Immunological evidence for the presence of B protein (apoprotein of β-lipoprotein) in normal and abetalipoproteinemic plasma

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ABSTRACT The antigenicity of β-lipoprotein that had been chemically altered by acetylation or arsanilation was compared with that of native β-lipoprotein, of lymph chylomicrons, of plasma proteins with d > 1.21, and of plasma from patients with abetalipoproteinemia. Chemical alteration causes structural changes in β-lipoprotein which render it immunologically identical with a protein that is present both in normal plasma and in plasma from patients with abetalipoproteinemia. This protein has been identified by immunoelectrophoresis as a β-globulin which does not stain for lipid. It is presumed to be the lipid-free apoprotein of β-lipoprotein (B protein).

The findings suggest that abetalipoproteinemia is not due to inability to synthesize B protein, but might instead be due to a defect in the formation of the complete β-lipoprotein macromolecule.

KEY WORDS β-lipoprotein · azo-β-lipoprotein · acetyl-β-lipoprotein · antisera · B protein · plasma 1.21 infranate · abetalipoproteinemia · lipid transport · man

IT IS GENERALLY ACCEPTED that the absence of β-lipoprotein in abetalipoproteinemia is due to the patient's inability to synthesize its protein moiety, the B protein (1–3). This conclusion is based on the findings that antisera to β-lipoprotein do not react with the plasma of patients with that disease and that antiserum against the plasma of such a patient does not precipitate β-lipoproteins (2). Since it has been assumed that the B protein is immunologically identical with β-lipoprotein, at least partially, the inability to find material reactive with anti-β-lipoprotein antisera has been equated with an absence of B protein and therefore with the failure to synthesize it.

The studies reported here were undertaken to test whether B protein is present in the plasma of patients with abetalipoproteinemia. The structure of normal β-lipoprotein was altered by chemical means, and antisera to these new antigens were prepared. Then the presence in plasma of a lipid-free protein immunologically similar to that of the altered β-lipoproteins was sought. The results reported here provide evidence not only for the synthesis of B protein in abetalipoproteinemia but also for its presence, free of lipid, in normal plasma.

Abbreviations: B protein, the protein moiety (apoprotein) of β-lipoprotein; EDTA, ethylenediamine tetraacetic acid; EDTA–saline, 0.9% sodium chloride solution containing 0.001 M EDTA and adjusted to pH 9 with ammonium hydroxide.

METHODS

Normal plasma was obtained from blood donors at the New York Blood Center through the courtesy of Dr. Robert L. Hirsch. Lymph chylomicrons were obtained by thoracic duct cannulation of a patient with carcinoma of the cervix uteri, through the cooperation of Dr. George L. Irvin, National Cancer Institute, Bethesda, Maryland. This patient had no evidence of disease of carbohydrate or lipid metabolism. She was fed two test meals of 100 g of corn oil emulsified in skim milk with a 3 hr interval between feedings. Lymph was collected into a solution of 5% disodium EDTA (0.02 ml/ml of lymph) at room temperature from 1 hr after the first meal until 6 hr after the second meal. The cells were removed by low-speed centrifugation.

Plasmas from five patients with abetalipoproteinemia were studied. As noted in Table 1, four of the five have been cited repeatedly in publications by several authors. The fifth patient, P.B., was referred to The Rockefeller

University by Dr. William Diamond, Wantagh, New York, with the diagnosis of acanthocytosis; he is, to my knowledge, the youngest patient in whom the diagnosis of abetalipoproteinemia has been made. The clinical and chemical data for this patient are summarized in Table 2.

Blood samples were drawn after a 12–14 hr fast into 5% disodium EDTA solution (0.02 ml/ml of blood). Plasma was separated by low-speed centrifugation at 4°C and stored at that temperature until used. Most analyses or fractionations were begun within 3 days of blood collection. A few samples from subjects with abetalipoproteinemia were stored frozen at −20°C and several months later were thawed for immunological study.

Chylomicron Isolation

Lymph was centrifuged for 10^5 *g*-min in an International RC-2 refrigerated centrifuge. The layer of packed chylomicrons was removed from the top of the tube and resuspended in EDTA–saline by repeated passage through a fine needle. In this manner the chylomicrons were then washed six times by ultracentrifugation for 10^5 *g*-min into overlying EDTA–saline. The final preparation was a creamy white emulsion which was stable for months at 4°C.

Lipoprotein Isolation

β-Lipoprotein was isolated by ultracentrifugal flotation between density 1.019 and 1.063 as described by Havel, Eder, and Bragdon (4). Each preparation was washed once by recentrifugation at density 1.063. β-Lipoprotein prepared in this way was free from contamination by other proteins as judged by paper electrophoresis, and showed a single peak on analytical ultracentrifugation (Fig. 1). However, immunoelectrophoresis performed with the antisera listed in Table 3 demonstrated that one of two such preparations contained a faint trace of α-lipoprotein, estimated at $\ll 1\%$. The second preparation contained no contamination detectable by immunoelectrophoresis or by double diffusion in agarose gel, and was therefore used for preparation of the chemically altered lipoproteins. For immunoprecipitation experiments it was used at concentrations of 5 and 40 mg/ml.

