notes on methodology

A rapid micromethod for apolipoprotein E phenotyping directly in serum

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Summary A new method for the apolipoprotein E phenotyping has been developed. The method is based on isoelectric focusing of either delipidated or guanidine-HCl-treated serum or plasma in a horizontal slab gel system followed by immunoblotting using either polyclonal or monoclonal anti-apolipoprotein E antibodies as first antibody. Apolipoprotein E phenotyping with this method in 200 serum samples that had been stored at −20°C for more than one year gave exactly the same results as obtained with the conventional method based on isoelectric focusing of delipidated very low density lipoproteins isolated from fresh serum followed by protein staining. Compared with the conventional method, the present method is less laborious because ultracentrifugation to isolate VLDL is not needed; it is suitable for large scale screening purposes; it needs only a few microliters of serum or plasma, and can easily be performed with samples with low concentrations of apolipoprotein E.—**Havekes, L. M., P. de Knijff, U. Beisiegel, J. Havinga, M. Smit, and E. Klasen. A rapid micromethod for apolipoprotein E. phenotyping directly in serum.** *J. Lipid Res.* 1987. **28:** 455–463.

Supplementary key words delipidation • guanidine-HCl treatment • cysteamine treatment • isoelectric focusing • immunoblotting

In normal subjects, chylomicron and very low density lipoprotein (VLDL) remnants are rapidly removed from the circulation by means of receptor-mediated endocytosis in the liver or conversion into low density lipoprotein (LDL) (1). The apolipoprotein E (apoE) present on lipoprotein remnants plays a central role in the hepatic metabolism of remnant particles as this apolipoprotein is recognized with high affinity by the hepatic receptors involved in remnant uptake (2).

As determined by isoelectric focusing, human apoE can be separated into three major isoforms, i.e., E2, E3, E4, and a number of minor glycosylated isoforms (3, 4). The major isoforms differ in pI by a single charge unit, apoE4 being the most basic and E2 the most acidic. ApoE3 is the most commonly occurring isoform.

At present, a number of less frequently occurring apoE isoforms have been described. Some of these variants are more basic than apoE4 (5, 6) or more acidic than apoE2 (7, 8), and some of them have the same electric charge as E2 (9) or E3 (8, 10–12).

Except for apoE3 and E4, apoE2 and most of the less frequently occurring apoE isoforms have reduced activity for binding to lipoprotein receptors and cause thereby the clinical picture of dysbetalipoproteinemia. The dysbetalipoproteinemia is the major defect in type III hyperlipoproteinemia (13, 14) where chylomicron and VLDL remnants accumulate. Most of the type III hyperlipoproteinemic patients are homozygotes E2/E2. However, only about four percent of E2/E2 homozygotes develop type III hyperlipoproteinemia (15). The accumulation of chylomicron and VLDL remnant particles in the plasma often results in premature coronary artery and peripheral vascular disease and in xanthomatous lesions (16).

Until now, the determination of the apoE phenotype in order to diagnose type III hyperlipoproteinemia is done by isoelectric focusing of delipidated VLDL (apoVLDL) followed by protein staining. This method needs a fairly high amount of serum; it is also laborious and expensive inasmuch as ultracentrifugation is required to isolate VLDL and thus it is less suitable for large scale purposes. In this report we present a new rapid method for apoE phenotyping. With this method, delipidated serum is applied directly to an isoelectric focusing slab gel whereafter the apoE polymorphism is visualized by immunoblotting using anti-apoE antibodies as first antibody. This method needs only very small amounts of serum or plasma and can be easily used for large-scale diagnosis and population studies even in less well-equipped laboratories.

MATERIALS AND METHODS

Materials

Serum was prepared by low-speed centrifugation (10 min; 1000 *g*) of clotted blood freshly obtained from hyperlipidemic donors. Ampholytes (pH 5–7) were obtained from LKB (Bromma, Sweden). Nitrocellulose paper (blotting paper) was purchased from Schleicher and Schüll (Dassel, FRG). Peroxidase conjugates of rabbit anti-goat IgG and goat anti-mouse IgG were obtained from Nordic Immunology (Tilburg, The Netherlands) and from Jackson Immunoresearch Laboratories (Avondale, PA), respectively. Dithiothreitol (DTT) was from

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; apo, apolipoprotein.

Aldrich (Brussels, Belgium). Guanidine-HCl and 4-chloro-l-naphthol were purchased from Merck (Darmstadt, FRG). All other chemicals were reagent grade.

Preparation of polyclonal and monoclonal anti-apolipoprotein E antibodies

For antibody preparation, apolipoprotein E was isolated from human VLDL derived from subjects with E3/3 phenotypes. For polyclonal antibody preparation, apolipoprotein E was isolated from VLDL exactly as previously described (11). A goat was immunized subcutaneously with 200 μg of apolipoprotein E mixed with 2 ml of complete Freund's adjuvant and then every 2 weeks with 200 μg of apolipoprotein E mixed with 2 ml of incomplete Freund's adjuvant. One hundred ml of blood was collected 7 days after each injection. After seven boosters the goat was bled.

