Human Very Low Density Lipoprotein Apolipoprotein E Isoprotein Polymorphism Is Explained by Genetic Variation and Posttranslational Modification†

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ABSTRACT: In a recent communication [Zannis, V. I., & Breslow, J. L. (1980) *J. Biol. Chem. 255*, 1759–1762], we demonstrated that very low density lipoprotein (VLDL) apolipoprotein E (apo-E) from different individuals occurs in one of two complex patterns when examined by two-dimensional polyacrylamide gel electrophoresis. These patterns have been designated class α and class β, and each one contains several apo-E isoproteins. Class α apo-E consists of two major isoproteins and at least ten minor isoproteins. In contrast, class β apo-E consists of only one major isoprotein and at least eight minor isoproteins. Three of the minor isoproteins of both class α and class β apo-E have the same molecular weight as the major apo-E isoprotein(s) (38 000), and the remaining minor isoproteins range in molecular weight from approximately 38 500 to 39 500. Mixing experiments of VLDL obtained from different subjects with either an α or a β pattern revealed that the α or β patterns were not always superimposable. In this manner, three subclasses of class α (αII, αIII, and αIV) and three subclasses of class β (βII, βIII, and βIV) have been identified. The difference between the α and β subclasses resides in the isoelectric point of some or all the isoproteins which comprise the α or β pattern. The examination of the apo-E subclasses in subjects with type II, type IV, and type VI hyperlipoproteinemia revealed that these individuals had the commonly occurring apo-E subclasses αII, αIII, αIV, and βIII. Subjects with type III hyperlipoproteinemia had only the apo-E subclass βIV. This apo-E subclass was not found in any subjects with other types of hyperlipoproteinemia or in control subjects. Further studies have provided insight into the biochemical explanation for the complex array of apo-E isoproteins observed. Posttranslational modification of apo-E was demonstrated by showing that treatment of VLDL with *Clostridium perfringens* neuraminidase prior to two-dimensional gel electrophoretic analysis resulted in the disappearance of the minor acidic isoproteins of mol wt 38 500 to 39 500 from the apo-E pattern. This indicated that this group of isoproteins arose by sialation of apo-E. These sialo apo-E isoproteins may play an important, but as yet undefined, role in the metabolism of plasma lipoproteins which contain apo-E. The genetic basis of the apo-E subclasses has also been demonstrated by examining the inheritance of the apo-E subclasses in families. These studies showed that the apo-E pattern is inherited at a single genetic locus with three common alleles, ϵII, ϵIII, and ϵIV. The β subclasses of apo-E represent homozygosity for two identical apo-E alleles. In contrast, the α subclasses of apo-E represent heterozygosity for two different apo-E alleles. This clarification of the mode of inheritance of apo-E will allow a better understanding of type III hyperlipoproteinemia, a human disease associated with premature atherosclerosis.

In 1973, apolipoprotein E (apo-E) was identified in normal human very low density lipoproteins (VLDL) (Shore & Shore, 1973). The reported molecular weight of apo-E is 33 000 (Shelburne & Quarfordt, 1974), 37 000 (Weisgraber & Mahley, 1978), or 39 000 (Utermann et al., 1975) in different laboratories, and its amino acid composition is enriched in arginine (Shelburne & Quarfordt, 1974). In addition to being present in VLDL, apo-E is also found in the HDL_2 density range (*d* 1.063–1.12 g/mL) (Mahley & Innerarity, 1977; Mahley, 1978; Innerarity & Mahley, 1978). A subfraction of HDL_2, designated HDL_C, which contains apo-E can be precipitated from plasma with heparin and manganese. In a manner thought to be mediated by apo-E, HDL_C has been shown to have a greater affinity than LDL itself for the LDL receptors of human skin fibroblasts (Mahley & Innerarity, 1977; Mahley, 1978; Innerarity & Mahley, 1978). Delipidated apo-E loses its affinity for the LDL receptor, but the affinity is restored when apo-E is mixed with phospholipid (Innerarity et al., 1979). The affinity of apo-E for the LDL receptor is diminished when the arginine residues of apo-E are chemically modified and also when apo-E has complexed through a disulfide bridge with apo-AII (Weisgraber & Mahley, 1978; Innerarity et al., 1978; Mahley et al., 1977). Plasma apo-E levels are increased in human type III (Havel & Kane, 1973; Kushwaha et al., 1977; Curry et al., 1976) and type V (Curry et al., 1976) hyperlipoproteinemias. Human apo-E has been shown to have isoprotein forms (Utermann et al., 1975, 1977a,b; Pagnan et al., 1977; Marcell et al., 1979; Warnick et al., 1979; Weidman et al., 1979), and it was recently suggested that a deficiency in one of these isoproteins is associated with type III hyperlipoproteinemia (Utermann et al., 1975, 1977a,b). Numerous nutritional and biochemical experiments in animals and humans indicate that apo-E may be intimately involved in regulating plasma cholesterol metabolism and perhaps in the atherogenic process (Mahley et al., 1974–1976; Shore et al., 1974; Rodriguez et al., 1976; Mahley & Holcomb, 1977; Breslow et al. 1977; Kushwaha & Hazzard, 1978; Wong & Rubinstein, 1978; Rudel et al., 1979; Noel et al., 1979; Zilversmit, 1979; Sherrill et al., 1980).