α-Lipoprotein was isolated in a similar manner by ultracentrifugal flotation between density 1.063 and 1.15. The latter density was selected rather than 1.21 because it provided consistently better separation of α-lipoprotein from albumin. The isolated α-lipoprotein was free from contamination as judged by immunoelectrophoresis and double diffusion in agarose gel.

The term "1.21 infranate" is used to designate the material remaining after sequential removal of the lipoproteins of density < 1.019, 1.019–1.063, and 1.063–1.21 (4), or the material prepared as follows. The density of plasma was adjusted to 1.21 by the addition of solid KBr and the plasma was ultracentrifuged in the Spinco Model L preparative ultracentrifuge for 48 hr at 114,000 *g*. The 1.21 infranate isolated by tube slicing was washed

TABLE 1 RELEVANT DATA ON FIVE PATIENTS WITH ABETALIPOPROTEINEMIA*

No.	Patient	Age	Sex	Present Clinical Status	References†
1	R.I.	14	M	Ataxia, weakness, retinal changes	1, 2, 36, 37
2	M.S.	12	M	Slight ataxia, absent reflexes	1, 37–39
3	R.B.	10	M	Ataxic gait, early retinal changes	1, 37, 38
4	A.V.	9	F	Absent deep tendon reflexes	1, 38
5	P.B.	1	M	See Table 2	this publication

* Plasmas from patients 1, 2, and 4 were obtained through the courtesy of Dr. Herbert J. Kayden, New York University Medical Center.
† To recent reviews and reports concerning these patients.

TABLE 2 PERTINENT CLINICAL AND LABORATORY DATA ON PATIENT P.B.*

Date of birth	3 September 1965	Hemoglobin (*g/100 ml*)	11.0
Age of diagnosis	4 months	Hematocrit (%)	32
Consanguinity in parents	No	Reticulocytes (%)	2.4
Organomegaly	None	Acanthocytes (%)	>50
Neurological impairment	None	Autohemolysis† (% *in 48 hr*)	46
Plasma cholesterol (*mg/100 ml*)	25–40	White cell count	5500
Phospholipid (*mg/100 ml*)	76–81	Neutrophils (%)	32
Triglyceride	0	Lymphocytes (%)	53
Vitamin E†	0	Monocytes (%)	12
Prothrombin time (*sec*)	77‡	Eosinophils (%)	2
Control	13	Basophils (%)	1

* Studied at 4 months and from 6 to 16 months of age.
† Performed by Dr. Herbert J. Kayden, New York University Medical Center (39).
‡ Corrected to normal by parenteral or high oral doses of Vitamin K.

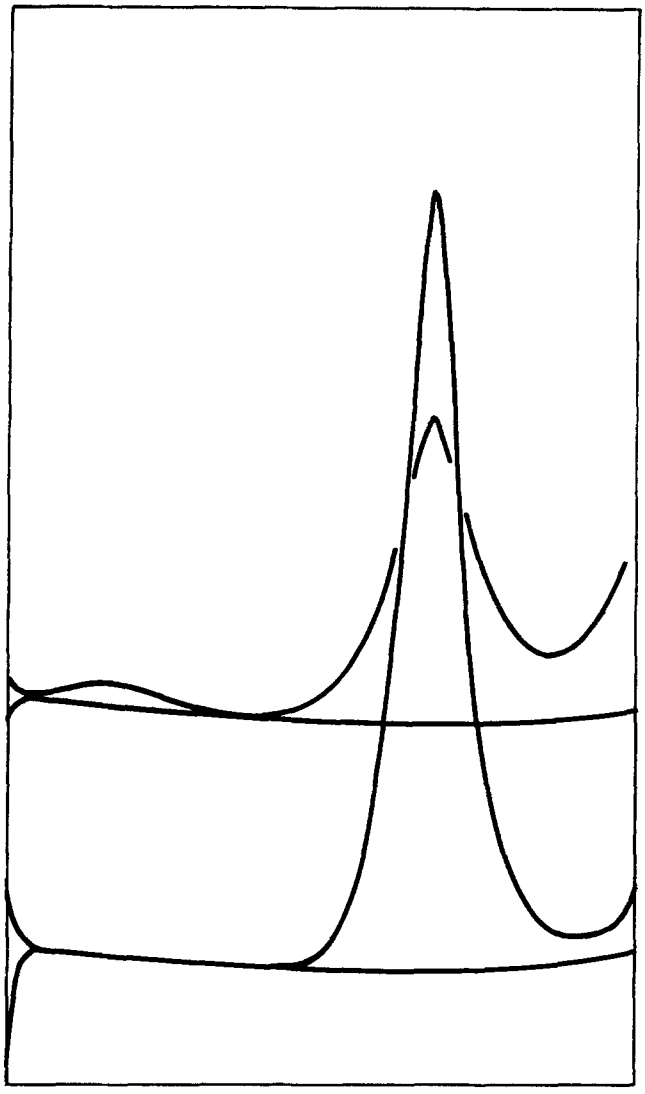

FIG. 1. Simultaneous analytical ultracentrifugation at 52,640 rpm of β- and azo-β-lipoproteins in EDTA–saline solution, density 1.006, with schlieren tracings at 112 min. The protein concentrations were 5.5 and 3.8 mg/ml, respectively. The single peak rising from the lower meniscus was β-lipoprotein. The preparation of azo-β-lipoprotein, analyzed in the upper tracing, contained a major component sedimenting at the same rate as β-lipoprotein (6S under these conditions) and a minor component sedimenting more slowly.

by recentrifugation at density 1.21 for 48 hr to rid it of traces of residual lipoproteins of d < 1.21. All samples were exhaustively dialyzed against EDTA–saline. 1.21 infranate contained traces of native α-lipoprotein and moderate amounts of partially delipidated α-lipoprotein (5, 6), but no immunologically detectable β-lipoprotein.

