

The Lipoprotein Abnormality in Tangier Disease

QUANTITATION OF A APOPROTEINS

G. ASSMANN, E. SMOOTZ, K. ADLER, A. CAPURSO, and K. OETTE

From the Kliniken der Universität Köln, Abteilung für Klinische Chemie, 5 Köln 41, West Germany

ABSTRACT In this study we have determined by radioimmunoassay and double immunoelectrophoresis the total quantities and distributions of A apoproteins in three adult patients affected with Tangier disease (hereditary α -lipoprotein deficiency). Compared with normal plasma, the total quantities of apoproteins A-I and A-II in Tangier plasma were determined to be <1% and 5–7%, respectively. In Tangier patients, approximately 90% of the apoprotein A-I sedimented when ultracentrifugations of plasma were carried out at density 1.21 g/ml KBr. By contrast, more than 95% of the apoprotein A-II floated under those conditions. In normal plasma, approximately 90% of both apoproteins A-I and A-II is found in the 1.063–1.21-g/ml KBr density fraction. These findings suggest that complete dissociation of A apoproteins occurs in Tangier plasma. This dissociation of apoproteins was confirmed by double immunoelectrophoresis with monospecific antisera. Immunochemical and electrophoretic experiments did not provide evidence for a structural abnormality of apoprotein A-I in these patients. The results taken together strongly suggest that normal high-density lipoproteins are absent from Tangier plasma.

INTRODUCTION

Tangier disease is a rare disorder of plasma lipid transport characterized by the absence of normal high-density lipoproteins (HDL)¹ and storage of cholesteryl esters in foam cells in many tissues (1). As of today, 21 patients have been discovered, representing 15

kindred, none of whom are known to be related (2–12).^{2,3} Prominent clinical features include enlarged orange-colored tonsils, splenomegaly, and frequently, peripheral neuropathy. Histological and ultrastructural observations have provided evidence that the intracellular lipid storage is mainly confined to reticulo-endothelial cells (foam cells) in tonsils, bone marrow, skin, and jejunal submucosa; Schwann's cells in peripheral nerves; and myenteric plexus and nonvascular, smooth muscle cells (12).

In the past, most of the attention given to this disease has been focused on the nature of the plasma lipoproteins, particularly HDL. It has been demonstrated that patients with this disease have small amounts of HDL in their plasma that are similar to, but not identical with, normal lipoproteins of this density class (13). The major apoproteins of HDL are apolipoprotein A-I (Apo A-I) and apolipoprotein A-II (Apo A-II) (14–16). These apoproteins are believed to play a major role in the structural and functional integrity of HDL. Apo A-I has been shown to stimulate lecithin-cholesterol acyltransferase, the plasma enzyme which synthesizes cholesteryl esters by transfer of the β -fatty acid from phosphatidyl choline to cholesterol (17, 18).

In Tangier disease, there is an absolute decrease in the amounts of Apo A-I and Apo A-II in plasma. Studies on the structures of the A apoproteins have failed so far to detect an abnormality in amino acid composition (13).

² The diagnosis of Tangier disease in the patient A. Bo. has been based upon investigations carried out by Dr. H. Heckers, Universität Giessen, Med. Klinik, who kindly provided plasma samples from this patient to our laboratory.

³ Assmann, G., H. E. Schäfer, J. Zach, T. Gheorghiu, and K. Oette. Clinical, morphological, and genetic findings in two cases of Tangier disease. Manuscript in preparation.

Gheorghiu, T., G. Assmann, and H. E. Schäfer. 1976. Endoscopic findings in Tangier disease, *Endoscopy*. 8: 164–169. These two manuscripts refer to patients J. Si. and E. Ga.

Received for publication 26 July 1976 and in revised form 4 October 1976.

¹ *Abbreviations used in this paper:* The apoprotein components are designated by the term Apo: Apo A, apolipoprotein A, consisting of A-I and A-II polypeptides; Apo C, apolipoprotein C, consisting of C-I, C-II, C-III₁, and C-III₂ polypeptides; Apo HDL, protein moiety of HDL; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins.

Accurate determinations on the distribution and quantities of Apo A-I and Apo A-II have not yet been performed. In the present study, we have applied a double-antibody radioimmunoassay technique and double immunoelectrophoresis to further characterize the distribution and quantities of these apo-proteins in Tangier plasma.

METHODS

Patients. Three patients homozygous for Tangier disease (A. Bo., a 56-yr-old man; J. Si., a 42-yr-old man; and E. Ga., a 46-yr-old woman, sister of J. Si.) were donors of the Tangier plasma.^{2,3} Besides the results given in this report, the diagnosis has been further based on the following data.

Patient A. Bo. The patient was referred to the Department of Medicine, Universität Giessen, in November 1974, at which time hypocholesterolemia (60 mg/100 ml) was discovered. Subsequent investigation revealed the near absence of HDL from the patient's plasma as assessed by ultracentrifugal, electrophoretic, and immunologic techniques. Repeated analyses in the following years showed plasma cholesterol levels of 60–80 mg/100 ml, HDL cholesterol levels of 4–8 mg/100 ml, and triglyceride levels at or near 100 mg/100 ml. Secondary causes of HDL deficiency have been excluded. The patient's only daughter presented with plasma cholesterol levels of 120–140 mg/100 ml and HDL cholesterol levels of 20–22 mg/100 ml on repeated determinations.

Tonsillectomy was performed at age 20 yr; however, yellow-orange mucosal tags observed in the pharynx of this patient were similar in appearance to lesions in the pharynx described in other tonsillectomized patients with Tangier disease (3, 4). Microscopic examination of lymphoid tissue obtained by biopsy revealed the presence of large numbers of foam cells. The patient was found to have hepatomegaly, splenomegaly, flocculent infiltration throughout the corneal stroma, and recurrent polyneuropathy. Foam cells similar in appearance to those reported previously (12) were present in the spleen, the periportal fields of liver, and lamina propria of the colonic mucosa. Chemical examination of biopsy specimens of the colon revealed a five-fold increase in cholesteryl esters.

Patient J. Si. The patient originally consulted his physician because of persistent abdominal pain which was found to be caused by extreme splenomegaly. Upon sternal marrow aspiration, large numbers of lipid-containing foam cells were discovered, and the patient was then referred to the Department of Medicine, Universität Köln, in October 1975, with a tentative diagnosis of Niemann-Pick disease. Upon admission, he was found to have hepatomegaly (hepatic edge palpable 3–4 cm below the costal margin) and extreme splenomegaly (palpable 15 cm below the costal margin). Tonsillectomy had been performed at age 37 yr after recurrent tonsillitis since childhood; however, orange-yellow mucosal tags in the pharynx were present on inspection. Careful neurological examination was negative; there was no evidence of lymphadenopathy and there were no other significant physical abnormalities.

Routine laboratory data were unremarkable except for hypocholesterolemia (45 mg/100 ml), hypertriglyceridemia (300 mg/100 ml), thrombocytopenia (40,000/mm³), and routine lipoprotein analyses (heparin-M precipitation of the 1.006-g/ml infranate and agarose electrophoresis) which revealed the absence of HDL.

Histologic and ultrastructural examination of tissue specimens demonstrated that lipid storage was mainly confined to reticuloendothelial cells (foam cells) in spleen, periportal fields of liver, bone marrow, colon and rectal mucosa, Schwann's cells in peripheral nerves, and myenteric plexus and nonvascular, smooth muscle cells (jejunal submucosa). The morphological findings in this patient were very similar to those recently described by Ferrans and Fredrickson (12). Chemical analyses of biopsy specimen of colon and spleen indicated a 10–20-fold increase in cholesteryl esters; otherwise, there were no other obvious tissue lipid abnormalities.