To better understand apo-E metabolism and any possible association of this apoprotein with type III hyperlipoproteinemia, we studied human plasma apo-E using high resolution, two-dimensional gel electrophoresis. In this report we show that human apo-E appears in one of two complex patterns, each of which consists of several isoproteins that differ in size and/or charge. These complex electrophoretic patterns of apo-E result partly from posttranslational modification of an original apo-E polypeptide and partly from a commonly occurring genetic variability in the apo-E gene of humans. We also demonstrate that patients with clinically diagnosed type

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III hyperlipoproteinemia do not have a deficiency (as previously suggested) of any of the apo-E isoproteins. Instead, eight type III patients possess an acidic subclass of apo-E, designated βIV, which was not found in any other type of hyperlipoproteinemia or in normal controls. This genetic apo-E variant may constitute a molecular marker that can be used for unequivocal early diagnosis and proper dietary management of type III hyperlipoproteinemia.

Experimental Procedures

Materials. Materials were purchased from the following sources: bovine serum albumin, ovalbumin, lysozyme, trypsin inhibitor, neuraminidase (*C. perfringens*), Tris, glycine, sodium bisulfite, and agarose from Sigma Chemical Co.; acid and alkaline phosphatase from Worthington Biochemical Corp.; neuraminidase (*V. cholerae* and influenza virus), rabbit muscle pyruvate kinase, bovine glutamate dehydrogenase, and rabbit muscle aldolase from CalBiochem; bovine spleen purine nucleoside phosphorylase from P-L Biochemicals, Inc.; *Staphylococcus aureus* V8 protease from Miles Laboratories, Inc.; ampholines (pH 2.5–4, 3.5–5, and 5–8) from LKB Instruments, Inc.; Nonidet P-40 from Particle Data laboratories, Ltd.; acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylenediamine, ammonium persulfate, Coomassie brilliant blue, bromophenol blue, and biolites (ampholines, pH 4–6) from Bio-rad; urea ultrapure grade from Schwarz/Mann; ^{125}I (17 Ci/mg) from New England Nuclear; Chloramine T from Eastman Kodak; Chronex 4 X-ray film from Du Pont. All other materials were of the purest grade commercially available.

Plasma Lipid and Lipoprotein Determination and VLDL Separation. Plasma was obtained from normal volunteers and from patients attending a clinic for individuals with plasma lipid disorders. After a 12-h fast, subjects resting in the sitting position for 5 min underwent venipuncture, and blood was obtained free flowing without tourniquet constriction. Specimens were collected in EDTA tubes and placed on ice. The red blood cells were separated by centrifugation at 2500 rpm for 20 min in a refrigerated centrifuge at 4 °C. The plasma was used for simultaneous determination of total cholesterol and triglycerides by the Technicon AutoAnalyzer II method as specified by the Laboratory Manual of the Lipid Research Clinics, sponsored by the NHLBI, and standardized by the Lipid Standardization Program of the Center for Disease Control in Atlanta (Technicon Instruments Corp., 1972; DHEW, 1974). Another aliquot of plasma was used for lipoprotein fractionation by ultracentrifugation also following the guidelines of the manual. During this procedure, VLDL was isolated from 5 mL of plasma after the specimen was overlayed with 1 mL of normal saline and centrifuged in a Beckman L5-65 ultracentrifuge for 18 h at 120000*g* in a 40.3 rotor. Each VLDL specimen was dialyzed overnight against 4 L of water at 4 °C, lyophilized, and used directly as a source of apo-E for two-dimensional gel electrophoretic analysis. In control experiments, delipidation of VLDL prior to analysis with chloroform–ether (2:1) did not affect the observed apo-E isoprotein patterns.

Enzymatic Treatments of Apo-E. Lyophilized VLDL was dissolved in 0.5 mL of the appropriate buffers at a concentration of 0.4 mg of VLDL protein/mL. The enzymatic treatments were performed as specified in the instructions for each commercial enzyme preparation used: (a) VLDL in 0.1 M sodium acetate buffer (pH 5.0) was treated with two units of *C. perfringens* neuraminidase at 37 °C for 2 h; (b) VLDL in 0.1 M sodium acetate buffer (pH 5.6) was treated with 4 units of influenza virus neuraminidase at 37 °C for 4 h; (c) VLDL in 0.1 M sodium phosphate buffer (pH 7.0) was treated with 4 units of *V. cholera* neuraminidase at 37 °C for 4 h; (d) VLDL in 0.1 M sodium acetate buffer (pH 5.0) was treated with 250 μg of acid phosphatase at 25 °C for 2 h; (e) VLDL in 0.1 M Tris-HCl buffer (pH 8.0) was treated with 2 units of alkaline phosphatase at 25 °C for 2 h. The treated VLDL samples were dialyzed against water, lyophilized, dissolved in the sample buffer or lysis buffer, and analyzed either by one-dimensional NaDodSO$_4$ or two-dimensional polyacrylamide gel electrophoresis, respectively.