For monoclonal antibody preparation, apolipoprotein E was isolated from human VLDL by preparative polyacrylamide gel electrophoresis (Desaga Apparatus) in SDS gels (13%) according to Neville (17). A female Balb/C mouse was immunized by two intraperitoneal injections (with complete and incomplete Freund's adjuvant, respectively) and a final intravenous injection in the tail vein was given 3 days before the cell fusion. The spleen cells (including the lymphocytes) were isolated and fused with myeloma cell line $P_3 \times 63$ Ag 8 U_1. The cells were plated in 96-well plates in HAT-DMEM (Gibco) (U. Beisiegel et al., unpublished results). A solid phase indirect binding assay with apoE coated in microtiter plates was used to detect anti-apoE in the cell supernatant. ^{125}I-labeled goat-anti-mouse IgG was used as second antibody (18). The culture supernatants that gave positive results in this assay were tested for recognition of the apoE isoforms on immunoblots of apoVLDL, separated by isoelectric focusing. The hybrid cell line EE7 (producing an IgG) was used for the immunoblot experiments shown in this paper.

Sample preparation

Two methods to dissociate apolipoprotein E from lipoprotein lipids were used.

Method A. Delipidation with chloroform–methanol. A 10-μl serum sample was diluted 1:10 with distilled water and then delipidated once with chloroform–methanol 2:1 (by volume) and once with ethyl ether. The protein pellet was stored at −20°C or used immediately. The pellet was resuspended in 40 μl of buffer (0.03 M Tris-HCl, 6 M urea, pH 8.2). After 10 min of shaking, the suspension was centrifuged (10 min, 10,000 *g*), whereafter the supernatant was diluted fourfold, unless otherwise indicated (see Fig. 4) with the same buffer.

Method B. Addition of guanidine-HCl. To avoid extensive sample manipulation, we tested the addition of guanidine-HCl in order to dissociate apolipoprotein E from lipoprotein lipids. Therefore, serum samples were diluted twofold with 1 M guanidine-HCl for 15 hr at 37°C. Thereafter the samples were diluted fivefold in the buffer used in method A.

To 30 μl of diluted sample, 10 μl of DTT (15 μg/ml) and 10 μl of ampholytes (pH 5–7, 10%) were added. Aliquots of 30 μl of this mixture were applied to the gel.

Cysteamine treatment

When cysteamine treatment was used, 10 μl of plasma was diluted 10-fold with freshly prepared 0.4 M cysteamine solution and incubated at 37°C for 15 hr. Thereafter the samples were delipidated as in method A and treated likewise. To 30 μl of delipidated and diluted sample, 10 μl of Tris–urea buffer was added instead of 10 μl of DTT solution. After addition of 10 μl of ampholytes (pH 5–7; 10%), aliquots of 30 μl of this mixture were applied to the gel.

Experimental

Isoelectric focusing with horizontal slab gels. The polyacrylamide gel (5% by weight) was composed exactly as described by Warnick et al. (19) containing 2% (by weight) ampholytes (pH 5–7).

The polyacrylamide gel solution was poured into a cassette of two perspex plates (10 × 24 cm) separated by a 2.3-mm spacer. On the inner side of one of the two perspex plates, 24 perspex squares (4 × 6 mm; 1 mm thick) were mounted in order to obtain individual slot formers **(Fig. 1A).** After polymerization, the cassette was put onto a horizontal LKB 2117 Multiphor cooling plate, with the 24 slot-formers at the upper side. Thereafter the upper plate was removed carefully (Fig. 1B). Both long sides of the gel were brought in contact with electrode buffers by means of strips of Whatman 3 MM filter paper hanging over in reservoirs containing the electrode buffer (0.02 M NaOH at the cathode; 0.01 M H_3PO_4 at the anode) **(Fig. 2).** The gel between both strips of filter paper (electrodes) was covered with a perspex plate (1 mm thick) perforated at the position of the slots. The slots, located near the cathode, were completely filled with 30 μl of sample using a microsyringe and then closed with a sheet of plastic. The electrophoresis was run subsequently at 100 V for 30 min, 300 V for 2 hr, and at 500 V for another 15 hr. During the isoelectric focusing, the gel was cooled at a temperature of 6°C.

Immunoblotting. After isoelectric focusing, the slab gel was washed in a blot buffer (192 mM glycine, 25 mM Tris-HCl, 20 volume % methanol) for 30 min, followed

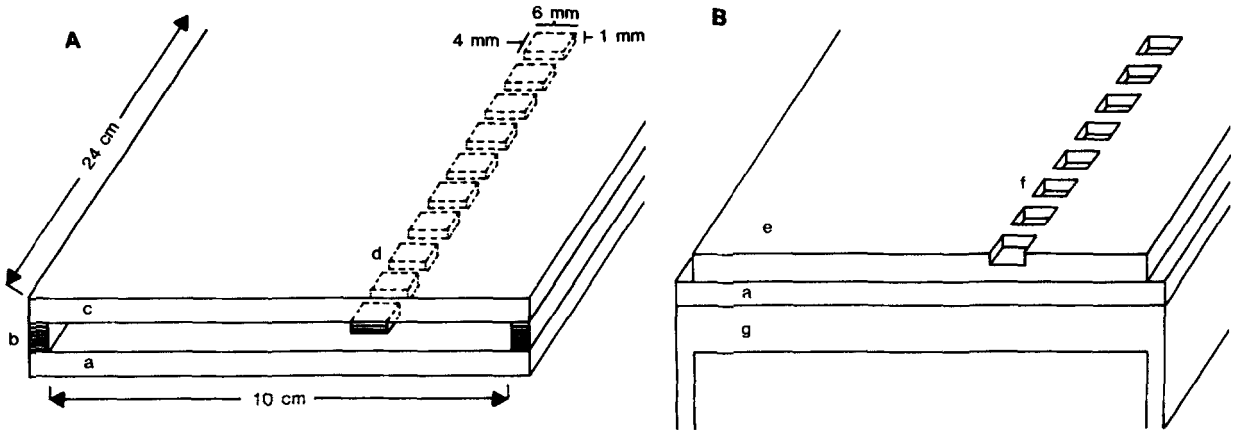

Fig. 1. Schematic presentation of the preparation of the polyacrylamide slab gel. A) a, Lower perspex plate; b, rubber spacer (2.3 mm); c, upper perspex plate; d, perspex slot former. B) a, Lower perspex plate; e, polyacrylamide gel; f, slot; g, cooling plate.