The patient has three children 9, 4, and 3 yr of age. Plasma lipid and lipoprotein analyses in these children revealed hypocholesterolemia (105–120 mg/100 ml) and HDL deficiency (15–25 mg/100 ml HDL cholesterol).

Patient E. Ga. The 46-yr-old sister of J. Si was not suspected of having any abnormality until examination during a family survey in January 1976. Orange-yellow mucosal tags on the pharynx were visible although a tonsillectomy had been performed on the patient at age 9 yr. Hypocholesterolemia (75 mg/100 ml), hypertriglyceridemia (320 mg/100 ml), and the absence of HDL on agarose electrophoresis led to further careful examination. Upon ultracentrifugation, HDL cholesterol levels were found to be less than 5 mg/100 ml.

Histological and histochemical examination of sternal marrow aspirate and rectal biopsy revealed the presence of large numbers of lipid-containing foam cells. Lipid deposits in Schwann's cells of small cutaneous nerves and dermal histiocytes were observed upon electron microscopic examination of skin which grossly appeared normal. Further clinical studies on this patient are still in progress.

In the patient's children (16-yr-old boy; 14-yr-old girl) plasma cholesterol (110 and 115 mg/100 ml), plasma triglyceride (225 and 170 mg/100 ml), and HDL cholesterol (15 and 18 mg/100 ml) values were consistent with a genetic defect causing HDL deficiency in this family.

Electroimmunoassays (rocket electrophoresis) for human serum apolipoprotein A (Apo HDL) applied to native sera from the three Tangier homozygotes failed to produce immunoprecipitation; sera from several obligate heterozygotes produced only partial responses (Fig. 1).

Control normolipidemic subjects included medical students and hospital personnel, 15 males and 15 females, ranging in age from 18 to 35 yr. Physical examination and appropriate laboratory data did not give evidence of either diabetes mellitus, or liver, kidney, gastrointestinal, heart, or thyroid disease.

Preparation of lipoproteins and apoproteins. Plasma obtained by plasmaphereses on patients or normal subjects who had fasted overnight, was collected in 0.01% EDTA. Tangier and normal plasmas were carried through all of the subsequent separations in parallel. Lipoprotein fractions were separated in the preparative ultracentrifuge by the method of Havel et al (19). The density of the plasma of the lipoprotein isolates was raised by addition of solid KBr and checked by pycnometry at 20°C. Four fractions were usually isolated, subsequently designated as very low density lipoprotein (VLDL) ($d < 1.006$ g/ml), low-density lipoprotein (LDL) ($d 1.006$ to 1.063 g/ml), HDL ($d 1.063$ to 1.21 g/ml), and the fraction of density >1.21 g/ml. Radioimmunoassay determinations were performed on the respective lipoprotein fractions without further recentrifugation. The lipoproteins were dialyzed exhaustively against 0.9% NaCl/0.01% EDTA solution, pH 7.4, and concentrated by ultrafiltration.

To ensure maximal exposure of antigenic sites on Apo A-I and Apo A-II (20), native sera and isolated lipopro-

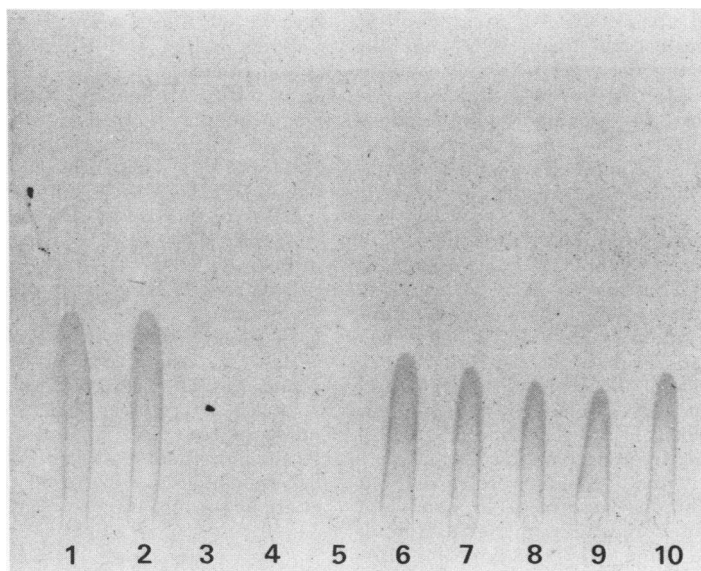


FIGURE 1 Immunoprecipitation ("rockets") of normal and Tangier serum. HDL antigenicity of native serum has been evaluated by Laurell electroimmunoassay employing anti-HDL. 1 μ l serum, 1:4 diluted, has been applied to the wells; electrophoresis buffer, 0.05 M diethylbarbiturate, pH 8.6; HDL antiserum was mixed with 0.5% agarose at 55°C; a field strength of 10 V/cm⁻¹ has been applied to the electroimmunoassay plate for 6 h; 0.1% Coomassie blue stain. 1, 2, normolipidemic sera; 3–5, sera of patients J. Si., A. Bo., and E. Ga., no visible reaction; 6–10, sera of patients obligate heterozygote for Tangier disease (children of J. Si. and E. Ga.) show diminished HDL reactivity.

tein fractions were delipidated by ethanol-ether (21). The protein precipitates were dried under N₂ and redissolved in 10 volumes of 0.9% NaCl, pH 7.4, or 10 mM NH₄HCO₃. Radioimmunoassay determinations on native and delipidated samples were run in parallel. Since different conditions of plasma storage and dilution may affect the degree of immunosassayable apoproteins (22), only fresh plasmas at constant dilution (10⁻³) were used in our studies.

To prepare Apo A-I and Apo A-II, HDL was delipidated with chloroform:methanol (2:1) according to Lux et al (23). The resulting apo HDL was fractionated by Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) chromatography as described by Scanu et al (24). The Sephadex column fractions corresponding to Apo A-I and Apo A-II were further purified by chromatography on DEAE-cellulose in 6 M urea, employing a linear gradient (0.03 M Tris/HCl, pH 8.6, 0.1 M Tris/HCl, pH 8.6) (23–25). To retard carbamylation of proteins (26), urea solutions were passed over a mixed bed ion-exchange resin (Rexyn I-300, Fisher Scientific Co., Pittsburgh, Pa.); all protein isolations were conducted at 4°C.

Homogeneity of the apoproteins used in these studies was determined by polyacrylamide electrophoresis in urea (27) and sodium dodecyl sulfate (28), by amino acid analysis, and by immunodiffusion techniques that utilize specific antisera against these apoproteins. To determine the specificity of radioimmunoassay and immunoelectrophoresis procedures, human albumin (Serva, Heidelberg, W. Germany), LDL (isolated between *d* of 1.020 and 1.050 g/ml (KBr) and C-apoproteins were tested as antigens. C-apoproteins (Apo C-I, Apo C-II, Apo C-III₁, Apo C-III₂) were purified from VLDL by a combination of Sephadex G-200 gel filtration and DEAE chromatography, respectively (29).

Preparation of antisera. In 1 ml H₂O, 1–2-mg amounts of Apo A-I or Apo A-II were dissolved and emulsified in an equal amount of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) (30). Four rabbits were injected subcutaneously on three occasions 14 days apart and bled 7 days after the last injection. The antisera were tested by double immunodiffusion and immunoelectrophoresis against different antigens and appeared to be monospecific. Anti-LDL anti-HDL, and anti-IgG antisera (produced in goats) were purchased from Behring Werke/Marburg, W. Germany. Antisera were stored frozen in small lots. Equivalence points were determined semiquantitatively with the device described by Piazzini (31).