NaDodSO$_4$–Slab Gel Electrophoresis and Apo-E Molecular Weight Determination. Electrophoresis was performed on a vertical gel apparatus constructed by a modification of the design described by Studier (1973). The slab gels were 17 × 20.5 cm with a thickness of 0.75 mm. The separating gel [11.7% acrylamide and 0.32% bis(acrylamide)] and the stacking gel [4.4% acrylamide and 0.12% bis(acrylamide)] were prepd. according to the method of Davis (1964). Lyophilized VLDL was dissolved in NaDodSO$_4$ sample buffer and one-dimensional separation performed (Davis, 1964). The molecular weight of apo-E was determined by NaDodSO$_4$–polyacrylamide gel electrophoresis with proteins of known molecular weight as standards (Shapiro et al., 1967; Weber & Osborn, 1969).

Two-Dimensional Polyacrylamide Gel Electrophoresis. Lyophilized VLDL was dissolved in a solution of 9.5 M urea, 2% (w/v) Nonidet P-40, 2.1% ampholines of the same composition as the focusing gels, and 5% β–mercaptoethanol. Two-dimensional polyacrylamide gel electrophoresis was carried out by using O'Farrell's method (O'Farrell, 1975) with previously described modifications (Zannis et al., 1978). The ampholines used were 1.2% (pH 5–8), 0.5% (pH 2.5–4), and 0.4% (pH 4–6). The samples were loaded in the first dimension without prerunning, and focusing was carried out for a total of 9500 V h with the equilibration time between the two dimensions limited to 15 min. For the second dimension, the focused cylindrical gels were placed on slab gels identical with those described above for one-dimensional NaDodSO$_4$–slab gel electrophoresis. After electrophoresis, the gels were fixed in 50% methanol–10% acetic acid for 1–2 h and stained in a solution of 0.25% Coomassie brilliant blue, 50% methanol, and 10% acetic acid for 1 h. Destaining was accomplished by sequential exposure of the gel for a period of 0.5–2 h to 100 mL of fixing solution followed by 400 mL of water until the proper stain background was achieved. Two such exchanges were usually sufficient.

Protein molecular weight markers used in NaDodSO$_4$–gel and two-dimensional gel electrophoresis were as follows: bovine serum albumin, 68 000; rabbit muscle pyruvate kinase, 57 000; bovine glutamate dehydrogenase 53 000; ovalbumin, 43 000; rabbit muscle aldolase, 40 000; bovine pure nucleoside phosphorylase, 30 000; human apo-AI, 28 000; trypsin inhibitor, 19 000; egg white lysozyme, 14 300.

Determination of Isoelectric Points and Estimation of Protein Concentration of Individual Apo-E Isoproteins. The apparent isoelectric points (PI) of the apo-E isoproteins identified by two-dimensional polyacrylamide gel analysis were estimated as follows: Six replicate focused gels representing the first dimension of a given two-dimensional analysis were cut in 6.5-mm slices. The slices were extracted with distilled water for 3 h, and their pH was measured with a radiometer Model PHM62 pH meter. The pH gradient was drawn by using an appropriate scale to correct for the expansion of approximately 10% which occurs during the destaining of the slab gel. The gradient was then superimposed on the dried

and stained two-dimensional polyacrylamide gel so that the origin and the end of the gradient coincided with the origin and end of the two-dimensional polyacrylamide gel, respectively. In this manner, the isoelectric points of the apoproteins were determined. The estimation of charge unit differences between apo-E isoproteins has been described previously (Zannis & Breslow, 1980). The relative concentrations of the major and minor apo-E isoproteins were estimated from the intensity of the Coomassie brilliant blue dye eluted from specific spots on the polyacrylamide gels (Fenner et al., 1975).

One-Dimensional Peptide Maps of Apo-E Isoproteins. Peptide maps of the isoproteins of apo-E were determined by cutting each isoprotein from a dried two-dimensional gel. The gel piece was then placed in a small siliconized tube, washed sequentially with distilled water and 10% methanol, and dried under vacuum. The iodination of the protein in the piece of gel was performed according to the method of Elder et al. (1977), except that only 100 μCi of ^{125}I was used per gel piece. After the iodination, the gel pieces were extensively washed with 10% methanol for 36 h followed by a 30-min incubation in 0.125 M Tris-HCl (pH 6.8), 0.1% $NaDodSO_4$, and 1 mM EDTA. Peptide mapping of these iodinated proteins was carried out as described by Cleveland et al. (1977) by loading gel pieces into sample wells and overlaying with 0.125 M Tris-HCl (pH 6.8), 0.1% $NaDodSO_4$, 1 mM EDTA, and 20% glycerol. *Staphylococcal aureus* V8 protease, 0.2 μg, or chymotrypsin, 0.5 μg, was added to each well in 10 μL of the Tris–$NaDodSO_4$–EDTA buffer containing 10% glycerol. Electrophoresis was then performed as described (Cleveland et al., 1977), but the current was turned off for 45 min when the bromophenol blue tracking dye reached the bottom of the stacking gel. After completion of the electrophoresis, the gel was dried and exposed to Cronex 4 X-ray film.