by electroblotting on nitrocellulose paper in the same blot buffer, but without methanol, for 2 hr at 400 mA. After blotting, the nitrocellulose paper (blot) was incubated with a solution of bovine serum albumin (3% by weight) in buffer A (0.15 M NaCl, 10 mM Tris-HCl, 0.05 volume % Tween 20, pH 7.4) for 1 hr at room temperature in order to saturate the blot with protein. Then the blot was washed (two times, 15 min) with buffer A followed by incubation at room temperature in buffer A in the presence of 0.1% (by volume) of goat anti-apoE antiserum. After 1 hr of incubation at room temperature, the blot was washed (two times, 15 min) in buffer A and further incubated in buffer A but in the presence of 0.1% (by volume) of rabbit-anti-goat IgG conjugated to horseradish peroxidase. After 1 hr of incubation at room temperature the blot was washed (two times, 15 min) and, eventually, developed by incubation at room temperature in a substrate solution consisting of 3 mM 4-chloro-1-naphthol, 3 mM H_2O_2, 200 mM NaCl, 50 mM Tris HCl, pH 7.4, and 17% (by volume) methanol. The enzyme reaction was stopped by washing the blot with distilled water. When monoclonal anti-apoE antibodies were used, the blot was incubated with supernatant medium from the EE7 clone (diluted fivefold with buffer A) for 18 hr at 4°C. For all further steps, the blot was kept at 4°C except for the incubation with the peroxidase substrate solution. Goat anti-mouse IgG conjugated to horseradish peroxidase was used as second antibody.

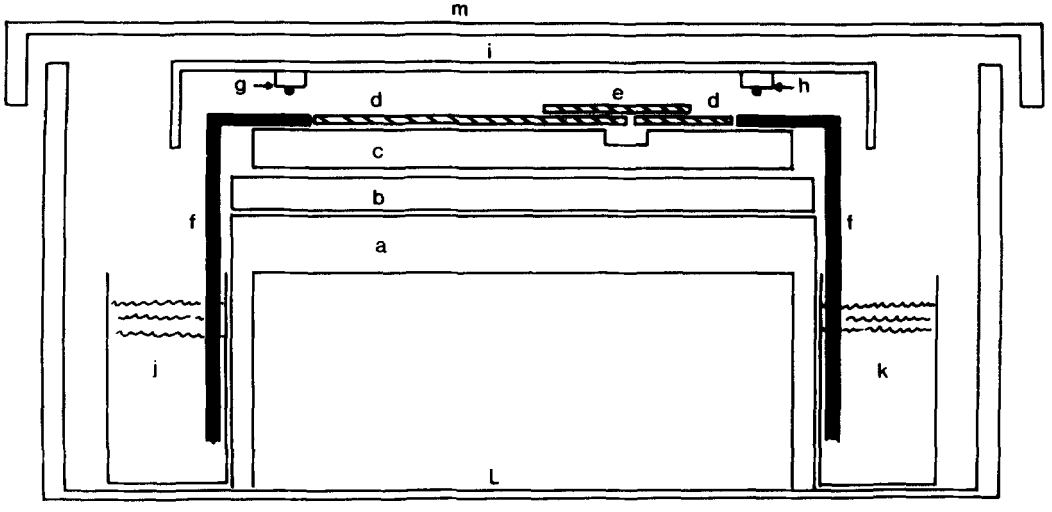

Fig. 2. Diagrammatic cross-section of the horizontal slab gel isoelectric focusing system. For text see Experimental section; a, cooling plate; b, lower perspex plate; c, polyacrylamide slab gel; d, perforated covering perspex plate (1 mm thick); e, plastic sheet; f, filter paper; g, anode; h, cathode; i, electrode holder; j, anode buffer reservoir; k, cathode buffer reservoir; l, electrophoresis box; m, cover.

Conventional isoelectric focusing. Isoelectric focusing of apoE using the conventional method was carried out as described by Menzel, Kladetzky, and Assmann (20) using delipidated VLDL.

RESULTS

Fig. 3 shows isoelectric focusing patterns of various delipidated VLDL samples performed by the conventional method described by Menzel et al. (20). In **Fig. 4,** isoelectric focusing slab gels loaded with various amounts of delipidated serum samples from hyperlipidemic donors are shown. Parallel isoelectric focusing slab gels were used for immunoblotting using either polyclonal (Fig. 4a) or monoclonal (Fig. 4b) anti-apoE antibodies as first antibody. After immunoblotting and peroxidase staining, the three major apoE isoforms were clearly visualized. With polyclonal anti-apoE antiserum as first antibody (Fig. 4a), a minor band appeared between E3 and E4 when higher amounts of delipidated serum were applied to the gel. With monoclonal antibodies (Fig. 4b), minor bands with apoE immunoreactive material were only found at the E1 position which are probably sialated and/or deamidated derivatives of the major apoE isoforms.