Whole plasma from patients with abetalipoproteinemia and 1.21 infranate from these and from normal subjects were concentrated by dry dialysis at room temperature against Sephadex G-50 (Pharmacia Fine Chemicals Inc., Piscataway, N.J.).

Chemical Modification of Lipoproteins

β-Lipoprotein was coupled with arsanilic acid as described by Margolis and Langdon (7). The resultant azo-β-lipoprotein was freed from excess reagent by exhaustive dialysis against EDTA–saline. It sedimented in the analytical ultracentrifuge at the same rate as the native lipoprotein (Fig. 1). In addition, a very small late-sedimenting peak appeared which was not present in the starting material.

β-Lipoprotein was acetylated by the method of Fraenkel-Conrat (8). This procedure caused precipitation of half of the lipoprotein protein. The supernatant acetyl-β-lipoprotein was slightly turbid even when freshly prepared; this turbidity rapidly increased with storage. It aggregated and precipitated completely after 10 days' storage at 0°C.

The accessible disulfide groups of β-lipoprotein were reduced by reaction with a 100-fold excess of dithiothritol (9) at 37°C for 20 min in an atmosphere of nitrogen. A 110-fold excess of iodoacetamide was added to alkylate the thiol groups produced by the reduction and the reagents were removed by dialysis against EDTA–saline.

Ether delipidation was performed at 0°C by the two-phase method of Avigan (10). Delipidation of β-lipoprotein by this method causes partial denaturation (11). The azo-β-lipoprotein and reduced β-lipoprotein were rendered insoluble, even in 6 M guanidine solution, by ether delipidation.

Preparation of Antisera

Antisera to the various protein preparations were made in New Zealand white or Rockefeller brown rabbits; the 15 antisera used are tabulated in Table 3. 5–20 mg of protein (for pure lipoprotein preparations) or 100–300 mg of protein (for plasma and 1.21 infranate) in 1 ml was emulsified with an equal volume of Freund's complete adjuvant just before injection. Acetyl-β-lipoprotein was freshly prepared for each injection as required and used while still soluble. The initial dose was divided between the four toepads. A booster dose was given intramuscularly in the back muscles 1 wk later, and blood was collected 7–10 days after the second injection. Antisera were preserved by the addition of 0.01% sodium merthiolate. Anti-α-lipoprotein antiserum was obtained commercially (Lloyd Bros. Inc., Cincinnati, Ohio). Anti-immunoglobulin G and anti-immunoglobulin M were donated by Dr. Mart Mannik, The Rockefeller University.

Immunoelectrophoresis was carried out in 1% agarose as described by Levy, Lees, and Fredrickson (11). Gel diffusion was performed at 4°C in Ouchterlony plates (12) in 1% agarose dissolved in barbital buffer $\mu/2 = 0.05$, pH 8.2. Phosphate-buffered saline solution at

TABLE 3 SOURCES AND REACTIVITY OF 15 ANTISERA USED IN THIS STUDY

Antiserum	Immunizing Antigen	Reactivity					
						B Protein in:	
		α*	β*	Azo-β*	Acetyl-β*	Normal 1.21 Infranate	AβLP† Plasma
R_4	β-Lipoprotein	tr.	+‡	+	+	−§	−
R_6	β-Lipoprotein	−	+	+	+	−	−
R_3	Ether-delipidated β-lipoprotein	−	+	+	+	−	−
R_5	Azo-β-lipoprotein	−	−	+	+	−	−
R_{10}	Acetyl-β-lipoprotein	−	−	+	+	−	−
R_1	Lymph chylomicrons	+	+	+	+	+	+
R_2	Lymph chylomicrons	+	+	+	+	+	+
R_9	Normal 1.21 infranate	+	−	+	+	+	+
R_{13}	Normal 1.21 infranate	+	−	+	+	+	+
R_{15}	Plasma from patient 2	+	−	+	+	+	+
R_{12}	Plasma from patient 4	+	−	+	+	+	+
R_{14}	Plasma from patient 5	+	−	+	+	+	+
R_8	1.21 infranate from patient 3	+	−	+	+	+	+
Anti-α-lipoprotein	α-Lipoprotein	+	−	−	−	−	−
Anti-immunoglobulin G	Immunoglobulin G	−	−	−	−	−	−

* α, β, azo-β, and acetyl-β refer to the respective lipoproteins, isolated or in whole plasma.
† AβLP = abetalipoproteinemia.
‡ +, reacts to give a precipitin line in gel diffusion or immunoelectrophoresis.
§ −, does not react.

pH 7 was initially used for the double diffusion experiments in 1% agarose, but it was readily apparent that the precipitin lines were denser and sharper in barbital buffer.

Analytical Methods

Protein assays were carried out by the method of Lowry, Rosebrough, Farr, and Randall (13); values for β-lipoprotein were corrected for their higher color yield relative to a bovine serum albumin standard, as suggested by Margolis and Langdon (14).