Results

Nomenclature and Description of Apo-E Classes and Subclasses. Apo-E is one of the major apoproteins of human VLDL and upon $NaDodSO_4$–slab gel electrophoresis appears as a broad protein band with an approximate molecular weight of 38 000–39 500 (Figure 1). Two-dimensional electrophoretic analysis of the partially or totally purified VLDL apo-E revealed that human apo-E exists in two major patterns designated class α and class β. In order to describe the complex electrophoretic patterns of apo-E, we have introduced new nomenclature (Zannis & Breslow, 1980). Class α apo-E consists of approximately 80% of two major isoproteins and 20% of at least ten minor isoproteins (Figure 2A,B). In contrast, class β apo-E consists of approximately 80% of only one major isoprotein and 20% of at least eight minor isoproteins (Figure 2C,D). Three of the minor isoproteins of both class α and class β apo-E have the same molecular weight as the major apo-E isoproteins (38 000), and the remaining minor isoproteins have a molecular weight ranging from approximately 38 500 to 39 500 as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis using proteins of known molecular weight as standards. Both class α and class β apo-E isoproteins are distributed in at least seven isoprotein groups numbered 1 through 7 (Figure 2A–D).

Two-dimensional polyacrylamide gel electrophoretic analysis of mixtures of VLDL obtained from different subjects with either class α or class β apo-E revealed that the α or β patterns were not always superimposable. A systematic analysis in hundreds of such mixing experiments allowed the identification to date of three subclasses of class α, designated αII, αIII, and αIV, and three subclasses of class β, designated βII, βIII, and βIV. Several such mixing experiments are shown in Figure

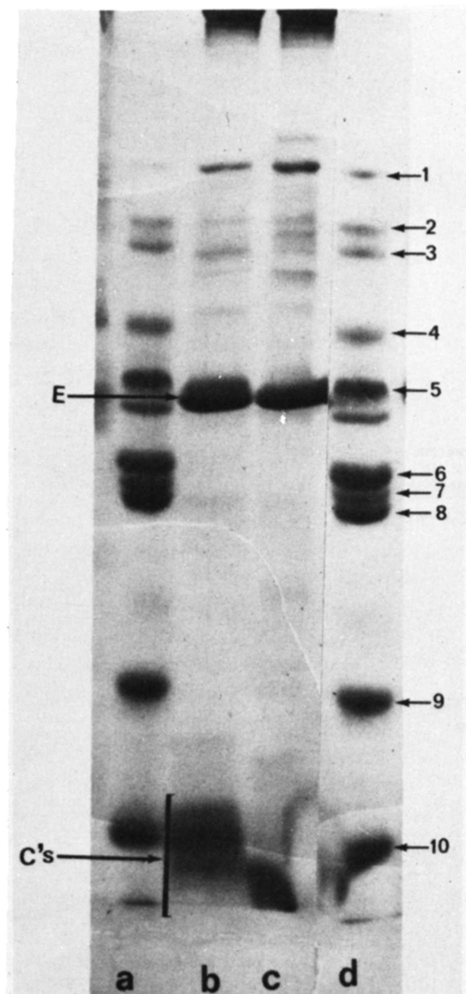

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of human VLDL before and after treatment with *C. perfringens* neuraminidase. (Lanes a and d) Molecular weight markers. (Lane b) Untreated VLDL (100 μg) and (lane c) VLDL (100 μg) after treatment with neuraminidase. The numbers 1–10 indicate molecular weight markers as follows: (1) bovine serum albumin, 68 000; (2) rabbit muscle pyruvate kinase, 57 000; (3) bovine liver glutamate dehydrogenase, 53 000; (4) ovalbumin, 43 000; (5) rabbit muscle aldolase, 40 000; (6) bovine purine nucleoside phosphorylase, 30 000; (7) apolipoprotein A-I, 28 000; (8) chymotrypsinogen, 25 700; (9) trypsin inhibitor, 19 000; (10) egg white lysozyme, 14 300. Note that neuraminidase treatment of VLDL in lane c not only removes the higher molecular weight band of apo-E but also causes a shift of the apo-C peptides, which are known to contain sialic acid, to lower molecular weight.

3A–E. The apo-E subclass αIV was only recently identified and had not been seen at the time of our recent communication. The apo-E subclass βIV was designated βIVa in our initial report (Zannis & Breslow, 1980). (See the section Stability of Apo-E Patterns for explanation.) A schematic representation of the apo-E subclasses and the relative differences in their isoelectric points are shown in Figure 4. In addition, the apparent isoelectric points and charge differences between isoproteins in each of the various apo-E subclasses are given in Table I. Our studies show that the apo-E isoprotein subclasses βII, βIII, and βIV are indistinguishable from each other without mixing experiments. Mixing experiments also allow one to see that the major apo-E isoprotein of apo-E subclasses βIII and βIV (along with the entire array of other apo-E isoproteins) is shifted by approximately 1 and 2 negative charges, respectively, relative to the major isoprotein of apo-E subclass βII (Figure 3A–C and Figure 4). The results also indicate that in the absence of mixing experiments, apo-E

