papers and notes on methodology

One-step screening method for the polymorphism of apolipoproteins A-I, A-II, and A-IV

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Abstract Apolipoprotein A-I exhibits a polymorphism that can be easily investigated in native serum by a simple method involving incubation of serum in the presence of decylsulfate and β-mercaptoethanol and subsequent isoelectric focusing. From six to eight proteins can be separated in a pH gradient from 4 to 6 and thus patients with apolipoprotein A-I variants can be distinguished from normal persons. This method also permits monitoring for polymorphic forms of apoA-II and apoA-IV as well as detection of C apolipoproteins. To verify the identity of the different apolipoproteins, a two-dimensional electrophoresis technique was applied, with an SDS system for the second dimension. In addition, monospecific antibodies for apolipoproteins A-I, A-II, and A-IV were used for the immunological identification. The method described here led to the discovery of three different familial apolipoprotein A-I variants.—**Menzel, H-J., R-G. Kladetzky, and G. Assmann.** One-step screening method for the polymorphism of apolipoproteins A-I, A-II, and A-IV. *J. Lipid Res.* 1982. **23:** 915–922.

Supplementary key words isoelectric focusing • two-dimensional electrophoresis • Tangier disease • apoA-I-Milano • apoA-I-Marburg

Polymorphic forms of apolipoproteins can be detected after ultracentrifugation of serum, delipidation of lipoproteins, and isoelectric focusing of apolipoproteins. Among the various apolipoproteins, polymorphic forms have been described for apoE (1), apoC-III (2), apoC-II (3), and apoA-I (4–6). The polymorphism of the apolipoproteins may be caused either by different carbohydrate moieties or differences in the primary structure. ApoC-III shows polymorphic forms due to a variation in sialic acid content, whereas the polymorphism of apoC-II[2] and apoE (7) resides with differences in the primary amino acid sequence of the respective apolipoproteins. The reason for the variation in the pI values of apoA-I-Marburg (6) is unknown at present, whereas apoA-I-Milano seems to contain one cystein molecule instead of none, as in normal apoA-I (5). In this study we describe a simple one-step method suitable for screening large populations for variants of apoA-I, A-II, and A-IV. Two-dimensional electrophoretic techniques and immunochemical procedures were applied to identify the nature of the apolipoproteins under investigation.

METHODS

Patients

Normal sera derived from 1,000 factory employees were collected in connection with a prospective epidemiological study, carried out in Münster ("Prospective cardiovascular study, Münster") (8, 9). In addition, 1,000 sera were collected from patients who underwent coronary angiography (Departments of Internal Medicine, Universities of Münster and Düsseldorf, FRG). The patients with the homozygous and heterozygous forms of Tangier disease have already been described (10, 11). Serum from a patient with the apoA-I-Milano variant was a gift of Dr. Franceschini (Milano) and the apoA-I-Marburg variant was a gift from Dr. Utermann (Marburg). Alpha-1-antitrypsin, acid glycoprotein, and human albumin (Fraction V) were from Sigma.

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; SDS, sodium dodecylsulfate; TEMED, N,N,N′,N′-tetramethylethylenediamine; TCA, trichloracetic acid; Tris, tris-(hydroxymethyl)aminomethane; C-II, apolipoprotein C-II.

[1] R-G. Kladetzky.

[2] Menzel, H-J., R. J. Havel, and J. D. Kane. Unpublished data.

Sample preparation

Four μl of serum was incubated with 50 μl of 0.01 M Tris–HCl, pH 8.2, containing 1% decylsulfate (Kodak) and 2% Ampholine (pH range 3.5–7, LKB), and 5 μl of mercaptoethanol for 1 hr at room temperature. About 10 μl of 80% sucrose was added to increase the density, and the sample was applied to the gel.