Fig. 5 shows the immunoblot of the apoE isoelectric focusing patterns of 13 different hyperlipidemic serum samples using monoclonal antibodies as first antibody. These serum samples had been stored at − 20°C for more than 1 year.

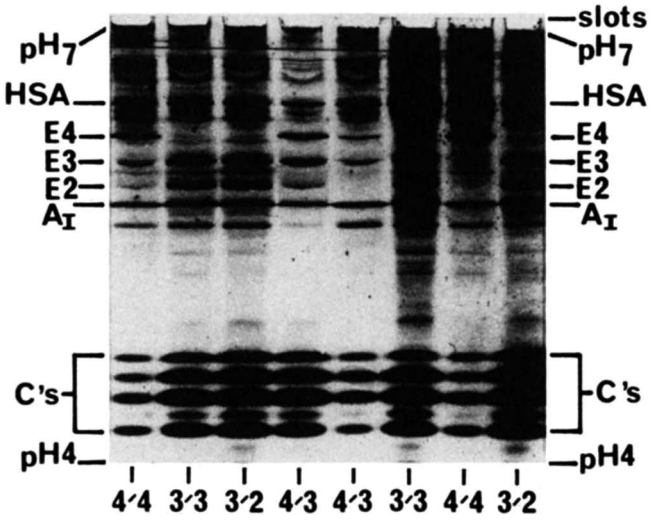

Fig. 3. Isoelectric focusing of apoVLDL from eight different serum samples by the conventional method (20). VLDL was isolated by density gradient ultracentrifugation (d < 1.006 g/ml). The pH gradient was 4–7. The gel was stained with Coomassie Brilliant Blue after fixation with trichloroacetic acid and sulfonic acid. The positions of the different apolipoproteins and of residual human serum albumin (HSA) are indicated.

Fig. 6 shows apoE isoelectric focusing patterns of 20 different fresh hyperlipidemic serum samples using polyclonal antibodies as first antibody. It is obvious from Fig. 6 that the dissociation of apoE from lipoprotein lipids by incubation of the serum samples in 0.5 M guanidine-HCl (Fig. 6b) leads to apoE isoelectric focusing patterns similar to those obtained after delipidation of the serum samples with chloroform-methanol (Fig. 6a). Incubation of the serum samples in 0.5 M guanidine-HCl instead of delipidation with chloroform-methanol requires much less extensive sample manipulation.

Interpretation of one-dimensional isoelectric focusing pattern of apoE may be confounded by a partial sialation or deamidation of apoE isoforms. In order to validate our method relating to the possible confounding effects of sialation and or deamidation, we have adapted our method to split-sample analysis with and without cysteamine treatment. After cysteamine treatment the major part of E2 and E3 focus at the position of E4 **(Fig. 7),** suggesting that at least the major parts of E3 and E2 do not represent monosialated or deamidated derivatives of E4 and E3, respectively. Fifty plasma samples (phenotype frequency: 21, 26, 3 for E3/E3, E3/E2 and E2/E2, respectively) were analyzed with and without cysteamine treatment. For 49 plasma samples, the results obtained after cysteamine treatment were in accordance with the results obtained without cysteamine treatment. However, for one E3/E2 plasma sample the apoE isoelectric focusing pattern was classified as E4/E3 instead of E4/E4 after cysteamine treatment (Fig. 7). This would suggest that in this sample a major part of E3 is monosialated or deamidated and focuses therefore at the position of E2 when analyzed without cysteamine treatment, and thus the sample would be falsely scored as E3/E2. However, DNA hybridization using synthetic oligonucleotides showed that this sample is an E3/E2 ($lys_{146} \rightarrow gln$) phenotype (M. Smit et al., unpublished results). E2 ($lys_{146} \rightarrow gln$) contains only one cys residue and will focus therefore at the position of E3 after cysteamine treatment.

DISCUSSION

In order to prepare immunoblots, isoelectric focusing was carried out with slab gels. Several slab gel systems could be used. In our laboratory, the vertical slab gel system as used by Menzel et al. (20) (Fig. 3) did not result in sharp isoelectric focusing patterns of apoE after immunoblotting. This is probably due to capillary leakage of the sample between the gel and the two glass plates. Capillary leakage of the sample is easily visualized by the highly sensitive immunoblotting technique. In the horizontal slab gel system as schematically drawn in Figs. 1