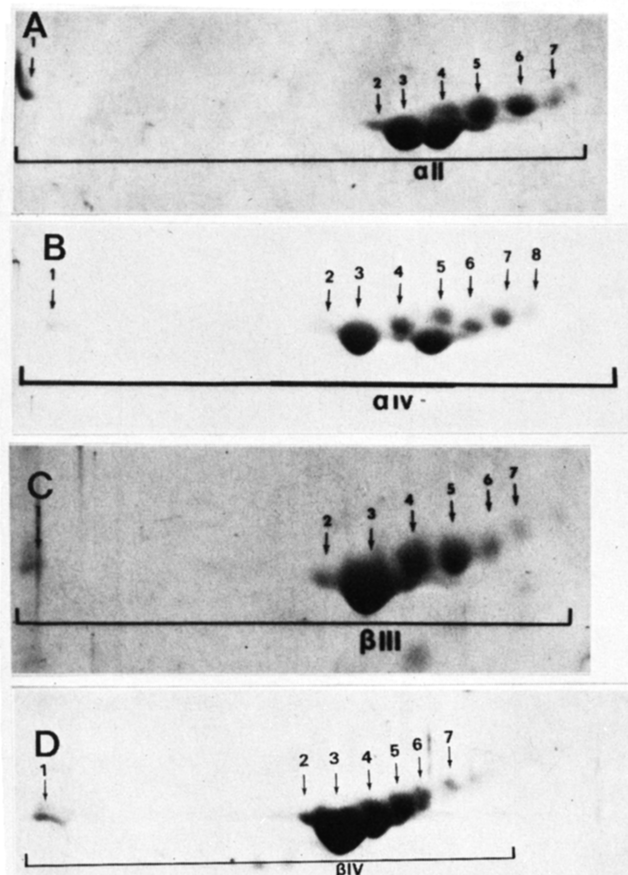

FIGURE 2: Two-dimensional gel electrophoretic patterns of VLDL apo-E are shown. Only the area of the gel in the vicinity of apo-E is presented. (Panel A) apo-E subclass αII (40 μg); (panel b) apo-E subclass αIV (40 μg); (panel C) apo-E subclass βIII (40 μg); (panel D) apo-E subclass βIV (40 μg). The apo-E subclass αIII is indistinguishable from αII, and the subclass βII is indistinguishable from βIII and βIV. Note that both the α and β apo-E subclasses contain numerous protein components (isoproteins) which differ in isoelectric point and/or molecular weight. These isoproteins are distributed in at least seven isoprotein groups numbered 1 through 7. (In this and all subsequent photographs, the cathode is on the left and the anode is on the right.)

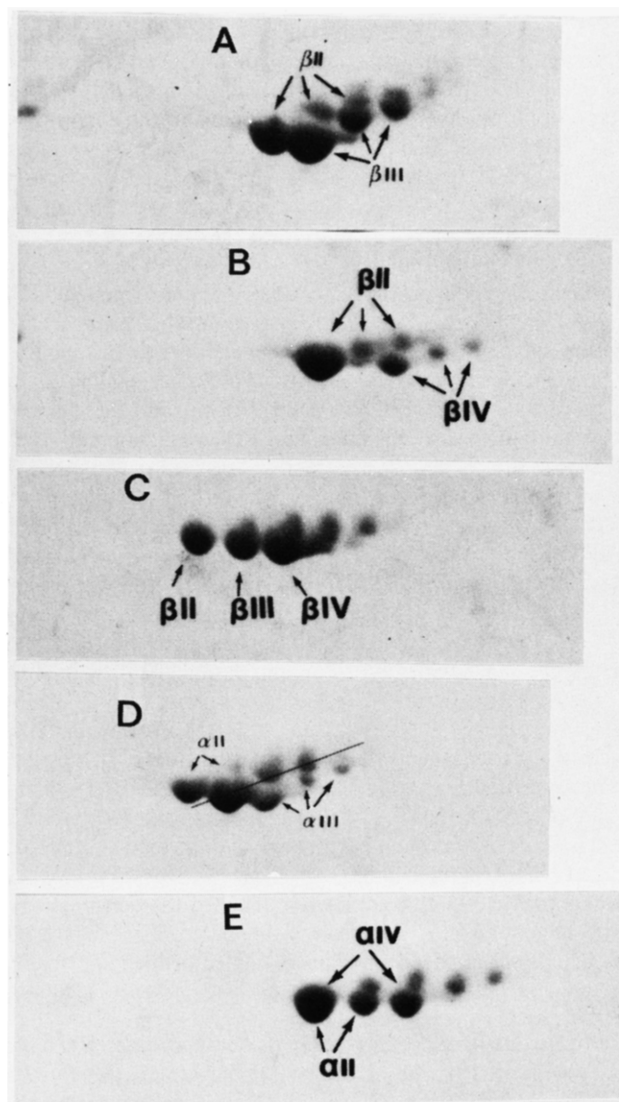

FIGURE 3: Two-dimensional gel electrophoresis patterns of mixtures of VLDL fractions obtained from individuals with different apo-E subclasses are shown. (Panel A) Mixture of 25 μg of βII and 30 μg of βIII. (A qualitatively similar pattern is obtained by mixing apo-E subclasses βIII and βIV—data not shown.) (Panel B) 25 μg of βII and 10 μg of βIV. (Panel C) 15 μg of βII, 15 μg of βIII and 15 μg of βIV. (Panel D) 30 μg of αII and 20 μg of αIII. (Panel E) 20 μg of αII and 20 μg of αIV. Note that the contribution of each subclass to the overall pattern is indicated. However, several components result from merging of isoproteins belonging to both apo-E subclasses.