Absorption of Apo A-I from *d* 1.21-g/ml plasma fractions of Tangier patients and normals was carried out with sufficient antibody to produce an antigen-antibody ratio at equivalence. The precipitates were allowed to stand 72 h at 4°C before the absorbed apoprotein was harvested by centrifugation. Precipitates were washed five times with 10 ml 0.9% NaCl.

Preparation of ¹²⁵I-labeled Apo A-I and Apo A-II. A apoproteins (0.2–0.5 mg) dissolved in 3 ml 0.1 M NH₄HCO₃ (pH 8), containing 0.01% EDTA, were separately treated with a ¹²⁵I-labeled acylating reagent, iodinated 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester (sp act 1,700 Ci/mmol; Amersham Buchler, Braunschweig, W. Germany) which reacts with free amino groups in the protein molecule to attach the ¹²⁵I-labeled groups by amide bonds (32).

The ¹²⁵I-labeled apoproteins were purified from the reaction products by Sephadex G-75 gel chromatography (Pharmacia Fine Chemicals, Inc.) and eluted, each in one major peak, after the void volume. The calculated sp act of the various preparations ranged between 0.5 and 1 mCi/mg protein.

The native and labeled apoprotein A-I gave identical immunoprecipitation arcs against rabbit anti-HDL and rabbit anti-Apo A-I antibodies. It did not react with antiserum to LDL, Apo A-II, or albumin. In the presence of excess antibody, 85–95% of [125 I]Apo A-I was precipitated in the radioimmunoassay; more than 95% of the radioactivity was precipitable by 10% TCA. Apo A-I competed with the [125 I]Apo A-I for limiting amounts of antibody, displacing 90–95% of the labeled antigen.

As evaluated by immunodiffusion and immunoelectrophoresis of both native and [125 I]Apo A-II against rabbit anti-HDL and anti-Apo A-II, the antigenicity of Apo A-II was not measurably influenced by the iodination procedure (sp act 0.3–0.75 mCi/mg protein). Native and labeled Apo A-II did not react with antiserum to LDL, Apo A-I, or albumin. The precipitability of the labeled Apo A-II and the degree of displacement by the unlabeled antigen were similar to those observed for Apo A-I. The labeled apoproteins were used immediately upon preparation.

Radioimmunoassay procedures. Assays were carried out in Eppendorf vials. All dilutions were made in a standard buffer containing 0.1% bovine serum albumin (Serva, Heidelberg) in phosphate-buffered saline (pH 7.2). A typical incubation mixture contained in a final volume of 500 μ l the following: 200 μ l of nonimmune rabbit serum (Behring Werke/Marburg; diluted 1:700), 100 μ l of diluted anti-Apo A-I (or anti-Apo A-II), 50 μ l [125 I]Apo A-I (or [125 I]Apo A-II) (20,000–30,000 cpm), and 10–150 μ l of diluted sample and sufficient buffer to bring the volume to 500 μ l.

For routine use, antisera were diluted to give precipitabilities of 80–85% in the absence of unlabeled A apoproteins. Control tubes which contained no anti-Apo A-I (or anti-Apo A-II) or label were also included to evaluate the non-specific precipitation and the absorption of label to the surface of the vial, respectively. In routine assays, all reactions were started at the same time. The vials were incubated at 4°C for 42–48 h, after which 200 μ l of goat anti-rabbit- γ -globulin (diluted 1:50) was added for another 18 h at 4°C (20).

The precipitates were isolated by centrifugation at 8,000

rpm for 20 min, washed three times with cold phosphate-buffered saline and counted in a Packard model 5230 gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). All experiments were performed in duplicate. The results are expressed as the percent of the total counts bound: % bound = (cpm of competed antibody-bound [125 I]Apo A-I – cpm in background)/(cpm of total antibody-bound [125 I]Apo A-I – cpm in background).

Other techniques. Electroimmunoassays to evaluate the concentration of HDL in plasma were performed according to Laurell's method (33). Double immunoelectrophoreses of plasma and isolated lipoprotein fractions were performed according to Clarke and Freemann (34). Analytical thin-layer electrofocusing was carried out on Ampholine PAG plates, pH 3.5–9.5 (LKB) using a LKB 2117 Multiphor chamber (LKB-Produkter, A.B., Bromma, Sweden) (35).

Silica gel thin-layer plates were developed in chloroform:methanol:H₂O (65:25:4) and exposed to 50% sulfuric acid to locate individual lipids and reference compounds. Protein determinations were carried out by the method of Lowry et al. (36) or by amino acid analysis.

RESULTS

Quantitation of A apoproteins by radioimmunoassay. The standard curve for the displacement of 2 ng of [125 I]Apo A-I by unlabeled apoprotein is shown in Fig. 2. A characteristic sigmoidal curve was obtained when the percent of [125 I]Apo A-I bound was plotted vs. apoprotein added. With four different antisera and three different apoprotein preparations, almost identical displacement curves were obtained. Maximum precision was in the range of 5–20 ng; the variability (SEM) of the displacement of [125 I]-labeled Apo A-I was $\pm 4.5\%$ for the different labeled Apo A-I and antibody preparations used.

The standard curve with purified Apo A-II showed

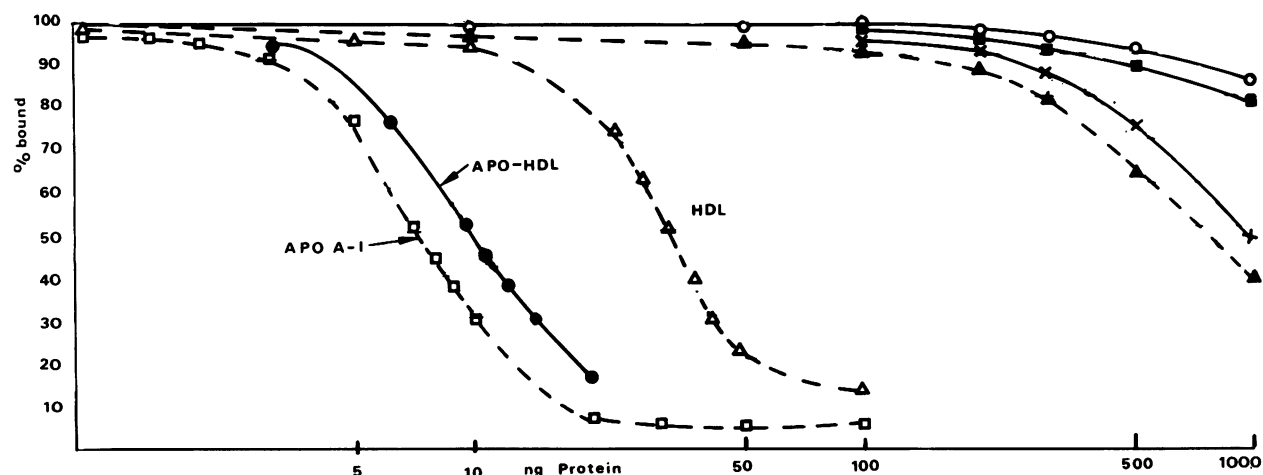


FIGURE 2 Standard curve for the radioimmunoassay of Apo A-I. The assay was performed as described in Methods. [125 I]Apo A-I was displaced from Apo A-I antiserum by Apo A-I, Apo HDL and HDL; Apo A-II, Apo C-III₁ (representative of Apo C), VLDL, and LDL displaced [125 I] counts only in amounts which were 1,000- to 2,000-fold greater than Apo A-I. □ — □, Apo A-I; ● — ●, Apo HDL; △ — △, HDL; × — ×, VLDL; ▲ — ▲, LDL; ■ — ■, Apo C-III₁; ○ — ○, Apo A-II.