Polyacrylamide gel isoelectric focusing

Isoelectric focusing was done according to Pagnan et al. (12) with some modifications. We used the slab gel system Model 220 from Bio-Rad and ampholytes pH 4–6 (LKB 4–6 Batch 48, Serva 4–6 Batch 23031). Gels were prepared with the following solutions. The acrylamide solution consisted of 30 g of acrylamide (Bio-Rad), 0.8 g of N,N′-methylene-bis-acrylamide (Bio-Rad), 36 g of urea, and water to a total volume of 100 ml. The TEMED solution contained 1 ml of N,N,N′,N′-tetramethylethylenediamine, 36 g of urea, and water to a total volume of 100 ml. The gel solution contained 8.3 ml of acrylamide solution, 1.38 ml of ampholytes (pH 4–6), 2.2 ml of TEMED solution, 22 ml of 8 M urea, and 22 mg of ammonium persulfate. The gel solution was added to the cassettes of a Bio-Rad Model 220 electrophoresis cell with 2-mm spacers. A 16-well comb was used to prepare the individual slots.

The focusing plates were run overnight (17 hr) at 250 V, starting with power limited to 3 watts per plate, and 600 V in the morning for 2 hr. For staining, we used a modification of the method from Malik and Berrie (13). Three grams of Coomassie Brilliant Blue G-250 (Bio-Rad) was solubilized in 150 ml of hot water (80°C) to which 75 ml of 3 N H_2SO_4 was added under vigorous stirring; heating was continued for 10 min at 80°C and afterwards the solution was filtered. The pH was raised to 5.5 with 10 N KOH and 0.25 g of decylsulfate and 50 g of TCA were added. The gels were stained at 60°C for 30 min and destained with 20% ethanol at 60°C. During staining the ampholytes, which are soluble in ~20% TCA, are removed.

Two-dimensional electrophoresis

For the two-dimensional electrophoresis of individual samples, strips from the focusing gels were cut and immersed in 0.002 M ethylmorpholine-HCl, pH 8.5, 0.2% SDS,[3] 0.1% β-mercaptoethanol, bromophenol blue solution, and 4% sucrose for 15 min at room temperature and applied to an SDS gel for electrophoresis in the second dimension. The SDS system was prepared according to Neville with 15% acrylamide concentration (14). The discontinuous system of Neville comprised two different gels. The lower gel solution was added to the cassettes of a Model 220 electrophoresis chamber (Bio-Rad) to a height of 9 cm (2.25 mm thick) and layered with water-saturated n-butanol. After polymerization the butanol was removed and the gel surface was washed with water, layered with lower gel buffer, and stored overnight at 4°C. The following morning the solution for the lower gel (5% acrylamide concentration) was mixed and poured onto the lower gel to a height of 1.5 cm and one slot was prepared for the molecular weight standard (Pharmacia). After applying the gel strips and the standard, the electrophoresis apparatus was assembled and electrophoresis was carried out at 15 mA per plate with an upper voltage limit of 200 volts. Gels were stained in 0.2% Coomassie G-250 (Serva) in 50% methanol–12% acetic acid for 1 hr at 60°C, and destained in 20% ethanol at 60°C.

Crossed immunoelectrophoresis

Identification of isoelectric focusing bands by immunological methods was done by crossed immunoelectrophoresis. After focusing, the gel strips were immersed in 20 ml of barbital buffer, pH 8.6, ionic strength 0.02 μ, for 5 min and then placed near the edge of a 9 × 12 cm glass plate. Twenty ml of melted 1.2% agarose (Bio-Rad) solubilized in the same barbital buffer containing 0.1% Triton X-100 was poured around the gel. After the agarose was stiff, a segment was cut out, vertical to the focusing gel, and filled with monospecific antiserum in melted agarose. Antisera for apoA-I and apoA-II were prepared in our laboratory (10); the apoA-IV antiserum was kindly supplied by Drs. Weisgraber and Mahley, San Francisco. Electrophoresis was run with the same barbital buffer at 4 V/cm for 5 hr. The plates were washed three times with 0.9% NaCl, dried, and stained with 0.2% Coomassie G-250 (Serva) in 50% methanol–12% acetic acid for 15 min, and then destained in 20% ethanol.