Cholesterol was determined with the AutoAnalyzer (15) by a modification of the method of Levine and Zak (16), phospholipids by the method of Stewart and Hendry (17), and triglycerides by difference (18), after the microgravimetric technique of Craig, Hausmann, Ahrens, and Harfenist (19) had been used to measure the total lipid content of a chloroform–methanol lipid extract (20).

RESULTS

The soluble material from partial ether delipidation of β-lipoprotein was antigenically identical with the starting material, in accord with previous experience (11). Antiserum made to it reacted strongly with β-lipoprotein. On double diffusion in Ouchterlony plates against this antiserum, ether-delipidated β-lipoprotein gave a single precipitin line which showed complete identity with the single line of native β-lipoprotein.

The chemically altered β-lipoprotein preparations were studied next in order to determine whether these alterations, unlike ether delipidation, changed the antigenicity of the molecule. This proved to be the case.

Antiserum made to the azo-β- or acetyl-β-lipoprotein gave a double precipitin line against its immunizing antigen (Fig. 2). In each case, a faster moving component was observed on immunoelectrophoresis which was present in lower concentration and stained less well with Oil Red O. These minor components did not cross-react with β-lipoprotein on double diffusion in Ouchterlony plates; they also failed to precipitate with anti-β-lipoprotein antisera (Fig. 2).

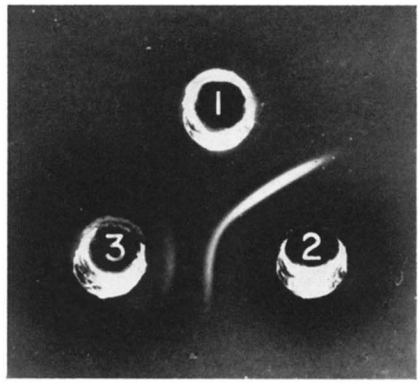

FIG. 2. Immunological heterogeneity of azo-β-liprotein. Double diffusion in agarose of azo-β-lipoprotein (well *2*) against anti-β-lipoprotein antiserum (R_4, well *1*) and anti-azo-β-lipoprotein antiserum (R_5, well *3*). The minor component of azo-β-lipoprotein is not precipitated by anti-β-lipoprotein antiserum.

Anti-azo- and anti-acetyl-β-lipoprotein antisera did not precipitate β-lipoprotein on immunoelectrophoresis or double diffusion in agarose. Anti-azo-β-lipoprotein precipitated the major but not the minor component of acetyl-β-lipoprotein. Anti-acetyl-β-lipoprotein likewise precipitated the major but not the minor component of azo-β-lipoprotein. With both antisera, a reaction of nearly complete identity was obtained for the major components of both altered lipoproteins, with only slight spur formation. (With anti-β-lipoprotein antisera there was complete identity between the two altered lipoproteins.)

Thus, the antigenicity of both azo- and acetyl-β-lipoproteins was different from β-lipoprotein, but each was similar to the other. Neither anti-azo-β- nor anti-acetyl-β-lipoprotein produced precipitin lines with plasma, from either normal patients, the five patients with abetalipoproteinemia (Table 1), or two patients with familial hyperlipoproteinemia, type II (21).

It could be shown, however, that the precipitation of azo-β-lipoprotein by anti-azo-β-lipoprotein was inhibited by a protein in abetalipoproteinemic plasma which migrated in the β-globulin region on immunoelectrophoresis. This suggested that a protein present in that region was immunologically similar to azo-β-lipoprotein. Partially reduced, alkylated β-lipoprotein was completely identical with the native lipoprotein when tested against all the antisera described in this report. Since the antigenicity of the partially reduced β-lipoprotein was so similar to that of the native lipoprotein, I elected not to use it as an immunizing antigen.

Antigenicity of Lymph Chylomicrons

Lymph chylomicrons were studied next, in order to determine the presence and antigenicity of B protein on these particles. The nature of the protein moiety of chylomicrons could not be studied in the native state by immunodiffusion techniques because these particles are too large to diffuse through agarose gel; delipidation of the chylomicrons was not attempted as this would be likely to alter the state of the protein moiety.

Antiserum to lymph chylomicrons precipitated both native and delipidated α-lipoprotein. It reacted with β-lipoprotein and weakly with a number of other plasma proteins. When plasma or 1.21 infranate from either normal subjects or patients with abetalipoproteinemia was subjected to immunoelectrophoresis and tested against anti-chylomicron antiserum, a previously undescribed β-globulin precipitin line of mobility similar to that of β-lipoprotein was noted, but it did not stain for lipid with Oil Red O (Fig. 3).

In order to identify this β-globulin, I subjected plasmas from patients with abetalipoproteinemia to electrophoresis in agarose. Azo-β-lipoprotein was placed in one antiserum trough and anti-chylomicron antiserum in the other: the line of precipitation of azo-β-lipoprotein sometimes showed partial and sometimes complete identity (Fig. 4) with the β-globulin line. On Ouchterlony gel diffusion of anti-chylomicron antiserum against either 1.21 infranate from normal subjects or patients with abetalipoproteinemia, or whole plasma from those with abetalipoproteinemia, a faint precipitin line which showed partial or complete immunologic identity with acetyl or azo-β-lipoprotein (Fig. 5) frequently appeared. Therefore, a lipid-free protein which is present in plasma is also present in some form in chylomicrons and is immunologically identical with chemically altered β-lipoprotein. These findings suggest the presence of B protein in plasma.

