bands was obtained with a specific anti-beta serum and the other with specific anti-alpha serum. The transformation of pre-beta to alpha and beta lipoproteins by ether extraction was demonstrated even more clearly on agarose (Figure 7).

The denatured protein remaining after ethanol-ether extraction of beta lipoproteins, when applied to the agar or agarose in a suspension, did not migrate on either medium, and thus no precipitation lines with any antisera could be clearly demonstrated (Figure 8). When pre-beta lipopro-

teins were similarly extracted, a single precipitation band could be seen, but only with antisera reacting with alpha lipoprotein (Figure 8). When the Ouchterlony technique was used, the antigen so exposed by the extraction of pre-beta lipoproteins formed a line of identity with ethanol-ether extracted alpha lipoproteins, the latter corresponding to the partially delipidated form of alpha lipoproteins, which was identified in a previous report as  $\alpha$ LP<sub>B</sub> (16).

The immunochemical studies are summarized in Table III. They demonstrate conclusively that pre-beta lipoproteins in normal subjects contain at least two antigens. One is identical to beta lipoproteins and the other, uncovered by solvent extraction, is identical to alpha lipoproteins.

Similarly, pre-beta lipoproteins isolated in the ultracentrifuge (as VLDL) from the plasma of each of 35 patients with hyperlipemia were demonstrated to contain both alpha and beta lipoproteins after decomposition with ether. These patients represented several genotypically different forms of hyperlipoproteinemia. Examples of Types I, IV, and V, as classified according to a system used in this laboratory (14), were included. The presence of a distinct pre-beta lipoprotein band

in fresh plasma was associated in all patients with elevated plasma triglycerides and concentrations of alpha lipoproteins (HDL) that were abnormally low or in the lower quartile of normal values (29).

*Studies in alpha lipoprotein deficiency.* From these findings arose a number of questions concerning the role that alpha lipoproteins might be serving in the pre-beta lipoproteins. Answers to several of these were sought by utilizing patients with severe deficiencies of plasma alpha lipoproteins.

*Familial deficiency.* Four patients with Tangier disease who had barely detectable concentrations of alpha lipoproteins and two of their alpha lipoprotein-deficient relatives were studied in a manner analogous to the normal subjects except that the dietary periods were shorter. Plasma triglyceride concentrations are abnormally high in those having either a single or double dose of the gene for Tangier disease while they are on a regular diet (13). When these patients were fed a low-carbohydrate diet for 2 to 3 days, the plasma glycerides were lowered significantly (Table IV). Isocaloric high-carbohydrate diets were then fed for 5 to 6 days, and the triglyceride concentrations