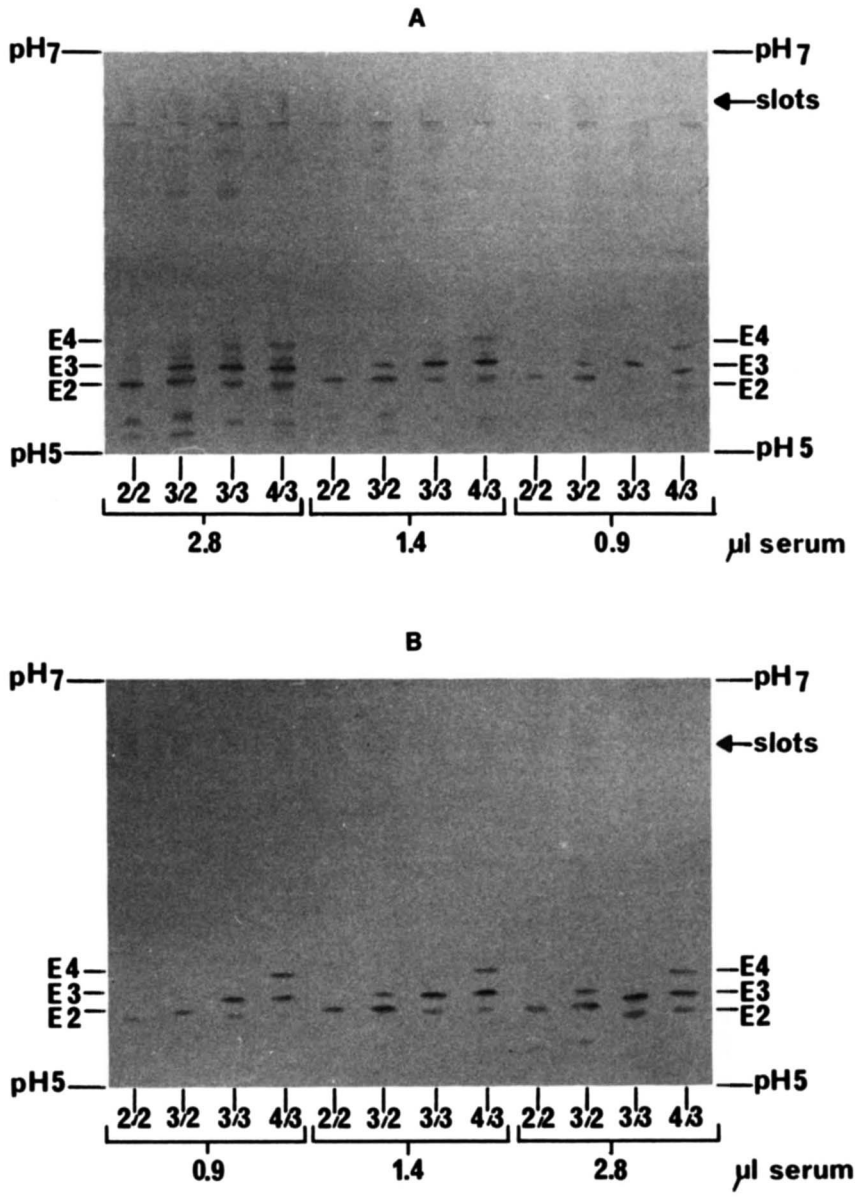

Fig. 4. Isoelectric focusing slab gels applied in parallel with increasing amounts of four different chloroform-methanol-delipidated serum samples. After delipidation, the protein pellet was dissolved in the Tris-HCl buffer (Materials and Methods) and diluted appropriately in order to apply the amount of serum as indicated. Serum samples had been stored at −20°C for more than 1 year. Gels were developed by immunoblotting using either polyclonal (A) or monoclonal (B) anti-apoE antibodies. ApoE phenotypes as indicated in the figure were determined with the conventional method for apoE phenotyping using isoelectric focusing of delipidated VLDL followed by protein staining immediately after collecting the blood.

and 2, capillary leakage between the gel and the lower perspex plate cannot occur.

However, a general disadvantage of horizontal slab gel systems is either condensation of water on the gel surface or drying of the gel during electrophoresis, depending on the relative humidity in the electrophoresis box. To prevent this phenomenon, we covered the gel with a perspex plate. This plate was perforated at the position of the slots in order to be able to fill the slots afterwards. In our experiments the sharp isoelectric focusing bands were obtained when the slots remained completely filled during the electrophoresis. Therefore, after application of the samples to the slots via the perforations, the perforations of the perspex plate were covered with a plastic sheet preventing diffusion of the sample solvent into the gel.

Our results show that an additional minor band was

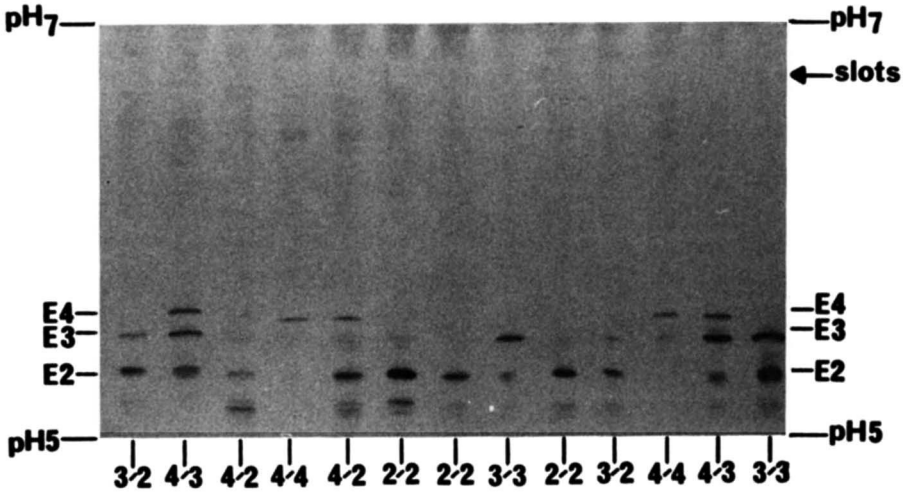

Fig. 5. ApoE immunoblot of an isoelectric focusing slab gel using monoclonal antibodies. Thirteen different serum samples that had been stored at −20°C for more than 1 year were applied. ApoE phenotypes as indicated in the figure were determined with the conventional method for apoE phenotyping using isoelectric focusing of delipidated VLDL followed by protein staining immediately after collecting the blood.