Antigenicity of Normal 1.21 Infranate

If B protein is present in plasma, it should sediment at density 1.21 and be concentrated by ultracentrifugation. It should inhibit the immunologic precipitation of azo-

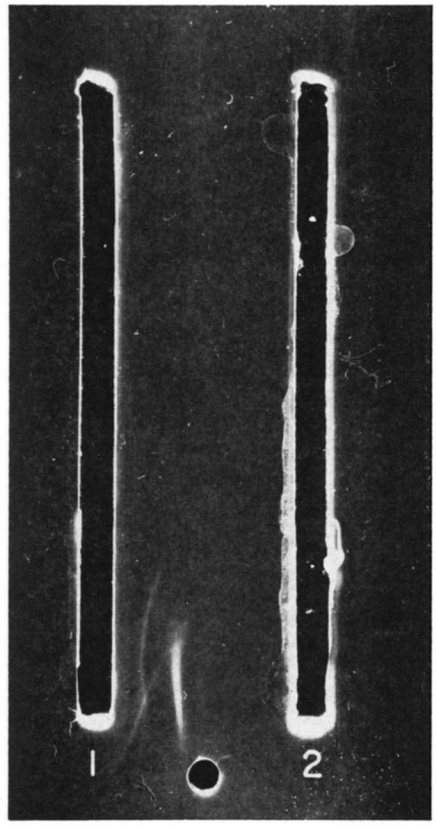

FIG. 3. Presence of B protein in abetalipoproteinemic plasma. Immunoelectrophoresis in agarose of plasma from patient 3, in the well; anti-chylomicron antiserum R_1 in trough *1*, and anti-β-lipoprotein antiserum R_6 in trough *2*. The dense β-globulin precipitin line produced by anti-chylomicron serum is not due to β-lipoprotein and did not stain for lipid. The faint lines near trough *1* represent slight reactivity of the anti-chylomicron antiserum with other unidentified serum proteins.

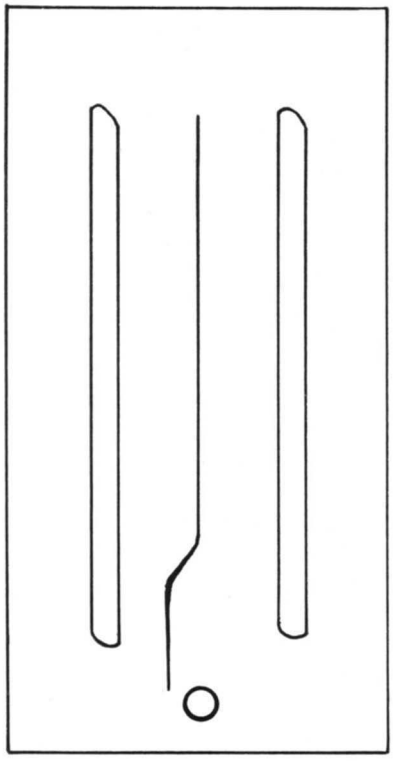

FIG. 4. Immunologic identity of B protein with azo-β-lipoprotein. Immunoelectrophoresis in agarose, similar to that shown in Fig. 3, with normal 1.21 infranate in the well. A tracing of a Polaroid photograph of this analysis is shown. Anti-chylomicron antiserum R_1 was in the left-hand trough and azo-β-lipoprotein in the right-hand trough. The vertical line of precipitation of azo-β-lipoprotein merges with the curved β-globulin precipitin arc, and this shows their immunologic identity. (The faint precipitin lines seen in Fig. 3 were omitted for the sake of clarity.)

and acetyl-β-lipoprotein, if they are antigenically similar to it.

No β-lipoprotein was found immunologically in 1.21 infranate even on 4-fold concentration. The 1.21 infranate did not precipitate with anti-azo-β- or anti-acetyl-β-lipoprotein, nor did it inhibit the precipitation of β-lipoprotein by anti-β-lipoprotein antisera. However, 1.21 infranate consistently caused strong inhibition of precipitation by anti-β-lipoprotein antisera of both azo- and acetyl-β-lipoprotein (Fig. 6).

If B protein is present in normal plasma, moreover, antiserum to 1.21 infranate should precipitate that B protein, and if the antigenicity of azo- and acetyl-β-lipoprotein is similar to that of B protein, antiserum to 1.21 infranate should also react with those altered β-lipoproteins. Antiserum to normal 1.21 infranate rarely and inconsistently gave a faint line of precipitation on gel diffusion against β-lipoprotein. However, it strongly precipitated acetyl-β-lipoprotein (Fig. 7) and, less strongly, azo-β-lipoprotein. On immunoelectrophoresis against whole normal plasma, it gave an array of lines representing most of the plasma proteins. Among these was the same β-globulin line seen with anti-chylomicron antisera (Fig. 3).