RESULTS

Isoelectric focusing of normal serum after incubation with 1% decylsulfate and β-mercaptoethanol in a pH gradient from 4 to 6 results in the resolution of 10–15 bands (**Fig. 1**). By a combination of isoelectric focusing and crossed immunoelectrophoresis, as well as by co-electrophoresis of authentic substances, bands 1 and 3 were identified as apoA-I, bands 2 and 4 as apoA-IV, and band 8 as apoA-II. The identity of these apoproteins was also confirmed by molecular weight determination in SDS gels using the two-dimensional technique (**Fig. 2**). Bands 1 and 3 (apoA-I-1 and apoA-I-2, respectively) were present in all normolipidemic sera investigated. In

[3] Recrystallization of SDS: 80 g of SDS was dissolved in 1 liter of boiling ethanol, filtered, cooled to 4°C overnight, and filtered again, after which the filtrate was air-dried.

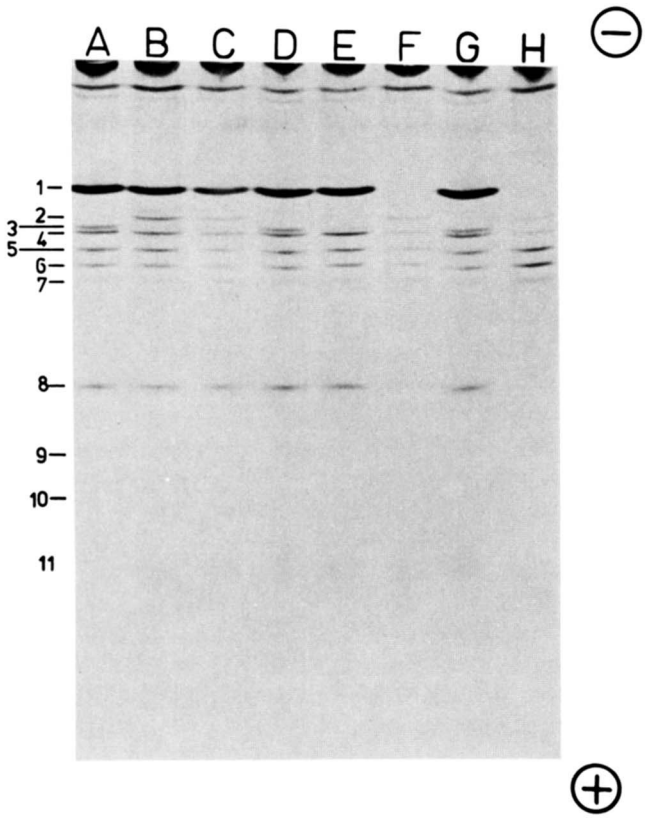

Fig. 1. Isoelectric focusing gel of serum in a pH gradient from pH 4 to 6. Bands are numbered from 1 to 11. Normal serum was applied in lanes A, D, and G; sera from heterozygous Tangier patients in lanes B, C, and E; and sera from homozygous Tangier patients in lanes F and H.

addition to these two major isoforms of apoA-I, three further minor apoA-I isoforms could be identified upon two-dimensional immunoelectrophoresis (**Fig. 3a**).

The applicability of the method described here to identify apolipoprotein A-I variants was first monitored with sera derived from patients with Tangier disease, and apoA-I-Milano and apoA-I-Marburg variants. In sera of homozygous Tangier patients (Fig. 1; lanes F and H), band 1 was extremely weak and band 3 was not visible; apoA-II was present only in trace amounts. In sera of heterozygous Tangier patients, however, band 1 and 3 as well as band 8 could be clearly visualized. As shown in **Fig. 4,** the apoA-I-Milano serum (lane B) exhibits a weak band in position 1, thus confirming the deficiency of the normal apoA-I isoform in this disease (5). An additional protein, however, could be visualized below band 5. This protein, upon two-dimensional electrophoresis (**Fig. 5**) and immunochemical investigation, exhibits the molecular weight and immunochemical properties of apoA-I. As shown by Weisgraber et al. (5), this abnormal apoA-I protein band (comigrating with band 5) differs from normal apoA-I in its amino acid composition. The apoA-I-Marburg serum is characterized by additional protein bands in positions 3 and 5 (comigrating with bands 3 and 5) (Fig. 4). The identity of these two additional proteins as apoA-I variants could be documented by SDS gel molecular weight determination (**Fig. 6**) and immunochemical investigation (not shown).