Table I: Apparent Isoelectric Points and Charge Differences of Apo-E Isoproteins

isoprotein group	apo-E subclasses (p*I*)			charge difference relative to isoprotein group 3
	αII, αIV, βII	αIII, βIII	βIV	
1	⩾6.5	⩾6.5	⩾6.5	
2	6.30	6.18	6.02	+1
3	6.18	6.02	5.89	0
4	6.02	5.89	5.78	−1
5	5.89	5.78	5.70	−2
6	5.78	5.70	5.61	−3
7	5.70	5.61	5.51	−4

subclass αIII is indistinguishable from apo-E subclass αII. However, as illustrated in Figures 3D and 4, both major isoproteins of subclass αIII are shifted by one negative charge unit relative to their counterpats of subclass αII. It is also notable that the major isoproteins of the apo-E subclass αIV differ by two charge units (Figure 2B) as opposed to the major isoproteins of apo-E subclasses αII and αIII which differ by one charge unit (Figure 2A). The isoelectric point relationship of the major isoproteins of subclass αIV, relative to those of αII and αIII, is shown in Figure 4.

The mixing experiments just described also provided a clue to a possible genetic relationship between the α and β patterns of apo-E. We noted that the αII pattern could be generated by mixing apo-E subclasses βII and βIII (Figure 3A), and the αIII pattern could be generated by mixing apo-E subclasses βIII and βIV. Similarly, the apo-E subclass of αIV (Figure 2B) could be generated by mixing apo-E subclasses βII and βIV (Figure 3B). These observations suggested that the α subclasses might be heterozygous for alleles which specify a β subclass of apo-E.

Stability of Apo-E Patterns. In our routine procedure, plasma collection and two-dimensional analysis are performed within 1 week of each other. Under these conditions, the apo-E patterns obtained are very clear and reproducible (Figures 2 and 3). Repeat plasma sampling of the same subject on different days always revealed the same apo-E subclass. However, if the plasma or VLDL are allowed to stand at 4 °C for over a week, this results in gradual smearing of the pattern and a gradual shift of apo-E isoproteins to more acidic isoelectric points. This phenomenon explains the βIVa phe-

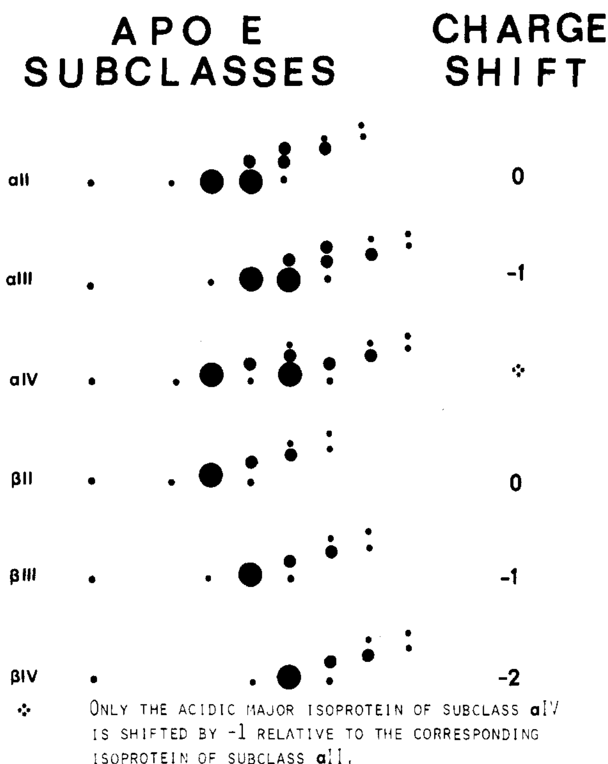

FIGURE 4: Schematic presentation of the charge relationship of human apo-E subclasses.

notype we originally observed with specimens from patients with type III hyperlipoproteinemia (Zannis & Breslow, 1980). The type III samples used in those analyses had been stored for a much longer period of time than the other samples tested. However, the repeat analyses of fresh samples from these initial patients revealed that their apo-E subclass βIV differs by 2 negative charge units from subclass βII, as opposed to βIVa which differed by 2.5 negative charge units from subclass βII. Prolonged storage also results in a decrease in the concentration of isoprotein 1 and of the minor isoproteins of molecular weight 38 500–39 500.

Characterization of Apo-E Isoproteins by One-Dimensional Peptide Mapping. To determine that all protein components that comprise the α or β subclasses were apo-E, the protein spots were cut from the gel and iodinated, and their one-dimensional chymotryptic peptide maps were determined (Elder et al., 1977; Cleveland et al., 1977). This analysis revealed that all protein spots of the α or β pattern of all six subclasses were apo-E. The chymotryptic peptide map of all the isoproteins of an α apo-E subclass has been presented before (Zannis & Breslow, 1980). A typical peptide map of the isoproteins belonging to a β apo-E subclass is shown in Figure 5A. A typical one-dimensional staphylococcal protease peptide map of the major apo-E isoprotein in each of the β subclasses of apo-E and the more basic of the two major apo-E isoproteins in each of the α subclasses of apo-E (apo-E isoprotein 3) are shown in Figure 5B.