Antigenicity of Plasma and 1.21 Infranate from Patients with Abetalipoproteinemia

Evidence was sought for the presence of B protein in plasma of five patients with abetalipoproteinemia. No β-lipoprotein was demonstrable immunologically in whole or concentrated plasma from any of the subjects. Neither whole plasma, concentrated plasma, nor concentrated 1.21 infranate inhibited the precipitation of normal native β-lipoprotein by anti-β-lipoprotein antisera on Ouchterlony plates. However, whole or concentrated plasma and concentrated 1.21 infranate from the subjects with abetalipoproteinemia caused strong inhibition of precipitation of both acetyl- and azo-β-lipoprotein (Fig. 6), which showed that all three fractions contained a protein antigenically very similar to azo-β-lipoprotein. (Fig. 6 also demonstrates the partial identity of azo-β- with native β-lipoprotein.)

Antisera to whole plasma, concentrated plasma, or concentrated 1.21 infranate from the patients with abetalipoproteinemia did not precipitate β-lipoprotein, but they strongly precipitated acetyl- and, less strongly, azo-β-lipoprotein. On immunoelectrophoresis of plasma from normal subjects or those with abetalipoproteinemia, these antisera, like those made to chylomicrons (Fig. 3) and to normal 1.21 infranate, showed a β-globulin precipitin line which did not stain for lipid.

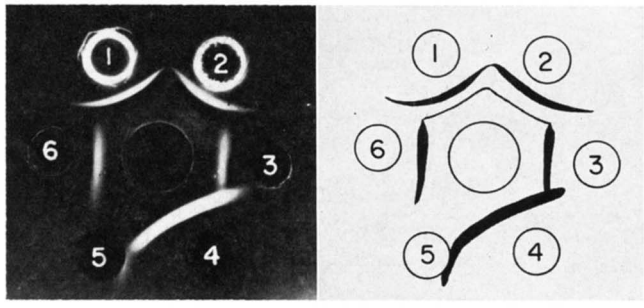

FIG. 5. Comparison of the antigenicity of plasma from two patients with abetalipoproteinemia with the antigenicity of native and acetylated β-lipoprotein. The picture and sketch are from an Ouchterlony double diffusion experiment in agarose gel. Anti-chylomicron serum R_1 was in the center well. Wells *1* and *2* contained plasma from patients 5 and 4 (Table 1), respectively. Wells *3* and *6* contained acetyl-β-lipoprotein. Well *4* contained native β-lipoprotein. Well *5* was empty.

Acetyl-β-lipoprotein is only partially identical with native β-lipoprotein but shows complete identity with a fine line of precipitation seen in both abetalipoproteinemia plasmas. (This line does not reproduce well on the photograph and is drawn in on the sketch.) The heavy precipitin lines at wells *1* and *2* are those of α-lipoprotein. Weak precipitin lines seen around the well on the photograph are due to unidentified plasma proteins and are not reproduced on the sketch.

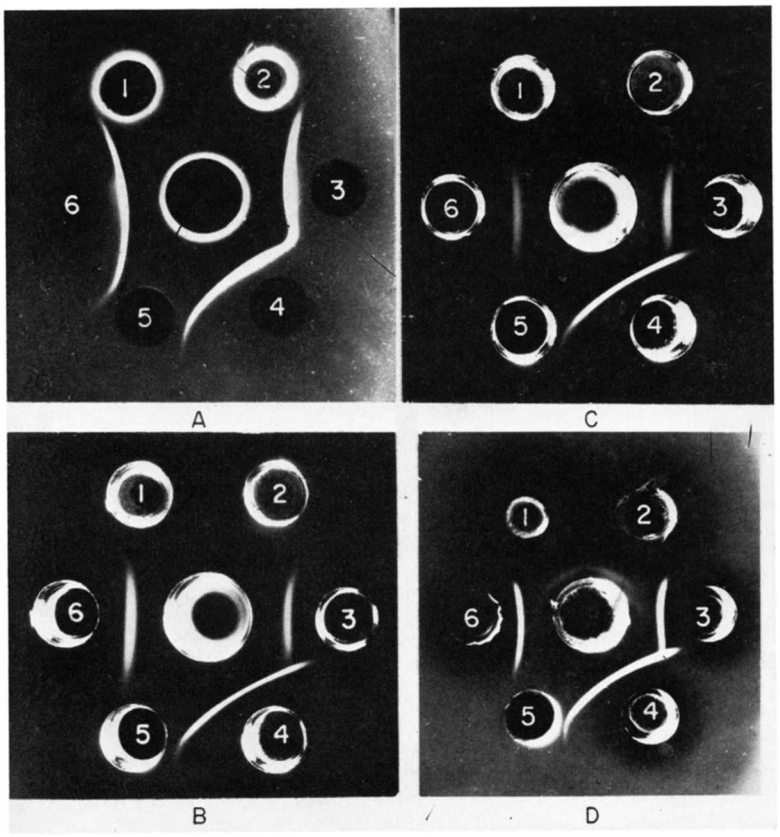

FIG. 6. Inhibition of precipitation of azo-β-lipoprotein by normal 1.21 infranate and by plasma from all five patients with abetalipoproteinemia. In the four Ouchterlony plates shown, the center well contained anti-β-lipoprotein antiserum (R_6 in A–C, R_4 in D), well *4* was filled with normal native β-lipoprotein, and well *5* was empty.

A. Well *1*, concentrated 1.21 infranate from a normal plasma; well *2*, plasma from patient 5 (Table 1); wells *3* and *6*, native β-lipoprotein. There is, as expected, a reaction of complete identity between the contents of wells *3* and *4*. No inhibition of precipitation is produced by the contents of wells *1* and *2*.