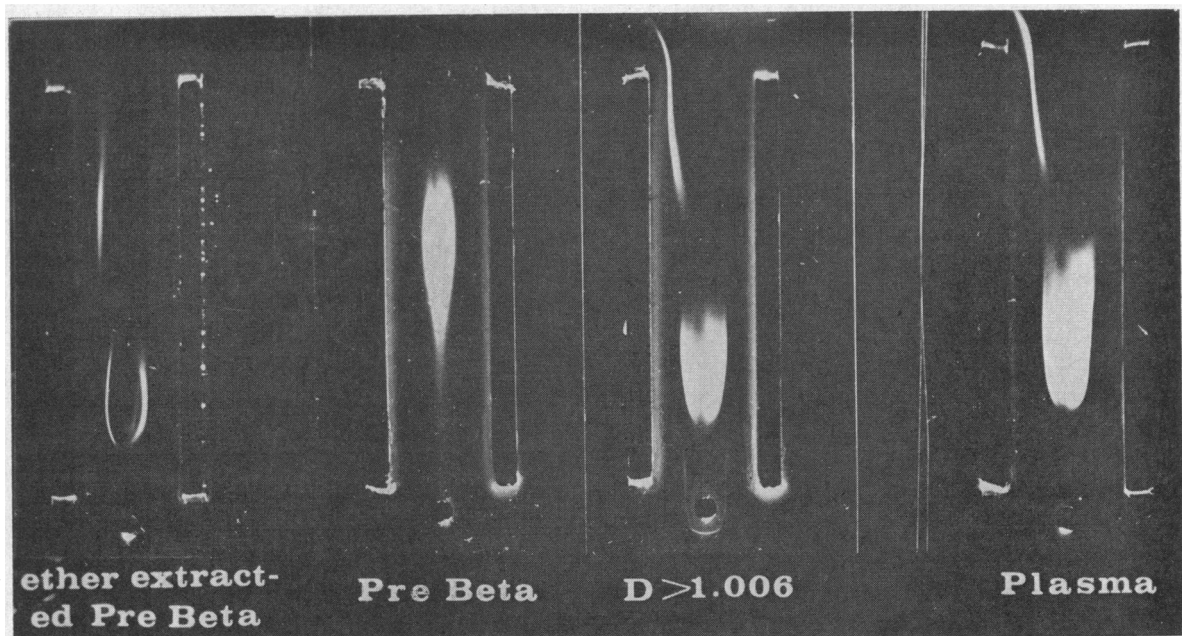


FIG. 7. IMMUNOELECTROPHORESIS ON AGAROSE OF WHOLE PLASMA AND PRE-BETA LIPOPROTEIN BEFORE AND AFTER ETHER DELIPIDATION. Antiserum S<sub>1A</sub> (combined anti-alpha and beta lipoprotein sera) was employed in the left-hand trough and antiserum R<sub>7</sub> (anti-beta lipoprotein serum) in the right-hand trough. All precipitin bands have been subsequently stained with Oil Red O.