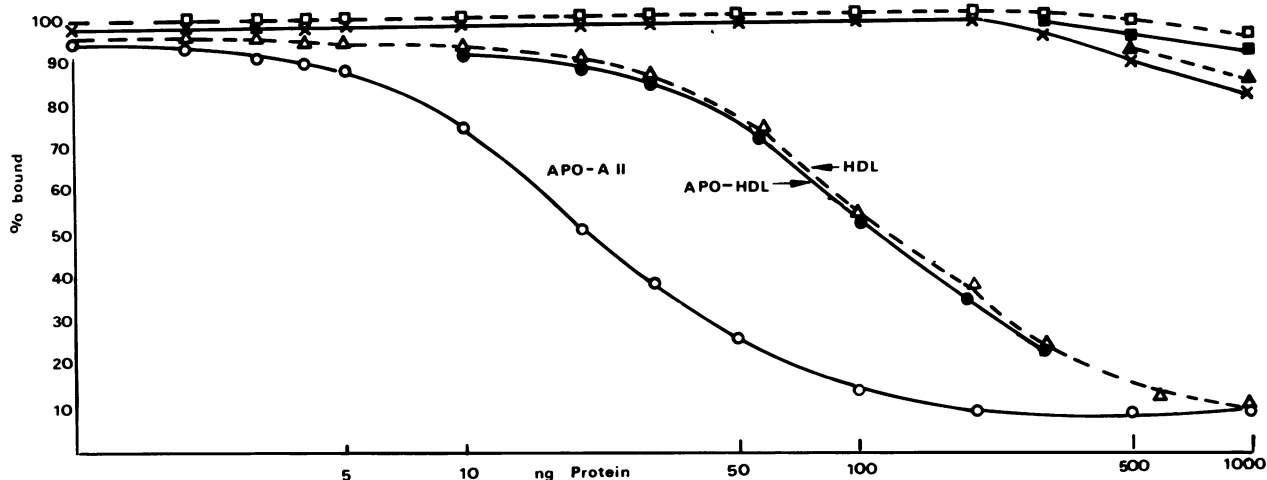


FIGURE 3 Displacement of [125 I]Apo A-II from Apo A-II antibodies. The assay was performed as described in Methods. (Symbols, see Fig. 2).

significant displacement at 5 ng (Fig. 3). The working range of this assay lay between 10 and 50 ng of Apo A-II. Maximal displacement (>90%) occurred with 200 ng protein.

HDL and delipidated HDL (Apo HDL) produced displacement curves which parallel those produced by Apo A-I and Apo A-II when analyzed by logit-log transformation. Assuming a molar Apo A-I: Apo A-II ratio of 3:1 in normal HDL (corresponding to a weight ratio of 4:1), 100 ng Apo HDL should contain 72 ng Apo A-I and 18 ng Apo A-II, together accounting for 90% of HDL protein (37). While the radioimmunoassay detected approximately 95% of Apo A-II in HDL, only approximately 30% of the calculated Apo A-I could be identified in fresh plasma or HDL preparations. An explanation of the greater effectiveness of Apo HDL and Apo A-I than HDL in displacing [125 I]Apo A-I has previously been thought to be in the partial coverage of antigenic determinants by protein-protein or protein-lipid interaction in the native HDL macromolecule (20, 22). By contrast, all of the Apo A-II was detected by the radioimmunoassay independent of delipidation (37). HDL and Apo HDL showed identical effectiveness in displacing labeled Apo A-II.

The plasma protein fraction of $d > 1.21$ g/ml was effective in displacing [125 I]Apo A-I and [125 I]Apo A-II. In contrast to Apo A-II, the Apo A-I reactivity of this fraction was significantly influenced by prior ether-ethanol or chloroform-methanol extraction. In several preparations, a 1.8- to 2.5-fold greater amount of Apo A-I could be detected after delipidation.

Specificity of radioimmunoassay. To determine the specificity of displacement, a variety of human plasma lipoproteins and apolipoproteins have been monitored by radioimmunoassay. VLDL, LDL, Apo

C-I, Apo C-II, Apo C-III₁, Apo C-III₂, and albumin did not displace 125 I-labeled Apo A-I or Apo A-II when added in amounts up to 200 ng protein (Figs. 2 and 3). At higher protein concentrations, curves parallel to the A apoproteins were obtained with both purified human VLDL and LDL. In various VLDL and LDL preparations isolated from normolipidemic plasma, the amount of A apoproteins accounted for less than 2% of the total protein, respectively.

A apoprotein levels and distribution in Tangier and normal plasma. To compare the A apoprotein content in Tangier and normal plasma, radioimmunoassay determinations were performed on native and delipidated samples. As shown in Table I, the absolute amounts of Apo A-I and Apo A-II in Tangier plasma ranged between 0.4–0.94 mg/dl and 1.9–2.8 mg/dl, respectively. Repeated sampling at monthly intervals over a period of 6 mo produced almost identical results. The apparent content of Apo A-I in Tangier plasma was increased about 1.3-fold by delipidation,

TABLE I
Quantitation of A Apoproteins in Tangier and Normal Plasma

Patient	Apoprotein A-I		Apoprotein A-II	
	Native plasma	Delipidated plasma	Native plasma	Delipidated plasma
	mg/dl	mg/dl	mg/dl	mg/dl
Control male (n = 15)	34.1±5.0	113±6.1	34±3.1	35±3.8
Control female (n = 15)	38.3±5.5	124±6.8	39±3.5	41±4.6
J. Si. (Tangier)	0.31	0.40	2.1	1.9
A. Bo. (Tangier)	0.45	0.61	1.9	1.8
E. Ga. (Tangier)	0.71	0.94	2.7	2.8

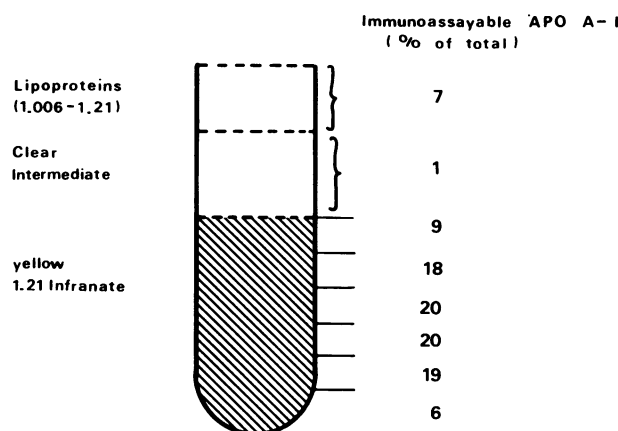


FIGURE 4 Distribution of Apo A-I in Tangier plasma after 48 h ultracentrifugation (60,000 rpm). Apo A-I has been quantitated by radioimmunoassay after delipidation of individual ultracentrifugal fractions.

whereas the increase in apparent content of Apo A-I in normal plasma was 3.3-fold. Since identical procedures for delipidation of Tangier and normal plasma have been employed, the observed difference reflects a greater accessibility of the Apo A-I antigenic sites to the antibody in Tangier plasma. Independent measurements by electroimmunoassay (Fig. 1) and double immunoelectrophoresis (Fig. 6) confirmed that the total plasma quantity of Apo A-I in all Tangier patients was less than 1% of normals.

In contrast to Apo A-I, the absolute values for Apo A-II in Tangier plasma were largely independent of the delipidation procedure. In the same individuals, absolute Apo A-II concentrations were 3–4.8 times higher than Apo A-I concentrations, approaching a total Apo A-II quantity of 5–7% of that observed in normals.