ApoA-IV migrates in these isoelectric focusing gels to

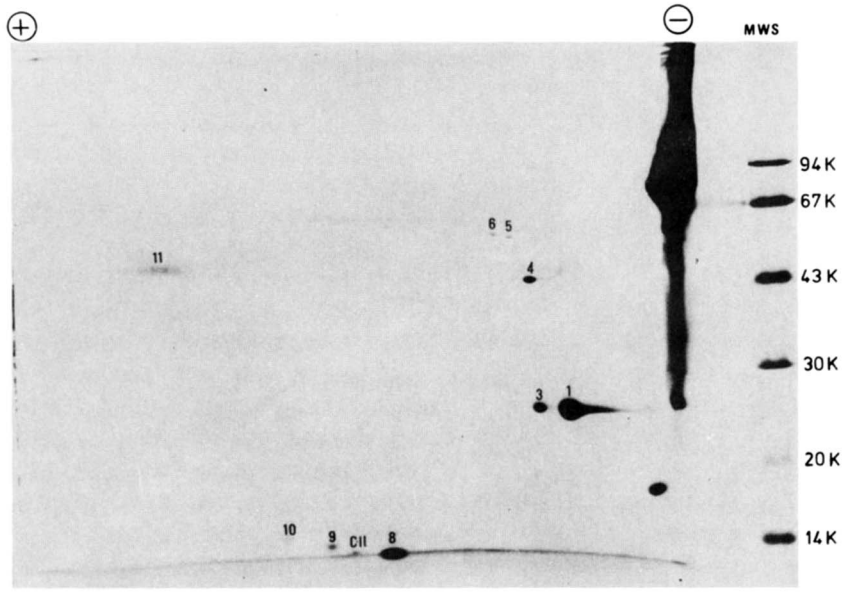

Fig. 2. Two-dimensional gel of normal serum. MWS is the molecular weight standard with six different marker proteins; their molecular weights are indicated. Numbering of the spots corresponds to the band numbers in Fig. 1. Spots numbered 1 and 3 are apoA-I, 4 is apoA-IV, 5 and 6 are alpha-1-antitrypsin, 8 is apoA-II, 9 and 10 are C-III_1 and C-III_2, respectively, and 11 is the acid glycoprotein. The plus and minus symbols indicate the direction of the first dimension.

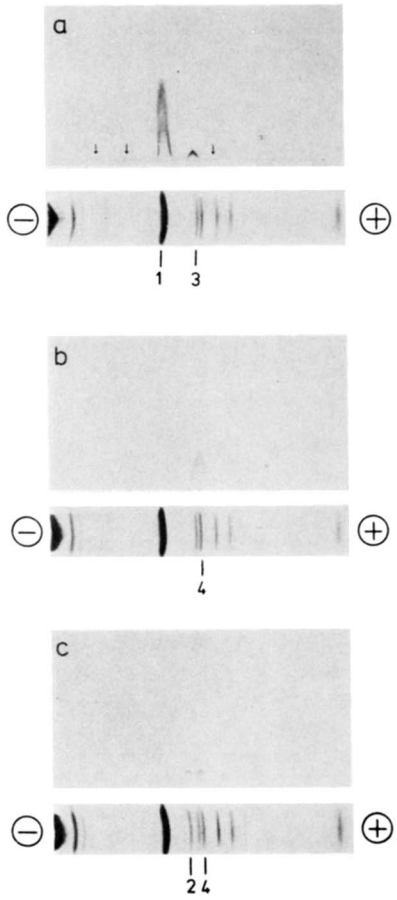

Fig. 3. Crossed immunoelectrophoresis after isoelectric focusing against anti-apoA-I (a), and anti-apoA-IV (b, c). Normal serum (a, b) and serum from a heterozygous Tangier patient (c) were used as serum samples. Arrows mark the minor isoproteins of normal apoA-I.