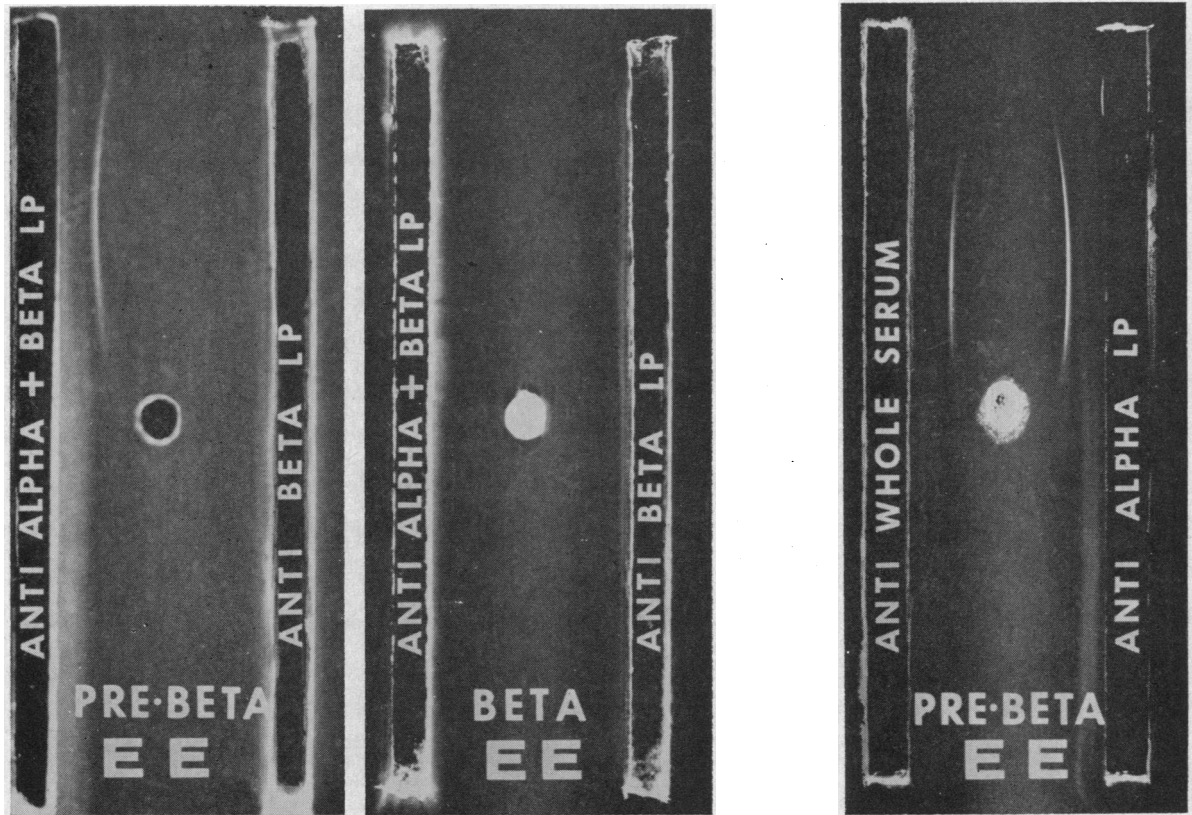


FIG. 8. IMMUNOELECTROPHORESIS ON AGAR OF BETA AND PRE-BETA LIPOPROTEIN (LP) AFTER ETHANOL-ETHER (EE) DELIPIDATION. Anti-alpha and beta lipoprotein sera = S1<sub>A</sub>; anti-beta lipoprotein serum = R<sub>7</sub>; anti-alpha lipoprotein serum = R<sub>1</sub>. Precipitin bands subsequently stained with Oil Red O. As noted in Methods, the immunoelectrophoresis was carried out longer and on higher current than in the other experiments.

rose to 300 to 600 mg per 100 ml within 3 to 4 days (Table IV and Figures 9, 10). These were higher than the concentrations reached during this same short time interval in 12 normal controls on similar diets.

In those heterozygous for Tangier disease, as in normal subjects, plasma alpha lipoprotein concentrations varied inversely with concentrations of triglycerides and pre-beta lipoproteins (Figures 9A and 10A). After induction of hyperlipemia, their alpha lipoprotein titers were decreased almost to the level seen in the Tangier homozygotes.

Patients homozygous for Tangier disease did not have pre-beta lipoproteins despite high plasma glyceride levels induced by carbohydrate feeding (Figures 9B and 10B). Their only detectable plasma lipoproteins were in a single beta-migrating band. This band widened and became more intensely stained as endogenous glyceride concentrations increased, but its forward boundary never

TABLE III  
*Immunochemical reactivity of lipoproteins\**

	Anti-alpha lipoprotein sera	Anti-beta lipoprotein sera
Pre-beta lipoprotein		
Native	±	+
Ether extracted	+	+
Ethanol-ether extracted	+	-†
Beta lipoprotein		
Native	-	+
Ether extracted	-	+
Ethanol-ether extracted	-	-†
Alpha lipoprotein		
Native	+	-
Ether extracted	+	-
Ethanol-ether extracted	+	-

\* Isolated in the preparative ultracentrifuge as summarized in Figure 1 and as defined in the text.

† Beta lipoprotein precipitated entirely by ethanol-ether.