obtained between E3 and E4 when relatively high amounts of serum were applied to the gel. It is our experience that this occurs only when old serum samples were applied, together with the use of a polyclonal anti-apoE antiserum instead of a monoclonal antibody as first antiserum (compare Fig. 4a with Figs. 4b and 5). With fresh serum samples this additional minor band did not appear (Fig. 6a). This suggests that this minor band between E3 and E4 is due to proteolysis during storage. The two minor bands at the E1 position were obtained with both monoclonal and polyclonal antibodies and their appearance is independent of the duration of the sample storage. These E1 bands were also obtained when apo VLDL was used with this immunoblot method. Since these bands partly disappeared after treatment of VLDL with neuraminidase, we strongly suggest that the presence of apoE immunoreactive material at the E1 position is due to sialation and deamidation of the major apoE isoforms. Also, with the conventional method of isoelectric focusing of apoVLDL followed by protein staining, minor bands at the E1 position are commonly found. In addition to sialated apoE isoforms that focus at position E1, mono- or di-sialated apoE4 and E3 isoforms exist and may confound the apoE phenotyping. For instance, homozygotes E4/E4 may falsely be scored as heterozygotes E4/E3 or E4/E2 and homozygotes E3/E3 may be classified as heterozygotes E3/E2. This problem of sialation arises in all one-dimensional apoE focusing methods and therefore, in this respect, the present method is as reliable as the conventional method. Usually, sialation of apoE isoforms occurs only at a minor portion, implying that a phenotype has to be classified as homozygote when the most basic band is clearly stronger than the more acidic bands. Reciprocally, when the most basic band is weaker than the more acidic one, the respective phenotype is scored as heterozygote (see Figs. 4–6). Only in those cases where two isoforms are about equally strong must special attention be paid in order to discriminate correctly between homozygote and heterozygote. With the present method, the problems of discriminating between homozygote and heterozygote in those particular cases are mostly solved by applying smaller amounts of sample (compare in Fig. 4a, phenotype 3/2 with 0.9 and 2.8 μl serum, respectively). Only with the very few samples where this is not the case, should phenotyping be repeated by a method of commonly accepted reliability such as the two-dimensional electrophoresis (4) or the split-sample cysteamine treatment technique (21). We found that the present method of immunoblotting can easily be adapted for analysis of serum samples that have been treated with cysteamine. We analyzed the apoE by isoelectric focusing in 50 plasma samples both in the presence and absence of cysteamine. The apoE phenotypes determined in the plasma samples without cysteamine treatment were in accordance with the results obtained with the respective cysteamine-treated plasma samples. This confirms the reliability of the present method.

All serum samples used in Figs. 4 and 5 had been stored at −20°C for more than 1 year. The phenotypes indicated in these figures are deduced from apoE phenotyping performed with the conventional method immediately after collecting the serum samples. Presently, we