position 4 (Fig. 1) and could be detected in all sera. An additional protein with the molecular weight and the immunochemical properties of apoA-IV (Fig. 3c) could be detected in approx. 10% of all sera investigated and migrates in position 2 (Fig. 1). This isoform of apoA-IV was also present in the serum of patients affected with Tangier disease (Fig. 1; lanes F and G). Epidemiological studies in company employees have shown that 85.6% of this population has exclusively one apoA-IV-band in position 4, which was designated apoA-IV-1. Only 0.6% of the sera shows the apoA-IV-band in position 2 (apoA-IV-2) and 13.8% exhibits both bands. Genetic studies revealed an autosomal codominant trait for this apolipoprotein polymorphism (**Fig. 7**).

In all sera investigated, apoA-II (band 8) was present and polymorphism was not detectable. Similarly, variations of the isoelectric focusing pattern of C apolipoproteins (Fig. 1 and **Fig. 8,** bands 9 and 10) could not be demonstrated. However, due to the low concentrations of the C apolipoproteins, they were barely visible and abnormalities may not have been discovered. Bands 5, 6, 7, and 11 (Fig. 1) do not correspond to any of the known apolipoproteins. They were identified as alpha-1 antitrypsin (band 5, 6, 7) and acid glycoprotein (band 11) by coelectrophoresis of authentic substances (Fig. 8). Albumin does not enter into the gel.

The screening procedure described here was also applied to sera derived from coronary angiography patients. This resulted in the discovery of three new variants of apoA-I (**Fig. 9**). The abnormal sera contained, in addition to normal apoA-I, an extra band in various positions that was identified as apoA-I by molecular weight and immunological properties (not shown). The variant forms of apoA-I were designated apoA-I-Münster-1, apoA-I-Münster-2, and apoA-I-Münster-3. Family studies indicated that all variant forms are transmitted by an autosomal codominant trait (**Fig. 10**).

DISCUSSION

Structural differences of apolipoproteins may have an impact on their functions regarding lipid binding, co-

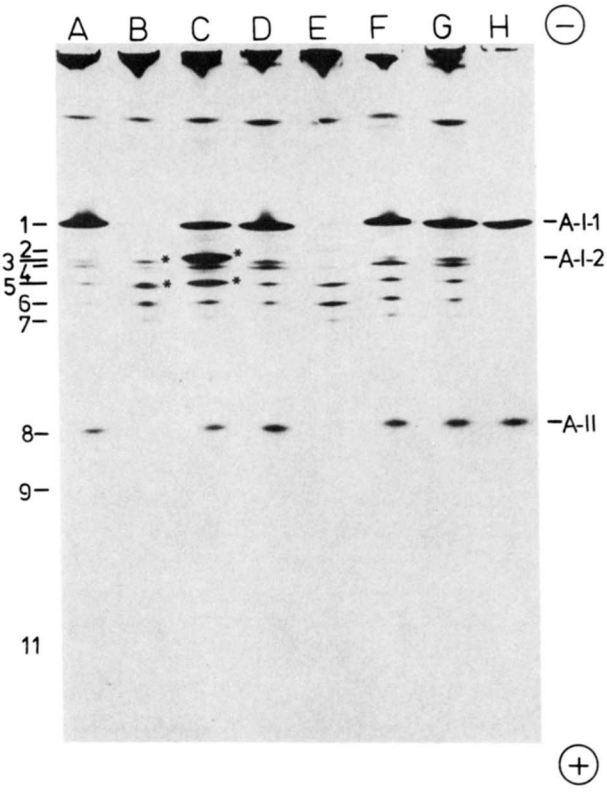

Fig. 4. Isoelectric focusing of normal serum (A, D, and G), serum from a patient with the apoA-I-Milano variant (B), serum from a patient with the apoA-I-Marburg variant (C), serum from homozygous (E) and heterozygous (F) Tangier patients, and apoHDL (H). Asterisks indicate additional bands characteristic for the two patients with the apoA-I variants. ApoA-I from apoHDL comigrates with bands 1 and 3 and apoA-II with band 8.