The distribution of Apo A polypeptides among lipoproteins was determined in unwashed fractions to minimize a possible artifact produced by repeated

ultracentrifugation. As shown in Table II, less than 1% of the Apo A-I quantity in Tangier patients can be determined in the HDL density area; 90% of the Apo A-I, however, occurs in the 1.21-g/ml bottom fraction. This distribution pattern was independent of the isolation procedure. Both sequential ultracentrifugations to prepare individual lipoprotein classes and single ultracentrifugation at $d = 1.21$ g/ml resulted in the preferential accumulation of Apo A-I in the 1.21- d bottom fraction. As demonstrated by 48-h ultracentrifugation of whole Tangier plasma at $d = 1.21$ g/ml, the Apo A-I was rather uniformly distributed throughout the yellow infranate fraction ($d > 1.25$ g/ml) (Fig. 4). The 1.21 g/ml supernate and the clear intermediate zone of the ultracentrifuge tube contained 7% and 1% of the total immunoassayable Apo A-I, respectively.

In further contrast to Apo A-I, 49–55% of the total Tangier Apo A-II in plasma could be identified in the HDL density region (Tables I and II). Whether the Apo A-II in the VLDL and LDL density areas of Tangier plasma occurs as an integral constituent of these lipoproteins or as a constituent of an abnormal lipoprotein has not yet been determined. The complete dissociation of Apo A-I and Apo A-II in Tangier plasma contrasts to the preferential accumulation of both apoproteins in the 1.063–1.21-g/ml lipoprotein fraction of normals (Table II).

Other immunological experiments. To further evaluate the lipoprotein abnormality in Tangier disease, fresh plasma has been subjected to double immunoelectrophoresis utilizing anti-LDL, anti-HDL, anti-Apo A-I, and anti-Apo A-II antisera (Figs. 5 and 6). Normal and Tangier plasma produced “rocket”-shaped precipitates in regular electrophoretic position, but to a different extent when tested against anti-LDL and anti-HDL (Fig. 5). Despite a twofold increase of antigen employed for analysis of Tangier lipoproteins, only a weak anti-HDL reactivity for α -migrating lipo-

TABLE II
Distribution of A Apoproteins in Tangier Plasma (Percent of Total Plasma A Apoproteins)

Patient	VLDL ($d < 1.006$)		LDL ($d = 1.006-1.063$)		HDL ($d = 1.063-1.21$)		1.21 Infranate	
	Apo A-I	Apo A-II	Apo A-I	Apo A-II	Apo A-I	Apo A-II	Apo A-I	Apo A-II
	%							
Control ($n = 15$, male)	0.5±0.08	2±0.04	1.5±0.03	1.0±0.02	87±2.4	89±3.1	11.0±1.2	8.0±0.9
J. Si. (Tangier)	5	18.5	2	24	<1	55	92	2.5
A. Bo. (Tangier)	6	26	4	24	<1	49	90	1
E. Ga. (Tangier)	8	27	3	19	<1	51	88	3

Quantitation of A apoproteins was carried out by radioimmunoassay on delipidated lipoprotein fractions (see Methods). The sum of A apoproteins of individual fractions accounted for >90% of A apoproteins contained in native serum.

proteins was observed. The anti-HDL reactivity in double immunoelectrophoresis was further tested employing monospecific Apo A-I and Apo A-II antibodies. Tangier Apo A-I yielded a major single arc which migrated more slowly anodally than regular Apo A-I as a constituent of HDL (Fig. 6). Despite a twofold increase of antigen employed in the electrophoretic separation (one dimension) of Tangier lipoproteins, only weak Apo A-I precipitin arcs were obtained confirming the relative deficiency of this apoprotein in Tangier disease. In similar experiments, the 1.21-g/ml infranant fraction derived from Tangier plasma gave rise to a single arc which migrated at about the same rate as Tangier Apo A-I from fresh plasma. These findings suggest that the majority of Apo A-I in Tangier plasma does not occur as an integral protein constituent of HDL, but behaves like a protein with abnormal electrophoretic migration and ultracentrifugal density properties. A slower migration of Apo A-I has been previously observed for HDL subjected to a variety of disruptive treatments (30). In the case of Tangier plasma, however, the abnormal migration of Apo A-I was independent of such manipulations and occurred in freshly drawn plasma subjected to electrophoresis. Due to the low Apo A concentrations in Tangier plasma, one-dimensional electroimmunoassays (Fig. 1) and double immunoelectrophoresis (Figs. 5 and 6) did not allow accurate estimation of the apoprotein concentration.

In contrast to Tangier Apo A-I, Tangier Apo A-II and normal A apoproteins migrated to the regular α -position when native plasma was taken as the antigen source (Fig. 6). When, instead of plasma, the isolated $d = 1.063 - 1.21$ g/ml ultracentrifugal fraction of Tangier plasma was monitored for the presence of A apoproteins by immunoelectrophoresis, Apo A-II and Apo HDL reactivities were observed in regular α -electrophoretic position (Fig. 7). Neither one-dimensional electroimmunoassays nor double immunoelectrophoresis revealed the presence of Apo A-I in this ultracentrifugal fraction. The absence of Apo A-I and presence of Apo A-II in the 1.063 - 1.21-g/ml density fraction was further confirmed by sodium dodecyl sulfate polyacrylamide electrophoresis. These data are in accord with the distribution pattern of A apoproteins as determined by radioimmunoassay.

Additional experiments were performed to determine whether the absolute decrease in the amount of immunochemically recognizable Apo A-I in Tangier disease was due to structural alteration of this apoprotein. Antigen-antibody precipitates of the Tangier 1.21-g/ml infranant fraction were subjected to urea and sodium dodecyl sulfate polyacrylamide electrophoreses (Fig. 8), as well as analytical isoelectric focusing in combination with immunoelectrophoresis (Fig. 9). In these experiments, Tangier Apo A-I

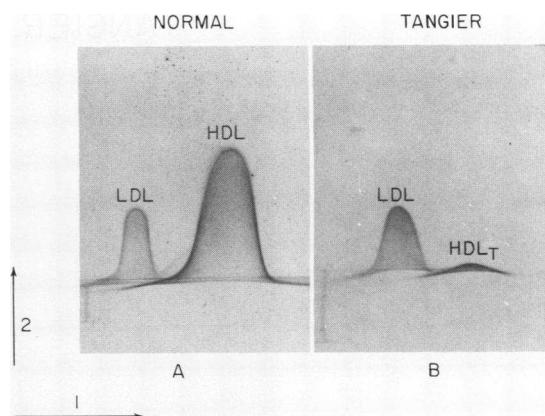


FIGURE 5 Double immunoelectrophoresis of normal (A) and Tangier plasma (B) (patient E. Ga.) (1) dimension: 150 μ l plasma has been mixed with 150 μ l 1% agarose in 0.1 M barbital buffer, pH 8.6 and 10 μ l bromphenol blue on a glass cover slip; 20 \times 1-mm strips were transferred to a microscope slide covered with 1 ml of 0.5% agarose, after which 2 ml of 0.5% agarose in 0.05 M barbital buffer was overlaid; buffer chambers contained 0.05 M barbital buffer, pH 8.6; field strength 20 V/cm⁻¹; running time 120 min; system cooled with running tap water. At the end of the electrophoresis 50 \times 5-mm thick strips were taken for II electrophoresis. (2) dimension: 5 \times 7.5-cm glass plates were layered with 1 ml of 0.5% agarose, dried and the strip (1. dimension) positioned 2.5 cm from the cathode; subsequently 1.5 ml of 0.5% agarose has been layered not covering the strip; 2.5 ml of 0.5% agarose containing 100–200 μ l of antiserum (LDL, HDL) was layered over the entire plate; field strength 6.6 V/cm⁻¹; running time 14–18 h; staining solution: 0.1% Coomassie blue. HDL_T: lipoprotein fraction of plasma from Tangier patients having HDL antigenicity.

exhibited identical properties to its normal counterpart. Thus, gross structural alterations affecting the size, the charge, or the immunoreactivity of Tangier Apo A-I, as isolated by the Anti-Apo A-I antiserum, could be excluded within the limitations of the methods used. It cannot be excluded that there may be present in Tangier plasma immunologically altered populations of Apo A-I which are not recognized by the antiserum prepared against normal Apo A-I. Detailed examination of the amino acid composition and tryptic peptide patterns of this apoprotein is still needed.