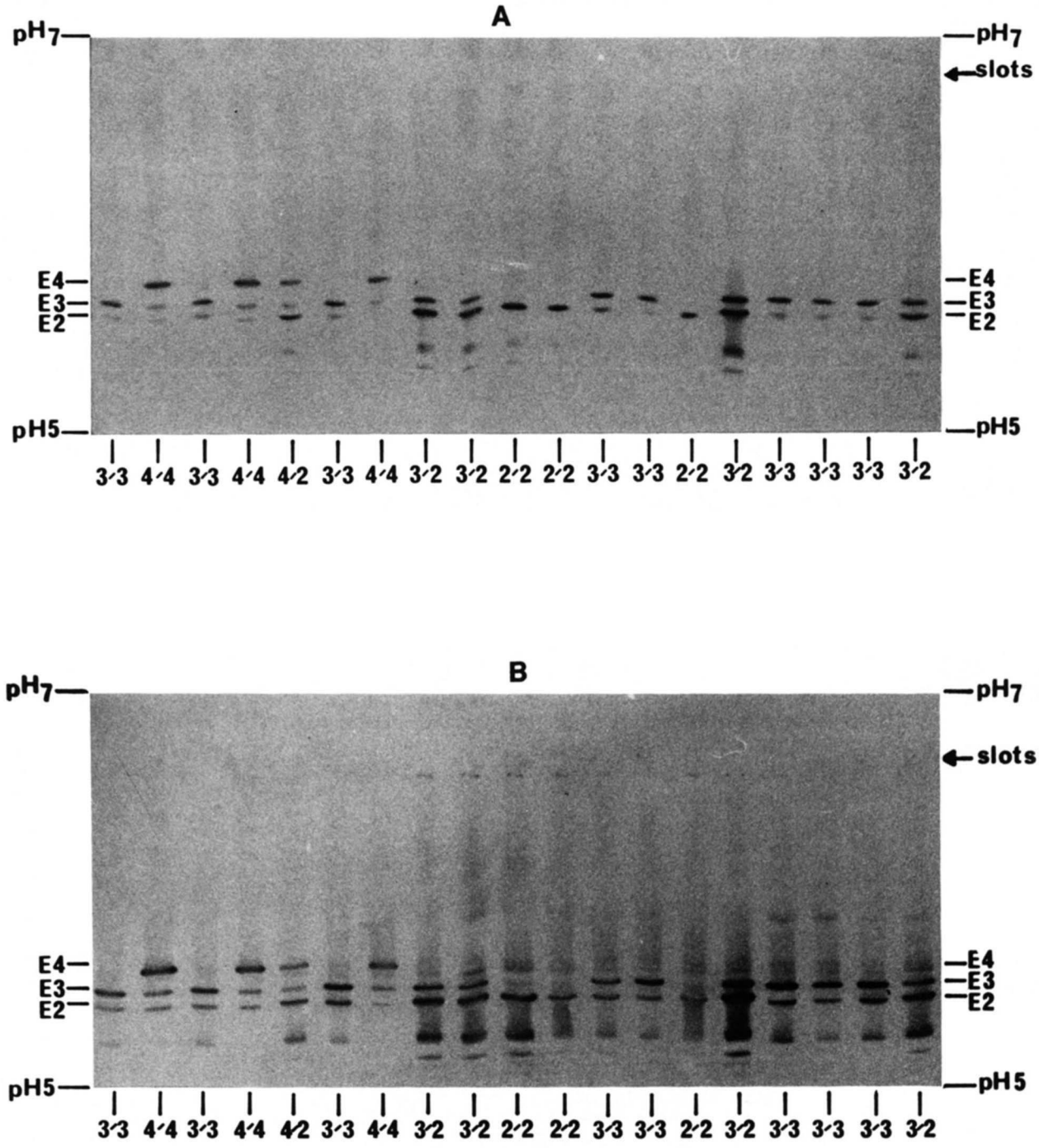

Fig. 6. ApoE immunoblot of isoelectric focusing slab gels applied in parallel with 20 different serum samples that had been delipidated either with chloroform–methanol (A) or by incubation in 0.5 M guanidine–HCl (B). Polyclonal anti-apoE antibodies were used as first antibody. The serum samples had been stored at −20°C for less than 2 weeks.

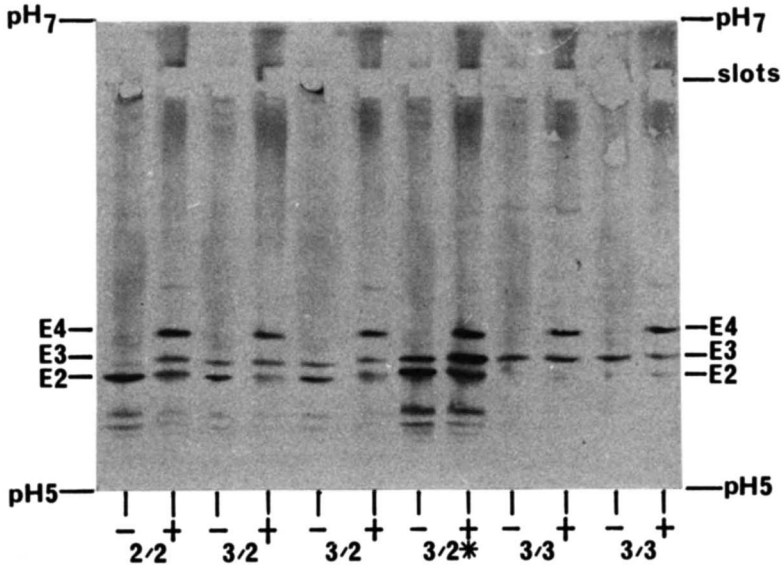

Fig. 7. ApoE immunoblot of isoelectric focusing slab gels of 6 of 50 different normolipidemic plasma samples with (+) and without (−) cysteamine treatment. After treatment the samples were delipidated with chloroform–methanol. Polyclonal anti-apoE antibodies were used as first antibody. The samples had been stored for 4 to 5 months at −20°C. *, Plasma sample with E3/E2 phenotype. DNA hybridization techniques using synthetic oligonucleotides revealed that this plasma contained E2 ($lys_{146} \rightarrow glu$) (M. Smit et al., unpublished results).

have determined the apoE phenotype with this immunoblot method in about 200 serum samples that had been stored at −20°C for 1 to 4 years and have compared the results with those obtained with the conventional method immediately after collecting the serum samples. Thus far, no false scores have been obtained. Thus, the method can safely be used with old serum samples; however, because of the possible appearance of some minor bands upon storage, we prefer the use of fresh serum samples, especially when polyclonal antiserum is used.

Although all results shown in this paper were obtained with serum samples collected from lipid clinic outpatients, the present method is also suitable for EDTA-plasma samples from either hyper- or normolipidemic subjects. With normolipidemic plasma samples we usually apply twice as high amounts of sample to the gel. With this method we have performed apoE phenotyping in plasma samples of 2000 apparently healthy and randomly selected 35-year-old men (M. Smit et al., unpublished results). We found that in this Dutch population the distribution of the apoE phenotypes was not significantly different from the expected Hardy-Weinberg distribution.

Taking these considerations of validation into account, we would like to conclude that the present method is of acceptable reliability.

In the present method, the delipidation of the serum samples with chloroform–methanol is the most laborious step. Therefore, we attempted to find a rapid and convenient method to dissociate apoE from lipoprotein lipids without the procedure of delipidation. Guided by the finding that exposure of human high density lipoproteins (HDL) to guanidine–HCl results in an irreversible dissociation of apolipoprotein A-I from HDL–lipid (22, 23), we treated serum with 0.5 M guanidine–HCl instead of delipidation with chloroform–methanol. Although some background staining was obtained, our results show that pretreatment of serum with 0.5 M guanidine–HCl offers a suitable and time-saving alternative to the conventional delipidation step (compare Fig. 6a with 6b).