Fig. 5. Two-dimensional gel of serum from a patient with the apoA-I-Milano variant. Legend is the same as in Fig. 2. Asterisks mark the additional bands in this variant serum.

factor activity, and binding ability to cell receptors. With the method described here it is possible to screen large populations without ultracentrifugation of lipoproteins or other laborious and economically unfavorable procedures that involve ultracentrifugation for several days, dialysis, and delipidation. In this communication we describe a method to check for polymorphic forms of apolipoproteins A-I, A-II, and A-IV. The procedure is very simple and includes only incubation of serum followed by isoelectric focusing. We have shown that the variant forms of apoA-I (apoA-I-Milano, apoA-I-Marburg) and the deficiency of apoA-I in Tangier disease can be demonstrated. We were also able to identify three new forms of apoA-I. The variant form apoA-I-Münster-2 resembles apoA-I-Marburg in its pI value, but since the nature of the mutation is unknown in both cases, we prefer to use our own nomenclature until the molecular defect has been resolved. An attempt to estimate quantitatively the

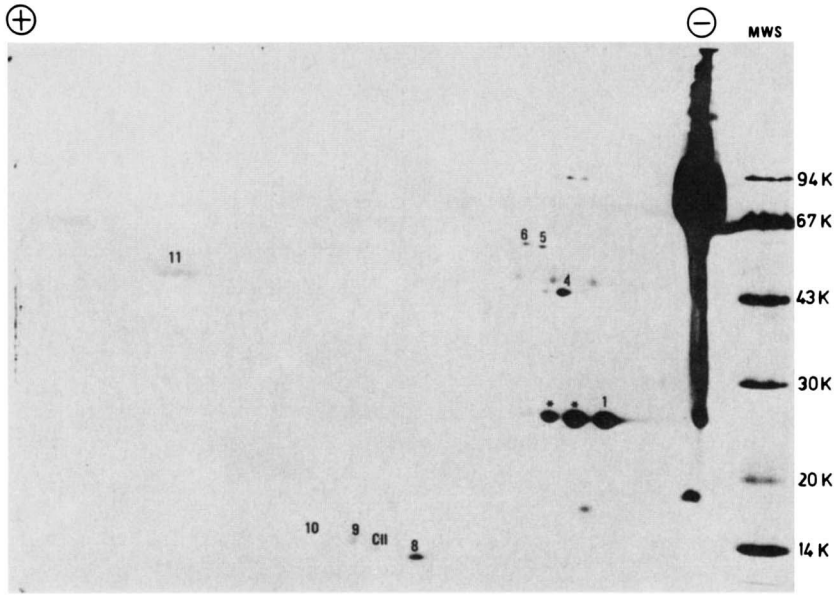

Fig. 6. Two-dimensional gel of serum from a patient with the apoA-I-Marburg variant. Legend is the same as in Fig. 2. Asterisks mark the additional bands in this variant serum.

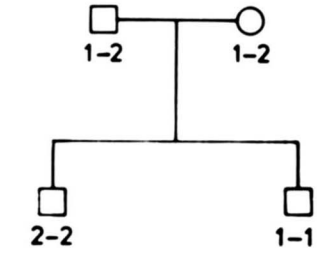

Fig. 7. Pedigree of a family with different isoforms of apoA-IV. Genotypes are indicated, with genotype 1-1 being characterized by the presence of apoA-IV-1 alone, genotype 2-2 by apoA-IV-2 alone, and genotype 1-2 by both isoforms.

recovery of the apolipoproteins was not made; the older methods are not even semiquantitative, and quantifying proteins by gel scanning requires a standard curve for each protein.