Delipidation of the Apo A-I-antibody complex, obtained from 500 ml of Tangier plasma, revealed that trace amounts of lipid were associated with Apo A-I (Fig. 10). Phosphatidyl ethanolamine and phosphatidyl choline appeared to be the major phospholipid constituents, cholesterol being the major neutral lipid component. The relative increase in phosphatidyl ethanolamine, as compared with control preparations, is as yet unexplained.

The presence of Apo A-II in the 1.063–1.21-g/ml ultracentrifugal fraction, as determined by radioimmunoassay and double immunoelectrophoresis

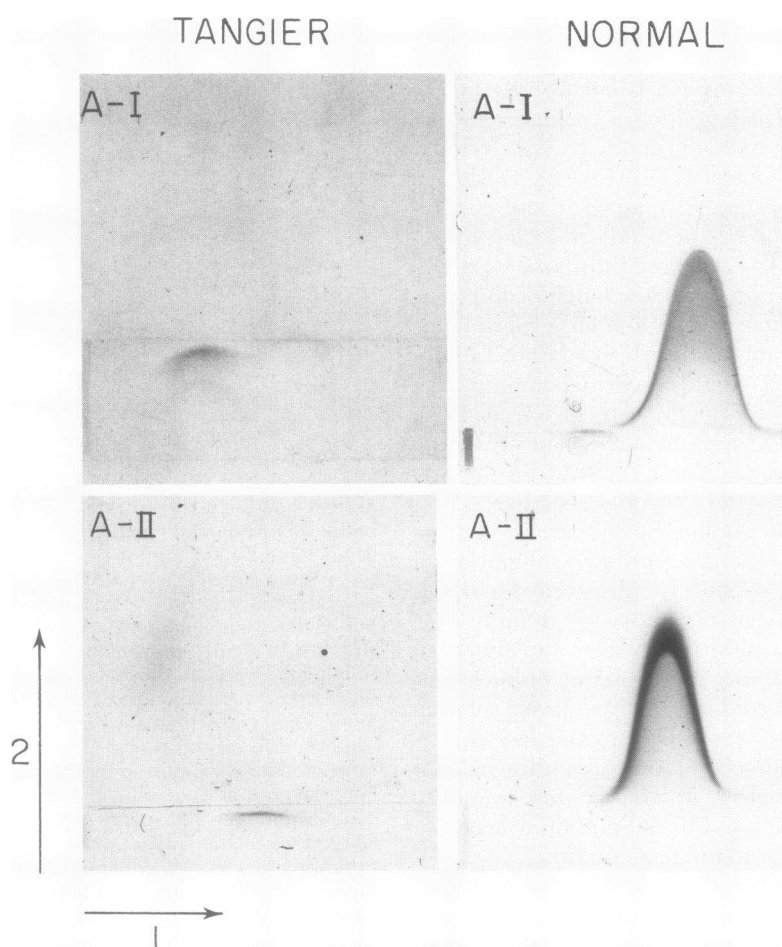


FIGURE 6 Double immunoelectrophoresis of Tangier and normal plasma. Details of electrophoretic separation are identical to those described in Legend to Fig. 5. Instead of LDL and HDL antisera, Apo A-I antiserum (upper) and Apo A-II antiserum (lower) have been employed for immunoprecipitation of A apoproteins contained in Tangier (left) and normal serum (right). Despite a twofold increase of Tangier serum employed, only weak Apo A precipitin arcs (arrows) were observed in pre- β -position (Apo A-I, upper left) and α -position (Apo A-II, lower left).

(α -migration), apparently accounts for residual α -lipoproteins previously observed in Tangier disease (13). A detailed morphological and physicochemical analysis of this lipoprotein fraction is given elsewhere.⁴

DISCUSSION

In the present study, we have further examined the apoprotein abnormality associated with the absence of normal HDL in Tangier disease. The data provide unequivocal evidence that the total amount of immunochemically recognizable A apoproteins in

Tangier plasma is significantly reduced. The total amount of Apo A-I is decreased to 1/100–1/200 of normal, and more than 90% of this residual apoprotein is associated with the plasma protein fraction of $d > 1.21$ g/ml. By contrast, the majority of the Tangier Apo A-II is confined to the d 1.063–1.21-g/ml KBr fraction. In three patients affected with Tangier disease, the concentration of this apoprotein in the HDL density area ranged from 5 to 10% of that in normals. Physicochemical and compositional studies have revealed that Apo A-II, isolated in the HDL density area from Tangier plasma, is the sole protein constituent of an abnormal lipoprotein representing Tangier α -lipoproteins (38).⁴ As shown in the present study, significant amounts of Apo A-II in Tangier plasma are also detectable in the lipoproteins of

⁴ Assmann, G., P. N. Herbert, T. Forte, and D. S. Fredrickson. An A-II lipoprotein particle in Tangier disease. Manuscript in preparation.

$d < 1.063$ g/ml. In normal plasma, small amounts of A apoproteins, the origin of which remains to be identified, can also be detected in these lipoproteins. The absolute amount of Apo A-II in Tangier VLDL and LDL was similar to that in normals; Tangier VLDL and LDL contained more Apo A-II than Apo A-I.

The results obtained for the total quantity of Apo A-I in normal plasma are in close agreement with those reported by others (20, 22, 39–41). However, the results obtained for the total quantity of Apo A-II in normals differ from data reported by Curry et al (39). These authors have determined, by an electroimmunoassay technique, a weight ratio of Apo A-I to Apo A-II in HDL of 1.7 corresponding to a molar ratio of these polypeptides that is close to unity. The difference in weight ratio of the major polypeptides in HDL, as assessed by electroimmunoassay and radioimmunoassay, is as yet unexplained. The relative quantity of A apoproteins determined by radioimmunoassay, however, equals results deduced from column chromatographic eluates (23, 24).

We have further substantiated that Apo A-I and Apo A-II antigenic sites are exposed to a different degree in HDL. Only approximately 30% of the calculated amount of Apo A-I could be detected in native plasma and HDL. In contrast, the total amount of immunoassayable Apo A-II could not be substantially increased by the delipidation procedure. Since in dyslipoproteinemia the degree of exposure of A antigenic sites may be variable, accurate determinations

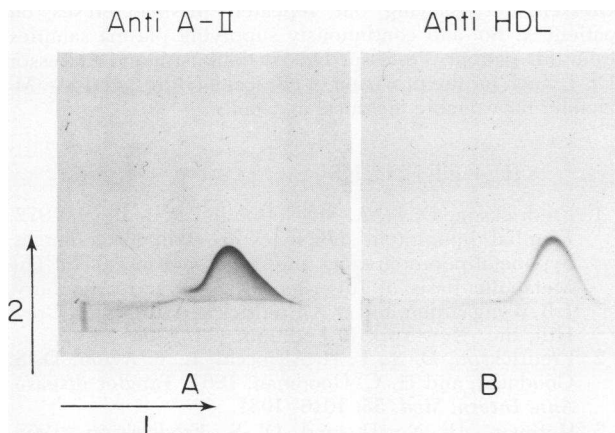


FIGURE 7 Double immunoelectrophoresis of the isolated $d = 1.063 - 1.21$ -g/ml fraction derived from Tangier serum (patient J. Si.). Details of electrophoretic separation are identical to those described in Fig. 5. Apo A-II antiserum (A, left) and Apo HDL antiserum (B, right) were used for immunoprecipitation of A apoproteins contained in the $d = 1.063 - 1.21$ -g/ml ultracentrifugal fraction of Tangier serum. In contrast to the Apo A-II and Apo HDL reactivity of these α -migrating lipoproteins, no Apo A-I reactivity could be observed.