Apo-E Subclass βIV Is Associated with Type III Hyperlipoproteinemia. The possible association of an apo-E subclass with a particular hyperlipoproteinemia pattern was studied by determining the apo-E subclass in 34 patients attending a clinic for individuals with plasma lipid disorders. Hyperlipoproteinemia phenotypes were defined as the following: type II, LDL cholesterol $\geq$ 170 mg/dL, TG < 140 mg/dL (Fredrickson et al., 1978); type III, cholesterol $\geq$ 230 mg/dL, TG $\geq$ 140 mg/dL, and the presence of β-VLDL of abnormal lipid composition (Hazzard et al., 1972; Mishkel et al., 1975; Fredrickson et al., 1975, 1978); type IV, LDL cholesterol < 170 mg/dL, TG $\geq$ 140 mg/dL (Frederickson et al., 1978); type VI, LDL cholesterol $\geq$ 170 mg/dL, TG $\geq$ 140 mg/dL (Fredrickson et al., 1978). Patients with types II, IV, and VI hyperlipoproteinemia had the commonly occurring apo-E subclasses αII, αIII, αIV, and βIII. In contrast, the type III hyperlipoproteinemia patients had only the apo-E subclass βIV. This apo-E subclass has not been seen in patients with other kinds of hyperlipoproteinemia or in any of 61 normal volunteers. Thus, this apo-E subclass may constitute a molecular marker for type III hyperlipoproteinemia.

Several of the Minor Apo-E Isoproteins Are Explained by Posttranslational Modification with Sialic Acid. VLDL was treated with three different preparations of neuraminidase prior to one-dimensional NaDodSO$_4$ and two-dimensional polyacrylamide gel electrophoresis. Figure 6A,B shows that treatment of VLDL with *C. Perfringens* neuraminidase resulted in the loss of the minor higher molecular weight apo-E isoproteins (38 500–39 500) of both α and β patterns. Since there was no observed change in the intensity of any of the major or minor apo-*E* isoproteins with mol wt 38 000, we assume that neuraminidase treatment affected only minor higher molecular weight apo-E isoproteins. In addition, two-dimensional polyacrylamide gel analysis of mixtures of neuraminidase-treated and untreated apo-E revealed that neither the molecular weight nor the charge of the major and minor apo-E isoproteins of mol wt 38 000 was affected by the neuraminidase treatment. These findings indicate that neuraminidase treatment is specific for the higher molecular weight apo-E isoproteins but has no effect on the apo-E isoproteins of mol wt 38 000. We also observed that *V. cholera* and influenza virus neuraminidases are less effective in removing the minor apo-E isoproteins, and treatment of VLDL with acid or alkaline phosphatase did not change the one-dimensional NaDodSO$_4$ or two-dimensional polyacrylamide gel electrophoresis patterns of apo-E.

The Genetic Basis of the Apo-E Subclasses. After neuraminidase treatment, the α subclasses of apo-E are composed principally of two isoproteins and the β subclasses of apo-E of one isoprotein. Furthermore, mixing experiments indicate homology between major isoproteins of one apo-E subclass and major isoproteins of another. On the basis of the mixing experiments, we hypothesized that the βII, βIII, and βIV apo-E subclasses represent homozygosity for one of three different apo-E alleles designated ϵII, ϵIII, and ϵIV, respectively, and that the α subclasses represent heterozygosity for those alleles. The ϵII and ϵIII alleles generate the apo-E subclass αII, the ϵIII and ϵIV alleles generate the apo-E subclass αIII, and the ϵII and ϵIV alleles generate the apo-E subclass αIV. This genetic hypothesis predicts the matings of certain apo-E subclasses can produce offspring with apo-E subclasses that differ from the apo-E subclass of either parent. Two such matings are shown schematically in Figure 7A,B. In family A, the mother was αII (ϵII, ϵIII) and the father was αIII (ϵIII, ϵIV). One of the children was αIII (ϵIII, ϵIV) and the other αIV (ϵII, ϵIV). The αIV child has inherited the ϵII allele from the mother and the ϵIV allele from the father. The probability of producing an αIV offspring in this family is 25%. In family B, both parents were αII (ϵII, ϵIII). One child was αII (ϵII, ϵIII) and the other βIII (ϵIII, ϵIII). according to our hypothesis, parents with αII (ϵII, ϵIII) have 25% probability of producing a βIII (ϵIII, ϵIII) offspring. Further studies in progress of apo-E inheritance in several large kindred are consistent with the hypothesis that the apo-E subclasses are determined at a single genetic locus with three common alleles,