Investigations from other groups (15, 16) in recent years about the polymorphism of apolipoprotein A-I have demonstrated the existence of four to five isoforms. We have demonstrated here the existence of two major apoA-I isoforms (apoA-I-1, apoA-I-2) in fresh serum; only the application of two-dimensional techniques showed three further isoforms of apoA-I which occur in minute concentrations. Two of these are more basic and one is more acidic than the major apoA-I isoforms. The minor isoproteins increased by varying amounts after ultracentrifugation, prolonged storage at room temperature, or with urea treatment[4] at the expense of the major isoproteins. Compared to the results of Zannis and Breslow (16), our pattern of apoA-I isoforms appeared identical, even in the quantitative distribution. Our band 1 (apoA-I-1) is identical to their spot number 4 and our band 3 (apoA-I-2) corresponds to spot 5 (16). The main isoforms described by Nestruck, Suzue, and Marcel (15), A-I-1 and A-I-2, correspond to band 1 and band 3, respectively, in our system. The more basic forms of apoA-I were not mentioned by these authors.

In all sera investigated (approx. 2,500) we were not able to detect any polymorphic forms of apoA-II, whereas

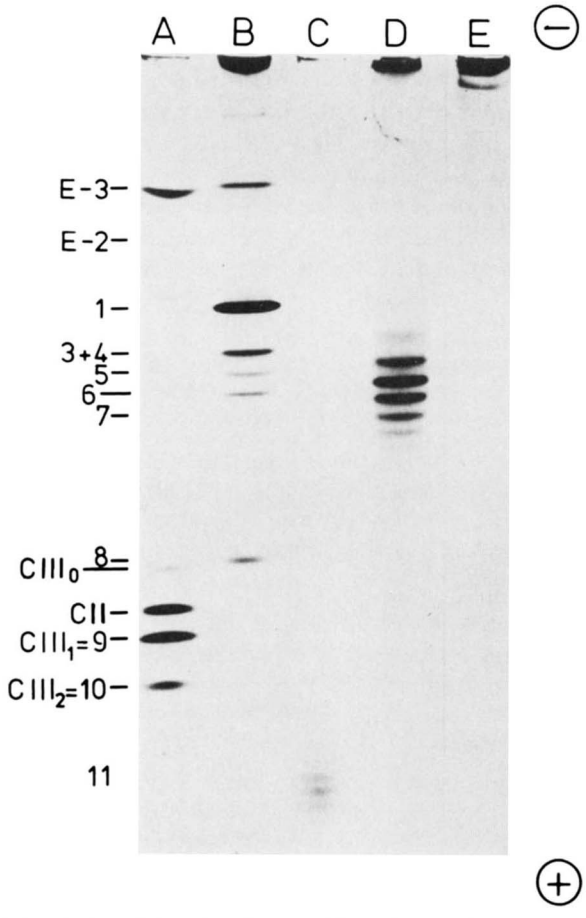

Fig. 8. Isoelectric focusing of apoVLDL (A), normal serum (B), acid glycoprotein (C), alpha-1-antitrypsin (D), and human albumin (E). Bands 9 and 10 comigrate with C-III$_1$ and C-III$_2$, respectively.

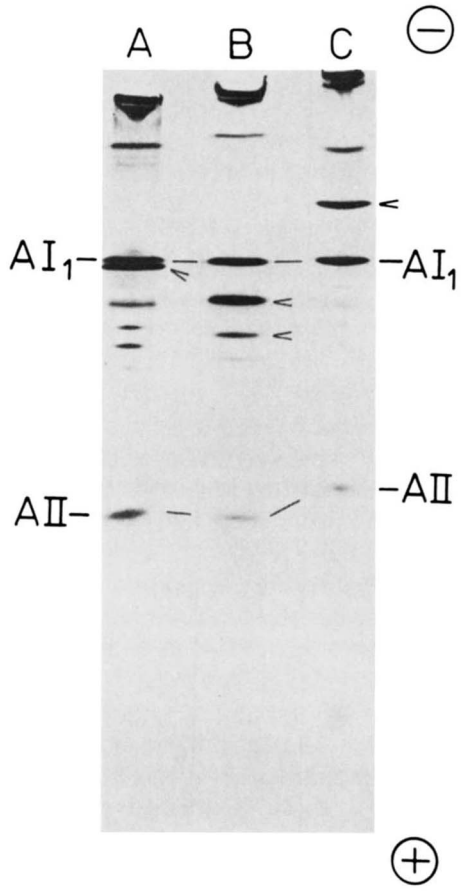

Fig. 9. Isoelectric focusing of sera with apoA-I variations. A), ApoA-I-Münster-1; B), apoA-I-Münster-2; and C), apoA-I-Münster-3. Arrows indicate apoA-I bands.