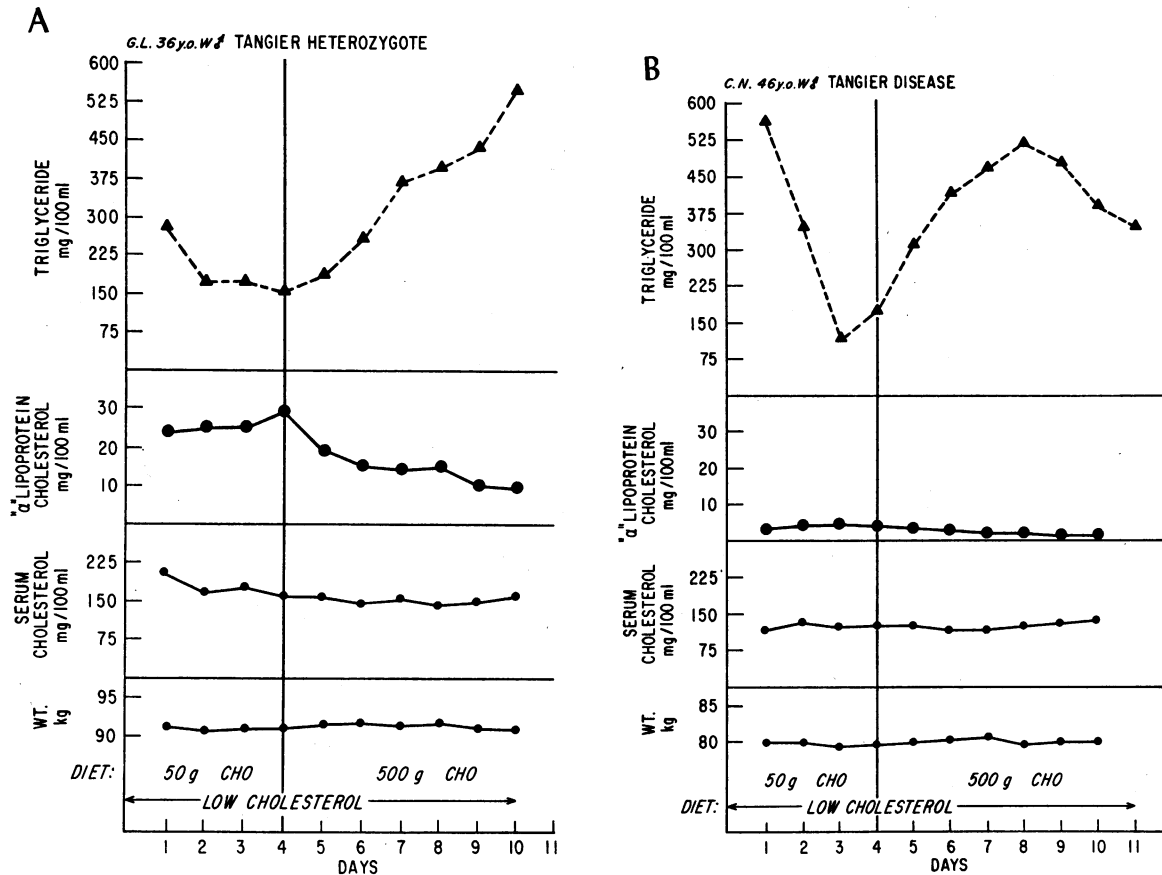


FIG. 9. A) CARBOHYDRATE INDUCTION IN A TANGIER HETEROZYGOUS SUBJECT. B) CARBOHYDRATE INDUCTION IN A TANGIER HOMOZYGOUS PATIENT. The patient in section B underwent numerous radiological studies during his last 3 days of study during which it was impossible to keep his diet isocaloric. He is the only Tangier patient with an apparent triglyceride maximum before day 5.

reached the "pre-beta" range of migration. Plasma obtained from the Tangier homozygous subjects on the fourth day of high carbohydrate feeding contained more than 200 mg per 100 ml of glyceride of density  $< 1.006$ , most of which normally should have been in pre-beta lipoproteins. However, these "VLDL" had beta mobility on paper electrophoresis.

*Acquired deficiencies.* Similar observations were made on two patients with acquired alpha lipoprotein deficiency, B.H. (NIH no. 05-79-61), a 53-year-old male who had a hepatoma; and M.E.W. (NIH no. 04-67-40), an 11-year-old female with total lipoatrophy (30) and severe portal cirrhosis, who 1 year earlier had a splenorenal shunt. Both had alpha lipoprotein cholesterol concentrations of less than 6 mg per 100 ml [minimal normal = 32 mg per 100 ml (29)] as de-

termined by both ultracentrifugation and precipitation. Their titers of immunoreactive alpha lipoproteins were only slightly greater than those seen in Tangier homozygous subjects. The clinical status of both these patients precluded dietary studies, but in six or more plasma samples obtained over a 2-month period from fasting patients, each consistently had plasma triglyceride concentrations above 200 mg per 100 ml. On paper electrophoresis, only a densely staining beta lipoprotein band was ever seen. There was never a pre-beta or an alpha lipoprotein band.

### Discussion

The experiments herein described leave little doubt that endogenous lipoproteins of density  $< 1.006$  and of  $S_f > 20$  are complexes that contain both alpha and beta lipoproteins in addition to tri-

glycerides. The products of the decomposition of the "very low density" or pre-beta lipoproteins suggest that the alpha and beta lipoproteins are probably present in these complexes as the intact lipoproteins themselves, although the immunochemical tests have ascertained beyond all reason-

able doubt only that the proteins of the lipoproteins are there.