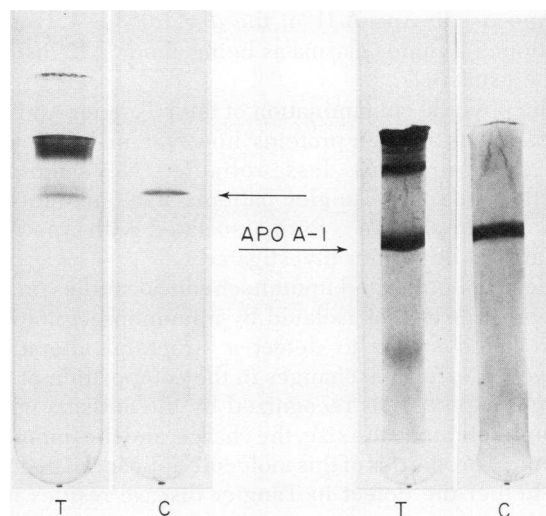


FIGURE 8 Polyacrylamide gel electrophoresis. Urea (left) and sodium dodecylsulfate (right) polyacrylamide gel electrophoresis of the Apo A-I immunoprecipitate (1.21 infranate) derived from Tangier patient J. Si. (T = Tangier) as compared to pure, isolated Apo A-I (C = Control). The identity of the protein band visible in the lower half of the sodium dodecyl sulfate gel (Tangier Apo A-I immunoprecipitate) could not be established and may be due to proteolytic activity contained in the antigen or antibody source.

cannot be achieved without prior delipidation of samples.

In immunoelectrophoresis, the majority of Apo A-I in Tangier plasma is confined to an abnormal position (pre- β). By contrast, the majority of Apo A-II in Tangier plasma electrophoretically migrates in regular α -position and is confined to the 1.063 – 1.21-g/ml density region upon ultracentrifugation. It cannot be excluded that the preferential segregation of Apo A-I to the 1.21-g/ml infranant fraction is partly due to an artifact induced by ultracentrifugation and (or) high salt concentration. However, the observation that Apo A-I and Apo A-II from fresh Tangier plasma electrophoretically migrate to different positions eliminates the possibility that the dissociation of the major A apoproteins is only produced artifactually. Instead, it appears that these apoproteins in Tangier disease are not integral constituents of HDL. Our data suggest that normal HDL macromolecules are absent from Tangier plasma.

Trace amounts of Tangier HDL of uncertain identification have been described in the original reports of this disease (13). In these earlier studies, antisera to HDL have been employed which cross-react with both Apo A-I and Apo A-II. Thus, the nature of the apoprotein composition of Tangier HDL remained unidentified in those studies.

On the basis of Sephadex G-200 chromatography experiments, Lux et al. (13) have estimated the ratio

of Apo A-I to Apo A-II in the $d = 1.063 - 1.21$ -g/ml fraction of Tangier plasma as being about 1:12 instead of the usual 3:1.

The possible contamination of their Tangier Apo A-I preparations by other proteins, however, makes chemical determinations less reliable. Nevertheless, whether in some Tangier patients trace amounts of Apo A-I circulate in a form associated with Apo A-II remains to be further investigated.

Electrophoretic and immunochemical studies on the Tangier Apo A-I, as isolated by immunoprecipitation, have failed so far to detect a structural alteration. However, extensive changes in the composition of the Tangier Apo A-I, as recognized by the antisera used, are unlikely since the size, the charge, and the immunochemical properties of this molecule appear unaffected.

Whether the defect in Tangier disease resides in a structural apoprotein abnormality or in a regulatory gene mutation affecting the synthesis of A apoproteins requires further investigation. The specific sites of protein-protein interaction in normal HDL have not yet been defined. A single amino acid replacement in Apo A-I or Apo A-II, at a position important for such specific interaction, conceivably may destroy the quaternary structure of HDL. Elucidation of the nature of protein-protein and protein-lipid interactions in normal HDL will promote the understanding of the origin of the dissociation of the A apoproteins in Tangier plasma.

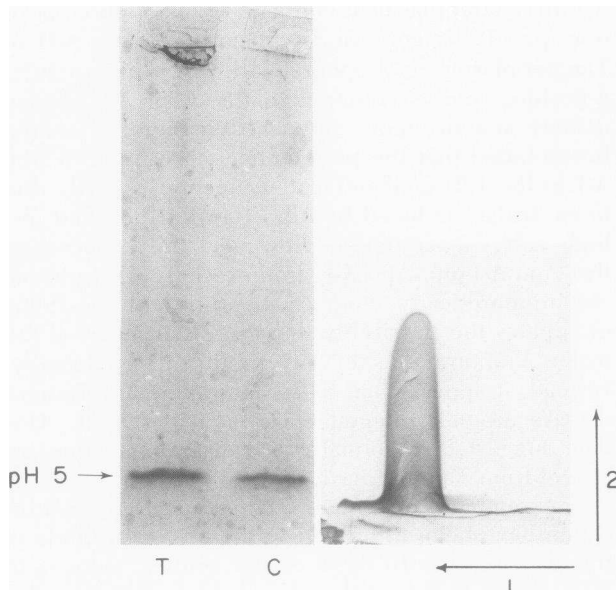


FIGURE 9 (Left) Isoelectric focusing of the Apo A-I immunoprecipitate (1.21 infranate) derived from Tangier patient J. Si. (T) as compared to the control immunoprecipitate (C). (Right) Isoelectric focusing of the Tangier Apo A-I immunoprecipitate in combination with subsequent electroimmunoassay (2. dimension), see also details of Methods, Fig. 5.

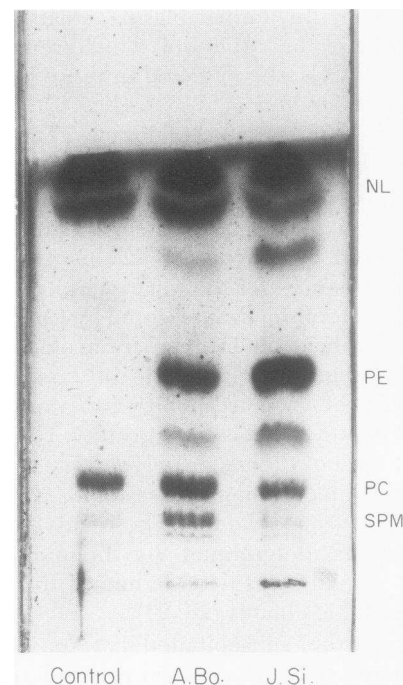


FIGURE 10 Thin-layer chromatography of Tangier (A. Bo and J. Si.) Apo A-I-associated lipids (derived from 1.21-g/ml infranate) vs. control. Solvent system-chloroform:methanol: H_2O (65:25:4). (NL, neutral lipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine, SPM, sphingomyelin).

ACKNOWLEDGMENTS

We wish to thank Dr. H. Heckers, Med. Klinik, Universität Giessen, for carrying out repeated plasmaphereses on patient A. Bo. and continuously supplying plasma samples from this patient; Professor Dr. W. Kaufmann and Professor Dr. J. Zach for the provision of clinical facilities; and Ms. M. Nyfeler for valuable technical assistance.