B. Well *1*, plasma from patient 2 (Table 1); well *2*, concentrated 1.21 infranate from a normal plasma; wells *3* and *6*, azo-β-lipoprotein. The normal 1.21 infranate and the abetalipoproteinemic plasma inhibit the precipitation of azo-β-lipoprotein (shown by shortening of the upper ends of its precipitin arcs).

C. Similar to B except that wells *1* and *2* contain plasma from patients 1 and 3 (Table 1), respectively.

D. Similar to B except that wells *1* and *2* contain plasma from patients *5* and *4* (Table 1), respectively, and antiserum R_4 is in the center well.

Lack of Reactivity with Other Antigens and Antisera

The following experiments were done to test whether the reactions observed were due to nonspecific inhibition of precipitation of the altered β-lipoproteins or to nonspecific precipitation by immune globulins. Pure human α-lipoprotein and immunoglobulin G were tested for inhibition of precipitation of acetyl- and azo-β-lipoprotein. There was no inhibition. In addition, anti-α-lipoprotein, anti-immunoglobulin G and anti-immunoglobulin M were tested for their ability to precipitate acetyl- and azo-β-lipoprotein. In no case was there any precipitation by the above antisera, either on immunoelectrophoresis or double diffusion in agarose.

The results of the immunologic studies are summarized in Tables 3 and 4.

DISCUSSION

The evidence presented above demonstrates that plasma from normal subjects and from patients with abetalipoproteinemia contains a β-globulin which does not stain for lipid, and which is antigenically similar to azo- and acetyl-β-lipoprotein. It seems probable that this β-globulin, which is present in the 1.21 infranate of normal and abetalipoproteinemic plasma, is the apoprotein of β-lipoprotein (or a fragment of it). Granda and Scanu have prepared soluble B protein stabilized by a detergent (22). Their B protein was not in a lipid-free state; its usual lipid complement was replaced by a small amount of a highly polar lipid, sodium lauryl sulfate. The authors demonstrated that antiserum to β-lipoprotein precipitated their detergent-stabilized material but did not show

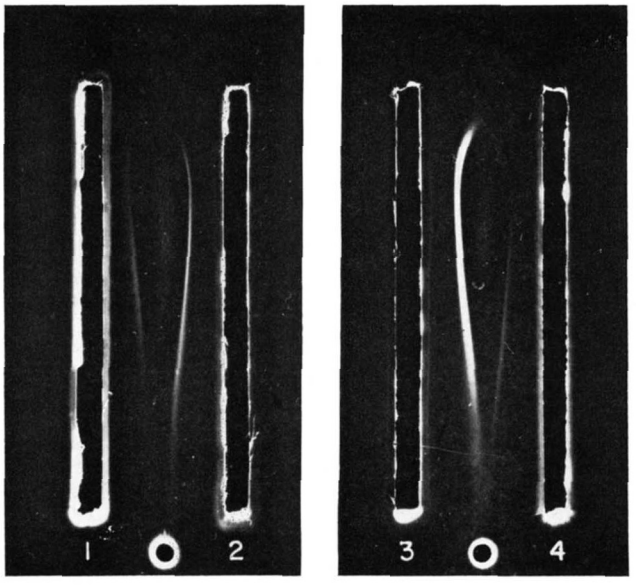

FIG. 7. Immunoelectrophoretic analysis of acetyl-β-lipoprotein. The acetylated lipoprotein was placed in each well, and was allowed to react with different antisera in troughs *1–4: 1*, anti-azo-β-lipoprotein (R_5); *2*, anti-1.21 infranate from patient 3 with abetalipoproteinemia (R_8); *3*, anti-β-lipoprotein (R_4); *4*, anti-normal 1.21 infranate (R_9). All four antisera precipitate the major component of the acetyl-β-lipoprotein.

with antisera to their preparation that it was antigenically identical with β-lipoprotein. It seems likely that their detergent-stabilized protein is also antigenically similar to naturally occurring B protein. Roheim, Miller, and Eder (23) have shown that rat plasma contains an apoprotein which on perfusion through the isolated rat liver is incorporated into lipoproteins. Their apoprotein is very probably B protein.

It has been shown repeatedly that there is no material in normal plasma 1.21 infranate or even in the whole plasma of patients with abetalipoproteinemia which is precipitated by anti-β-lipoprotein antisera (2, 5, 24–26). From this the conclusion has been drawn that there is no lipid-free B protein nor any subunit of it in human plasma, since it has been assumed that antigenicity of this protein was the same whether it is lipid-laden or in the delipidated state. Reasoning on that basis, several investigators (1–3) have concluded that the primary heritable defect in abetalipoproteinemia is the inability to synthesize the protein moiety of β-lipoprotein.

The experiments reported here have led me to the conclusion that the protein moiety of β-lipoprotein may exist in more than one antigenic state. In addition there is indirect evidence that β-lipoprotein is present in the chylomicron in a form different from that in the free state in plasma. Anti-chylomicron antisera react with a lipid-free β-globulin in plasma and 1.21 infranate from both normal subjects and those with abetalipoproteinemia. This β-globulin shows antigenic identity with acetyl- and azo-β-lipoprotein on gel diffusion (Fig. 5), but is antigenically different from native β-lipoprotein. The latter finding is not surprising, since the protein layer in the chylomicron membrane is only about 20 A in width, which is too thin to allow the presence in it of β-lipoprotein in the same conformation it usually occupies in plasma (27). One may perhaps imagine that the B protein fulfills its function of lipid transport by having a relatively elastic tertiary structure which enables it to exist lipid-free in plasma, or to solubilize lipids as β-lipoprotein or chylomicrons, in a somewhat different conformation in each instance.