There have been a number of earlier indications that alpha lipoproteins were present in the VLDL. The first proposal to this effect was a more or less hypothetical deduction based on the known lipid

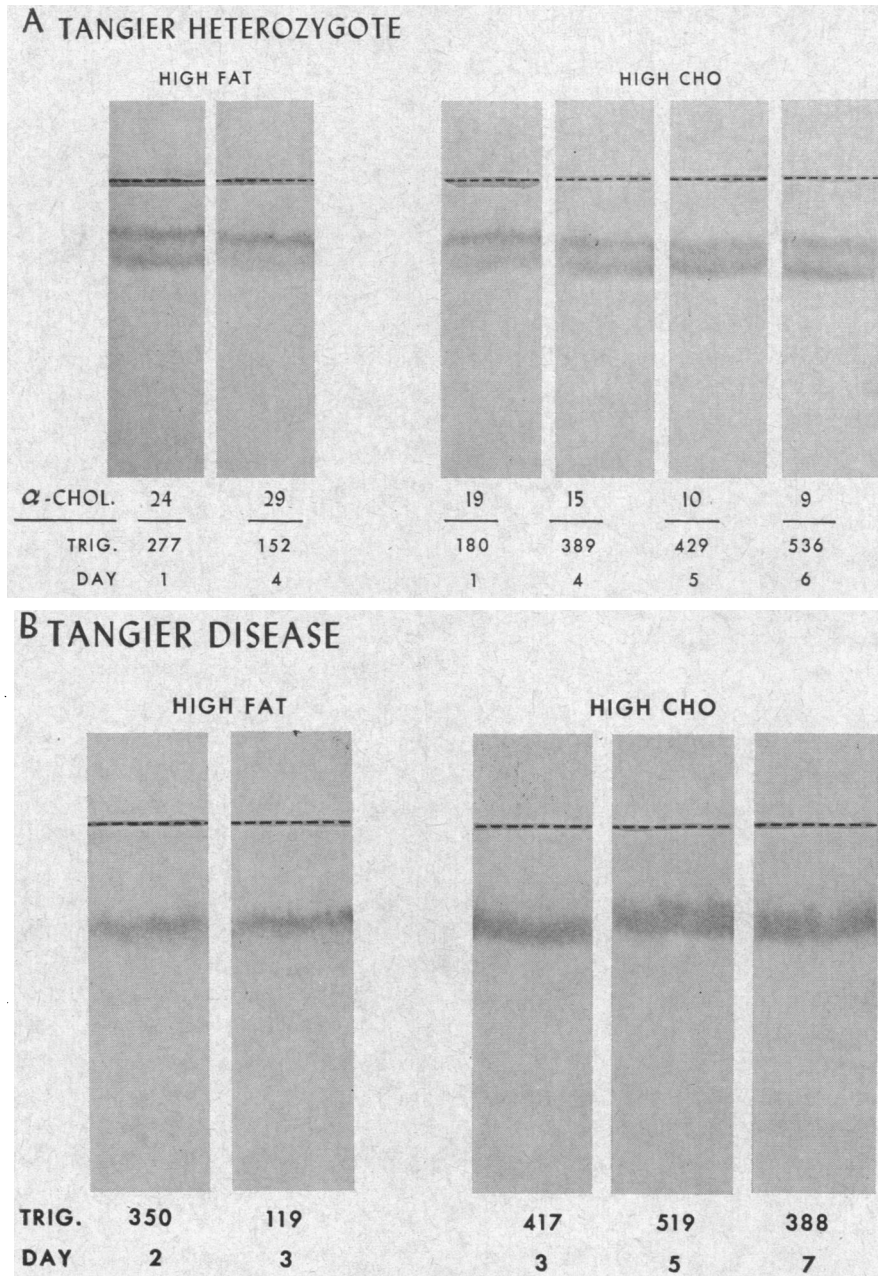


FIG. 10. A) ACTUAL ELECTROPHORETIC STRIPS SHOWING CARBOHYDRATE INDUCTION IN A TANGIER HETEROZYGOUS SUBJECT. B) ACTUAL ELECTROPHORETIC STRIPS SHOWING CARBOHYDRATE INDUCTION IN A TANGIER HOMOZYGOUS PATIENT.