[4] Menzel, H-J., R-G. Kladetzky, and G. Assmann. Unpublished observation.

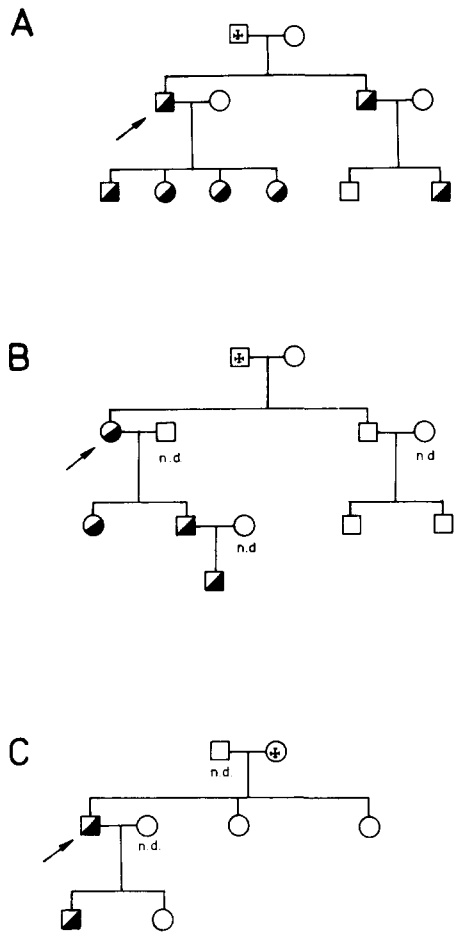

Fig. 10. Pedigree of three families with apolipoprotein A-I variations. A), Family with apoA-I-Münster-1; B), family with apoA-I-Münster-2; and C), family with apoA-I-Münster-3. ◪, ◑, affected members; □, ○, unaffected members; n.d., not determined.

about 10% of the normal sera showed an additional form of apoA-IV that has not previously been described. The amount of apoE and other apolipoproteins focusing between pH 4–6 is too small to give bands that allow a search for isoforms. It should also be possible to check for variations of the pattern of alpha-1-antitrypsin and the acid glycoprotein. The existence of microheterogeneity and genetic variations for these proteins revealed by isoelectric focusing has been demonstrated by several authors (17, 18).

Our method can easily be combined with two-dimensional gel systems and immunological applications. The two-dimensional gels exhibit fewer spots than the system described by Anderson and Anderson (19). This is due to a smaller amount of serum applied in the first dimension, a narrower pH gradient, and a higher acrylamide concentration (7.5% as opposed to 4%). In preliminary experiments we used the incubation methods of Anderson and Anderson (19) and O'Farrell (20) with SDS and NP-40, respectively, as well as our incubation and gel system (1% decylsulfate, 7.5% acrylamide, pH 4–6) for comparison. We obtained the same bands with all three detergents. Only the amount of alpha-1-antitrypsin was higher with SDS and NP-40. None of the other serum proteins of higher molecular weight or of more basic pI than albumin enter into the gel. In addition, the polypeptides of the haptoglobin β-chain, alpha-2-HS glycoprotein, fibrinogen alpha chain, alpha-1-antichymotrypsin, and the IgG light chains are present in negligible amounts and show only very diffuse spots in the second dimension (Fig. 2) in contrast to the two-dimensional system of Anderson and Anderson (19). Thus these proteins do not interfere in the identification of the bands we described.

The role of ampholytes (obtained from different sources and batches) in the separation of narrow bands must be considered. The difficulty has also been described by O'Farrell (20). We have observed that the apoA-I, apoA-IV, and the alpha-1-antitrypsin bands may differ in their position relative to each other. For example, the normal apoA-IV-band may comigrate with the apoA-I-2-band (band 3) or focus above or below depending on the source or batch of the ampholytes used. This may be due to association of protein with ampholytes resulting in an apparent change of the pI of the protein.

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