REFERENCES

1. Fredrickson, D. S., A. M. Gotto, and R. I. Levy. 1972. Familial lipoprotein deficiency (abetalipoproteinemia, hypobetalipoproteinemia and Tangier disease). In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, Inc., New York. 3rd edition. 493-530.
2. Fredrickson, D. S., P. H. Altrocchi, L. V. Avioli, D. S. Goodman, and H. C. Goodman. 1961. Tangier disease. *Ann. Intern. Med.* **55**: 1016-1031.
3. Hoffman, H. N., II, and D. S. Fredrickson. 1965. Tangier disease (familial high density lipoprotein deficiency). Clinical and genetic features in two adults. *Am. J. Med.* **39**: 582-593.
4. Fredrickson, D. S. 1966. Familial high-density lipoprotein deficiency: Tangier disease. In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill, Inc., New York. 2nd edition. 486-508.
5. Engel, W. K., J. D. Dorman, R. I. Levy, and D. S. Fredrickson. 1967. Neuropathy in Tangier disease. α -Lipoprotein deficiency manifesting as familial recurrent

- neuropathy and intestinal lipid storage. *Arch. Neurol.* **17**: 1-9.
6. Kocen, R. S., J. K. Lloyd, P. T. Lascelles, A. S. Fosbrooke, and D. Williams. 1967. Familial α -lipoprotein deficiency (Tangier disease) with neurological abnormalities. *Lancet*. **1**: 1341-1345.
 7. Kummer, H., J. Laissue, H. Spiess, R. Pflugshaupt, and U. Bucher. 1968. Familiäre Analphalipoproteinämie (Tangier-Krankheit) *Schweiz. Med. Wochenschr.* **98**: 406-412.
 8. Haas, L. F., and J. D. Bergin. 1970. Alpha lipoprotein deficiency with neurological features. *Australas. Ann. Med.* **19**: 76. (Abstr.)
 9. Huth, K., J. Kracht, W. Schoenborn, and W. Fuhrmann. 1970. Tangier-Krankheit (Hyp- α -Lipoproteinämie). *Dtsch. Med. Wochenschr.* **95**: 2357-2361.
 10. Bale, P. M., P. Clifton-Bligh, B. N. P. Benjamin, and H. M. Whyte. 1971. Pathology of Tangier disease. *J. Clin. Pathol. (Lond.)*. **24**: 609-616.
 11. Greten, H., T. Hannemann, W. Gusek, and O. Vivell. 1974. Lipoproteins and lipolytic plasma enzymes in a case of Tangier disease. *N. Engl. J. Med.* **291**: 548-552.
 12. Ferrans, V. J., and D. S. Fredrickson. 1975. The pathology of Tangier disease. A light and electron microscopic study. *Am. J. Pathol.* **78**: 101-158.
 13. Lux, S. E., R. I. Levy, A. M. Gotto, and D. S. Fredrickson. 1972. Studies on the protein defect in Tangier disease. Isolation and characterization of an abnormal high density lipoprotein. *J. Clin. Invest.* **51**: 2505-2519.
 14. Fredrickson, D. S., S. E. Lux, and P. N. Herbert. 1972. The apolipoproteins. *Adv. Exp. Med. Biol.* **26**: 25-56.
 15. Alaupovic, P., D. M. Lee, and W. J. McConathy. 1972. Studies on the composition and structure of plasma lipoproteins. Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. *Biochim. Biophys. Acta.* **260**: 689-707.
 16. Scanu, A. M., and C. Wisdom. 1972. Serum lipoproteins structure and function. *Annu. Rev. Biochem.* **41**: 703-730.
 17. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46**: 1493-1498.
 18. Soutar, A., C. W. Garner, H. N. Baker, J. T. Sparrow, R. L. Jackson, A. M. Gotto, and L. C. Smith. 1975. Effects of the human plasma apolipoproteins and phosphatidylcholine acyl donor on the activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **14**: 3057-3064.
 19. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345-1354.
 20. Schonfeld, G., and B. Fflegler. 1974. The structure of human high density lipoprotein and the levels of apolipoprotein A-I in plasma as determined by radioimmunoassay. *J. Clin. Invest.* **54**: 236-246.
 21. Gotto, A. M., and H. Kon. 1970. Observations on the conformation of human serum high density lipoproteins using infrared spectroscopy, circular dichroism, and electron spin resonance. *Biochemistry.* **9**: 4276-4283.
 22. Karlin, J. B., D. J. Juhn, J. I. Starr, A. M. Scanu, and A. H. Rubenstein. 1976. Measurement of human high density lipoprotein apolipoprotein A-I in serum by radioimmunoassay. *J. Lipid Res.* **17**: 30-37.
 23. Lux, S. E., K. M. John, and H. B. Brewer, Jr. 1972. Isolation and characterization of apo LP-Gln-II (Apo A-II), a plasma high density apolipoprotein containing two identical polypeptide chains. *J. Biol. Chem.* **247**: 7510-7518.
 24. Scanu, A., J. Toth, C. Edelstein, S. Koga, and E. Stiller. 1969. Fractionation of human serum high density lipoprotein in urea solutions. Evidence for polypeptide heterogeneity. *Biochemistry.* **8**: 3309-3316.
 25. Shore, B., and V. Shore. 1969. Isolation and characterization of polypeptides of human serum lipoproteins. *Biochemistry.* **8**: 4510-4516.
 26. Marier, J. R., and D. Rose. 1964. Determination of cyanate, and a study of its accumulation in aqueous solutions of urea. *Anal. Biochem.* **7**: 304-314.
 27. Davis, B. J. 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
 28. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412.
 29. Herbert, P. N., R. S. Shulman, R. I. Levy, and D. S. Fredrickson. 1973. Fractionation of C-apoproteins from human plasma very low density lipoproteins. Artfactual polymorphism from carbamylation in urea-containing solutions. *J. Biol. Chem.* **248**: 4941-4946.
 30. Levy, R. I., and D. S. Fredrickson. 1965. Heterogeneity of plasma high density lipoproteins. *J. Clin. Invest.* **44**: 426-441.
 31. Piazza, S. E. 1969. A simple method for preliminary immunodiffusion test of antigen-antibody systems having unknown ratios of reaction. *Anal. Biochem.* **27**: 281-284.
 32. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. Application to the radioimmunoassay. *Biochem. J.* **133**: 529-539.
 33. Laurell, C. B. 1972. Electroimmunoassay. *Scand. J. Clin. Lab. Invest.* **29** (Suppl. 124): 21-37.
 34. Minchin Clarke, H. G., and T. Freemann. 1968. Quantitative immunoelectrophoresis of human serum proteins. *Clin. Sci. (Oxf.)*. **35**: 403-413.
 35. Bours, J. 1971. Isoelectric focusing on lens crystallins in thin-layer polyacrylamide gels. A method for detection of soluble proteins in eye lens extracts. *J. Chromatogr.* **60**: 225-233.
 36. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
 37. Mao, S. J. T., A. M. Gotto, Jr., and R. L. Jackson. 1975. Immunochemistry of human plasma high density lipoproteins. Radioimmunoassay of apolipoprotein A-II. *Biochemistry.* **14**: 4127-4131.
 38. Assmann, G., D. S. Fredrickson, P. N. Herbert, T. Forte, and R. Heinen. 1974. An A-II lipoprotein particle in Tangier disease. *Circulation.* **49** and **50** (Suppl. III): 1000. (Abstr.)
 39. Curry, M. D., P. Alaupovic, and C. A. Suenram. 1976. Determination of apolipoprotein A and its constitutive A-I and A-II polypeptides by separate electroimmunoassays. *Clin. Chem.* **22**: 315-322.
 40. Fainaru, M., M. C. Glangeaud, and S. Eisenberg. 1975. Radioimmunoassay of human high density lipoprotein apoprotein A-I. *Biochim. Biophys. Acta.* **386**: 432-443.
 41. Albers, J. J., P. W. Wahl, V. G. Cabana, W. R. Hazzard, and J. J. Hoover. 1976. Quantitation of apolipoprotein A-I of human plasma high density lipoprotein. *Metab. Clin. Exp.* **25**: 633-644.