TABLE IV  
*Sample lipid values in Tangier disease*

Patient	Age	Sex	Regular diet				High fat diet*				High CHO diet†			
			Plasma		α LP		Plasma		α LP		Plasma		α LP	
			Chol.	Trig.	Chol.	PL	Chol.	Trig.	Chol.	PL	Chol.	Trig.	Chol.	PL
	<i>years</i>		<i>mg per 100 ml plasma</i>											
Heterozygous														
P.L.	29	F	182	467	18	51	204	90	33	76	131	403	13	46
G.L.	36	M	207	277	24	52	160	152	29	68	148	459	10	35
Homozygous														
C.N.	46	M	120	531	3	11	124	119	4	14	120	519	1	9
T.L.	9	M	80	178	3	17	87	75	4	20	81	348	2	13
E.L.	11	F	70	140	3	16	66	80	4	19	69	294	2	15

\* After 3 days of high fat diet.

† After 5 days of high CHO diet.

compositions of different segments of the density spectrum of the lipoproteins (31). Other evidence took the form of increases in alpha or high density lipoprotein concentrations in plasma after heparin induced lipolysis of VLDL (32, 33). Sometimes these were interpreted by the authors to be due to changing concentrations of plasma free fatty acids (8). It has also been suggested that high density lipoproteins seem to be dislodged from triglyceride-rich particles in plasma by sonic treatment (34), and in chemical analyses (11, 12), protein with amino-terminal aspartic acid, common to the alpha lipoprotein, has been found on several occasions.

Gustafson, Alaupovic, and Furman have reported finding three different proteins after delipidation, electrophoresis, and ultracentrifugation of lipoproteins or particles of density < 1.006 obtained from normal and hyperlipemic subjects (35). Two of these proteins are described as identical to the alpha and beta polypeptides, or A and B apoproteins. The third was designated as apoprotein C, considered chemically and immunochemically different from A or B. It is not known whether the starting materials used by Gustafson and co-workers corresponded to the endogenous pre-beta lipoproteins utilized here, chylomicrons not being specifically excluded. In our studies a third protein was not seen on paper electrophoresis nor was an unknown precipitation line detected by any of the antisera used.

As it has turned out, the considerable amounts of alpha lipoprotein present in VLDL cannot be clearly identified with specific antibodies until some of the triglyceride is removed. The easiest

method is mild solvent treatment followed by immunoelectrophoresis in agarose. There still are certain technical problems to be overcome in quantification of either the alpha or beta lipoproteins present in the pre-beta complex, for it has been repeatedly observed that some of the alpha proteins, at least, are soluble in the organic solvents and thus lost in estimates based on the aqueous phase. Precise measurement of the concentrations of the two major lipoprotein peptides, especially when the plasma glyceride concentration is increased, will be necessary to determine how often "true" alpha lipoprotein deficiency is present in patients with hyperlipemia, and whether such possible deficiency is a primary cause of abnormal triglyceride metabolism. Such measurements will also help in comparative studies of the metabolism of the peptides when complexed in pre-beta lipoproteins and when present in alpha or beta lipoproteins.

The bonds between the several lipoproteins and the glycerides in VLDL are sufficiently strong to resist dissociation in high centrifugal fields or during electrophoresis or precipitation with polyanions or polycations. It is noteworthy that the charge of the alpha lipoproteins is available and apparently accounts for the pre-beta mobility of the complexes on electrophoresis even though the configuration of the complexes is such as to prevent the full antigenic expression of this lipoprotein.

Only inferences, rather than certain conclusions, are available at this time about the possible functions of the alpha and beta lipoproteins, respectively, in the transport of glyceride. Patients with