We conclude that the present method of isoelectric focusing of delipidated or guanidine–HCl-treated serum or plasma, followed by immunoblotting using anti-apoE antibodies as first antibody, is a valid method for apoE phenotyping. When compared with the conventional method of isoelectric focusing of delipidated VLDL followed by protein staining, the present method offers the following advantages: *i*) the apoE isoelectric focusing patterns are easy to score since residual non-apoE proteins are not visualized (compare Fig. 3 with Figs. 4–7); *ii*) it is less laborious because an ultracentrifugation step to isolate VLDL is not needed; *iii*) it is suitable for large scale diagnosis and population studies even in less well-equipped laboratories; *iv*) it needs only a few microliters of serum or plasma that may have been stored for long

periods of time; and *v*) because of the high sensitivity, minor apoE isoforms can easily be detected in serum or serum density fractions.

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REFERENCES

1. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–655.
2. Sherrill, B. C., T. L. Innerarity, and R. W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J. Biol. Chem.* **255:** 1804–1807.
3. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinemia in man. *Nature.* **269:** 604–607.
4. Zannis, V. I., and J. L. Breslow. 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. *Biochemistry.* **20:** 1033–1041.
5. Yamamura, T., A. Yamamoto, K. Hiramori, and S. Nambu. 1984. A new isoform of apolipoprotein E—apo E5—associated with hyperlipidemia and atherosclerosis. *Atherosclerosis.* **50:** 159–172.
6. Yamamura, T., A. Yamamoto, T. Sumiyoshi, K. Hiramori, Y. Nishioeda, and S. Nambu. 1984. New mutants of apolipoprotein E associated with atherosclerotic diseases but not to type III hyperlipoproteinemia. *J. Clin. Invest.* **74:** 1229–1237.
7. Ghiselli, G., R. E. Gregg, and H. B. Brewer. 1984. Apolipoprotein E-Bethesda. Isolation and partial characterization of a variant of human apolipoprotein E isolated from very low density lipoproteins. *Biochim. Biophys. Acta.* **794:** 333–339.
8. Weisgraber, K. H., S. C. Rall, Jr., T. L. Innerarity, R. W. Mahley, T. Kuusi, and C. Ehnholm. 1984. A novel electrophoretic variant of human apolipoprotein E: identification and characterization of apolipoprotein E1. *J. Clin. Invest.* **73:** 1024–1033.
9. Rall, S. C., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
10. Havel, R. J., L. Kotite, J. P. Kane, P. Tun, and T. Bersot. 1983. Atypical familial dysbetalipoproteinemia associated with apolipoprotein phenotype E3/3. *J. Clin. Invest.* **72:** 379–387.
11. Havekes, L. M., J. A. Gevers Leuven, E. Van Corven, E. De Wit, and J. J. Emeis. 1984. Functionally inactive apolipoprotein E3 in a type III hyperlipoproteinaemic patient. *Eur. J. Clin. Invest.* **14:** 7–11.
12. Havekes, L. M., E. De Wit, J. A. Gevers Leuven, E. Klasen, G. Utermann, W. Weber, and U. Beisiegel. 1986. Apolipoprotein E3-Leiden. A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. *Hum. Genet.* **73:** 157–163.
13. Schneider, W. J., P. T. Kovanen, M. S. Brown, J. L. Goldstein, G. Utermann, W. Weber, R. J. Havel, L. Kotite, J. P. Kane, T. L. Innerarity, and R. W. Mahley. 1981. Familial dysbetalipoproteinemia. Abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits, and cows. *J. Clin. Invest.* **68:** 1075–1085.
14. Rall, S. C., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4700.
15. Utermann, G., K. H. Vogelberg, A. Steinmetz, W. Schoenborn, N. Pruin, M. Jaeschke, M. Hees, and H. Canzler. 1979. Polymorphism of apolipoprotein E. II. Genetics of hyperlipoproteinemia type III. *Clin. Genet.* **15:** 37–62.
16. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1980. The familial hyperlipoproteinemias. *In* The Metabolic Basis of Inherited Disease. 4th Ed. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 604–655.
17. Neville, D. M. 1971. Molecular weight determination of protein dodecyl sulphate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246:** 6328–6334.
18. Beisiegel, U., W. J. Schneider, J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monoclonal antibodies to the low density lipoprotein receptor as probes for study of receptor-mediated endocytosis and the genetics of familial hypercholesterolemia. *J. Biol. Chem.* **256:** 11923–11931.
19. Warnick, G. R., C. Mayfield, J. J. Albers, and W. R. Hazzard. 1979. Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type III. *Clin. Chem.* **25:** 279–284.
20. Menzel, H-J., R-G. Kladetzky, and G. Assmann. 1983. Apolipoprotein E-polymorphism and coronary artery disease. *Arteriosclerosis.* **3:** 310–315.
21. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518–2521.
22. Nichols, A. V., E. L. Gong, P. J. Blanche, T. M. Forte, and D. W. Anderson. 1976. Effects of guanidine hydrochloride on human plasma high density lipoproteins. *Biochim. Biophys. Acta.* **446:** 226–239.
23. Akazawa, S., Y. Ikeda, N. Kuriya, T. Nakanishi, K. Toyama, S. Miyake, and S. Nagataki. 1985. Radioimmunoassay of human plasma apolipoprotein A-I: pretreatment of plasma with guanidine hydrochloride. *Artery.* **12:** 388–398.