The antigenicity and probably the conformation of β-lipoprotein is changed by acetylation or arsanilation. The altered lipoproteins are different from native β-lipoprotein but quite similar to each other. A number of other proteins exhibit altered antigenicity with delipidation or other chemical change. For instance, the antigenicity of α-lipoprotein is greatly changed by delipidation (5); its protein moiety probably dissociates when lipids are removed (5). Many years ago Landsteiner (28) noted that arsanilation of proteins caused considerable loss of antigenic specificity. Perlmann showed that acetylation of pepsinogen changes its conformation (29). Relatively minor chemical changes in a number of proteins cause reduction or disappearance of their cross-reactivity with the native protein (30). More important, perhaps, is the demonstration that antigenicity is dependent on molecular conformation as well as on chemical composition (30).

The minor component seen in preparations of both acetyl- and azo-β-lipoprotein has not been further identified. These minor components do not precipitate antisera to β-lipoprotein nor does the minor component of acetyl-β-lipoprotein cross-react with that of azo-β-lipoprotein. They are not precipitated by antisera to chylomicrons, to

TABLE 4 SUMMARY OF INHIBITION EXPERIMENTS

Antigen	β	Azo-β	Acetyl-β
Normal 1.21 infranate	−	+	+
Plasma from patient 3	−	+	+
1.21 infranate from patient 3	−	+	+
Plasma from patient 5	−	+	+
1.21 Infranate from patient 5	−	+	+
Plasma from patient 4	−	+	+
Plasma from patient 1	−	+	+
Plasma from patient 2	−	+	+
α-Lipoprotein	−	−	−
Immunoglobulin G	−	−	−

Abbreviations as in Table 3; the tests for inhibition were performed by the Ouchterlony double diffusion method against antisera R_4 and R_6.

+, inhibits the precipitation of the β-lipoprotein derivative tested; −, does not inhibit the precipitation of the β-lipoprotein derivative tested.

1.21 infranate, or to abetalipoproteinemic plasma. Possibly the minor components are impurities adsorbed to β-lipoprotein which are released when the protein is chemically altered. It seems more likely, however, that they represent either smaller subunits released by the chemical reaction or portions of the β-lipoprotein so completely altered by the reagent that the original antigenic specificity is completely lost.

One may reason that the immunological findings are due to the presence of one of the many enzyme proteins known to be carried on the β-lipoprotein, a protein which might also be present in the free state in abetalipoproteinemic plasma. The results cannot be due solely to the presence of an adsorbed impurity in my preparations of "pure" β-lipoprotein, however, since it is the major, not the minor component of both azo- and acetyl-β-lipoprotein that is precipitated by antisera to abetalipoproteinemic plasma and whose precipitation is completely inhibited by abetalipoproteinemic plasma itself (Fig. 6).

Tangier disease or familial α-lipoprotein deficiency (31, 32) is due to production of a defective protein, rather than to total failure to synthesize that protein. The finding that the B protein may be present in plasma in abetalipoproteinemia makes it likely that this disease also is due to synthesis of a defective protein rather than total lack of synthesis. An alternative explanation is that the disease is caused by an inability to combine the lipid moiety with a preformed protein molecule of normal chemical structure. This latter possibility brings to mind the defect caused in rats by orotic acid feeding (33). It would also be consistent with the hypothesis that the recently described defect in man of partial deficiency of β-lipoproteins (34, 35) is due to a lesser degree of blocking of the "lipidation" of normal B protein.

It has been previously reported that disruption of the available disulfide bonds in β-lipoprotein caused no gross changes in the state of the lipoprotein, and it was suggested that those disulfide bridges are not essential for lipid binding (7). The present data confirm this and demonstrate further that such disruption of disulfide bonds does not alter the reactivity of the molecule with anti-β-lipoprotein antisera, although delipidation of the partially reduced and alkylated β-lipoprotein causes its rapid denaturation. These results, and the evidence that the antigenicity of the lipid-free B protein is very different from that of β-lipoprotein, are consistent with the hypothesis that the conformation of the protein in β-lipoprotein is maintained chiefly by its lipid moiety. This hypothesis would also explain why reduction of accessible disulfide bonds does not cause loss of lipid or change in antigenicity, and why delipidation makes previously buried disulfide bonds available to reducing agents (7). Acetylation, which alters the antigenicity in the direction of that of the lipid-free protein, causes instability of the molecule.

The complete lack of glyceride transport in the syndrome of abetalipoproteinemia (2) suggests that β-lipoprotein is essential for this process. The presence of soluble B protein in plasma would be consistent with the possibility that such transport occurs by repeated combination of B protein with lipid at sites of production such as the intestine and delivery of this lipid at sites of utilization. Further studies are now being carried out to isolate the B protein from plasma, to establish its size and structure, and to determine its concentration in normal plasma and in various abnormal states.

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