severe deficiency of alpha lipoproteins, whether heritable or acquired, cannot make pre-beta lipoproteins. In response to appropriate stimuli, such as carbohydrate induction, they do secrete endogenous glycerides, presumably from the liver. These glycerides circulate mainly in lipoproteins of  $S_r > 20$  that have an abnormal electrophoretic mobility. Both the heterozygotes and homozygotes for Tangier disease usually have higher than normal plasma glycerides in the postprandial state. These excess glycerides decrease rather than rise after the institution of a diet quite high in fat and are, therefore, presumably endogenous in origin. Alpha lipoprotein deficiency, then, appears to be compatible with secretion of glyceride from the liver, but either the rate of secretion of the glycerides is abnormally high or they are not removed as rapidly as in normal individuals, or both. Alpha lipoproteins have also been identified in chylomicrons, but there has not yet been agreement as to whether they are a constant accompaniment of these particles or have a specific function there (36). Patients with Tangier disease do absorb fat and form chylomicrons that have a peculiarly low content of cholesterol (13). These exogenous glycerides appear to be removed without significant delay by the patient with Tangier disease, although an early suggestion (37) that the handling of the cholesterol in the chylomicrons might be abnormal in a way that could lead to the tissue lipidosis characteristic of this disease is perhaps still the most plausible hypothesis concerning this phenomenon.

It is now well known that patients with abetalipoproteinemia cannot form chylomicrons although their intestinal mucosal cells may be engorged with fat (38). Evidence has recently been obtained (1, 39) that patients with abetalipoproteinemia cannot form pre-beta lipoprotein and have no response of plasma glycerides to high carbohydrate feedings. It has been shown elsewhere that the liver in such patients does appear to synthesize or at least to accumulate glycerides under these circumstances (40).

The available evidence favors the conclusion that beta lipoprotein or its characteristic polypeptide is necessary for the secretion of triglyceride from either intestinal or hepatic cells and that alpha lipoprotein is not required for this process.

Some shift of HDL cholesterol to VLDL on

incubation of hyperlipemic plasma has been reported by Nichols and Smith (41). In the present studies the estimates of HDL by electrophoresis and precipitation were both initiated within a few hours after plasma was obtained. If passive transfer or exchange of cholesterol were related to the changes in HDL concentrations observed, they must at least have begun *in vivo* and involved the alpha lipoprotein protein as well. Certain abnormalities in the metabolism of both exogenous and endogenous particles in the absence of a normal complement of alpha lipoprotein must be explained, however, before it can be assumed that alpha lipoprotein is merely adsorbed to these complexes without functional significance.

A suggestion has been presented in detail elsewhere (42) that there is declining usefulness in defining many subfractions of the lipoprotein "spectrum" on the basis of density and that there is merit in the more simplistic concept that the three important independent variables defining the state of fat transport by plasma lipoproteins are triglyceride and the alpha and beta polypeptides, the latter along with more or less stoichiometric amounts of phospholipid and cholesterol. Time will prove the usefulness of this concept, but in any event, it is apparent that one may no longer consider the alpha and beta lipoproteins as totally independent systems completely separated by both density limits and transport functions.

### Summary

1) The group of lipoproteins and particles transporting endogenous triglycerides and variously identified as very low density lipoproteins ( $D < 1.006$ ,  $S_r > 20$ ), or as pre-beta or alpha<sub>2</sub> lipoproteins, by electrophoresis on paper and starch, respectively, has been examined with regard to its relationship to alpha and beta lipoproteins.

2) In both normal subjects and patients with hyperlipoproteinemia, the plasma concentrations of the pre-beta lipoproteins have been manipulated by experimental diets. A reciprocal relationship between pre-beta and alpha lipoprotein concentrations was demonstrated.

3) Heparin-induced lipolysis *in vivo* caused concentrations of pre-beta lipoproteins to fall and alpha lipoproteins to rise.

4) With a variety of antigen-antibody test systems, including immunoelectrophoresis on agar

and agarose, isolated pre-beta lipoproteins in their native state cross-reacted only with antisera to beta lipoproteins. After solvent extraction, an antigen identical to alpha lipoproteins was also uncovered. This dissociation of pre-beta lipoproteins to alpha and beta lipoproteins by solvent extraction, presumably through removal of triglyceride, could also be demonstrated by paper electrophoresis.

5) Patients with hereditary (Tangier disease) or with acquired alpha lipoprotein deficiency could not produce pre-beta lipoproteins even though plasma glyceride concentrations could be increased by carbohydrate feeding.

6) The functional significance of alpha lipoproteins in the pre-beta lipoproteins is discussed; their role remains unknown.

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