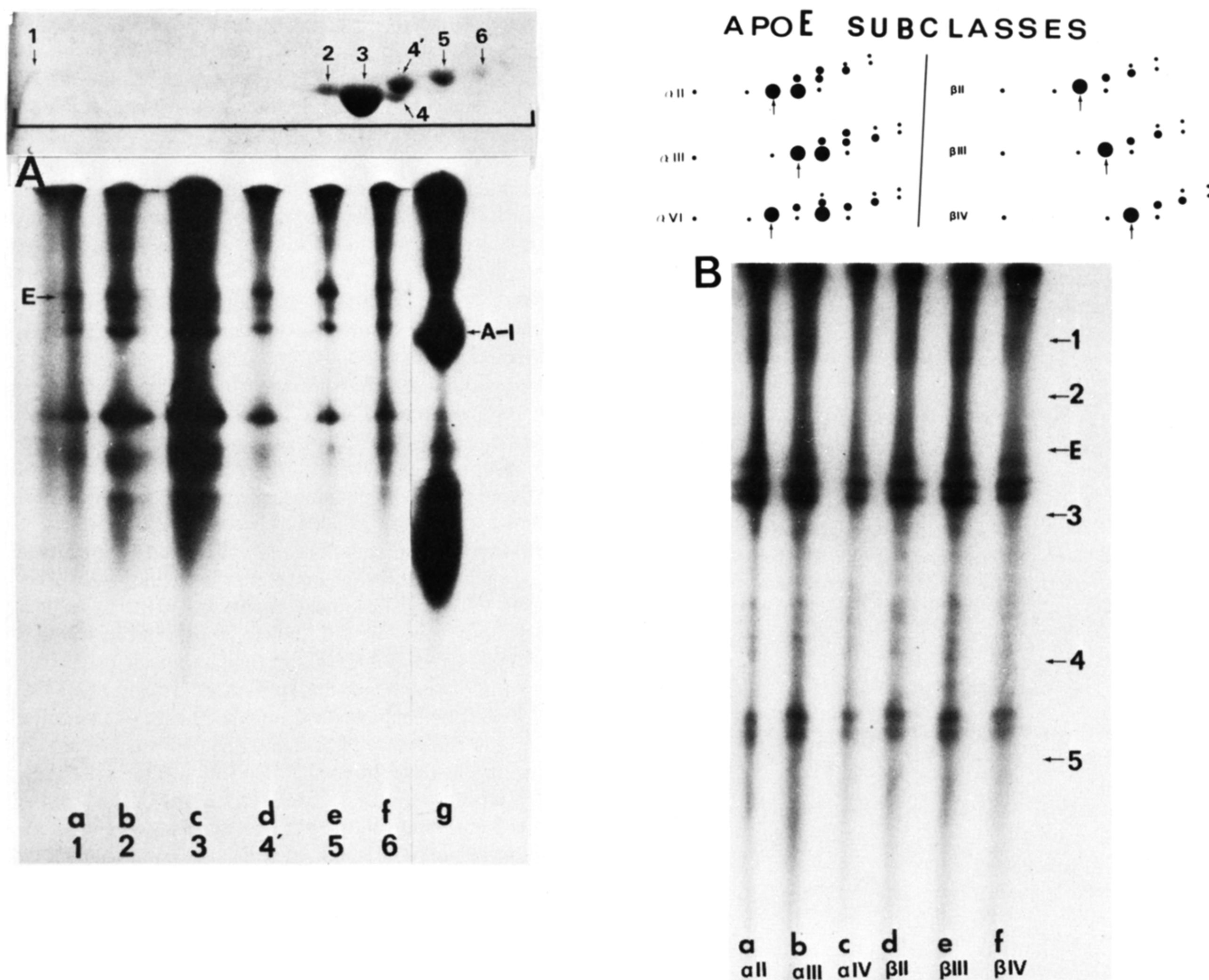

FIGURE 5: (Panel A) Autoradiogram of one-dimensional chymotryptic peptide maps of human apo-E isoproteins of class β. The isoproteins mapped are shown in the insert at the top of the photograph. Iodination and mapping apo-E isoproteins spots cut from the dried gel was performed as described under Experimental Procedures. The isoproteins are analyzed in lanes a–g as indicated in the figure: (lane a) isoprotein 1, (lane b) isoprotein 2, (lane c) isoprotein 3, (lane d) isoprotein 4′, (lane e) isoprotein 5, (lane f) isoprotein group 6, and (lane g) human apo-A-I. (Panel B) Autoradiogram of one-dimensional staphylococcal protease peptide maps of apo-E isoproteins obtained from the six different subclasses. The isoproteins mapped are indicated by the arrow in the insert at the top of the photograph. They represent the major isoprotein of each of the β subclasses or the major basic isoprotein of each of the α subclasses. The subclasses analyzed in lanes a–g are indicated at the bottom of the figure and are as follows: (lane a) αII, (lane b) αIII, (lane c) αIV, (lane d) βII, (lane e) βIII, and (lane f) βIV. The position of undigested apo-E is indicated. The numbers 1 to 5 indicate molecular weight markers as follows: (1) bovine serum albumin, 68 000; (2) ovalbumin, 43 000; (3) apo-A-I, 28 000; (4) trypsin inhibitor, 19 000; (5) lysozyme, 14 300.

ϵII, ϵIII, and ϵIV. The relationship of the apo-E subclasses to their genotypes is shown schematically in Figure 8.

The gene frequencies of the apo-E alleles ϵII, ϵIII, and ϵIV were estimated from the frequencies of the apo-E subclasses observed in a group of 61 normal unrelated volunteers excluding known hyperlipidemics or those with a family history of premature atherosclerosis. The observed frequencies of the apo-E subclasses were as follows: βII = 2%, βIII = 49%, βIV = 0%, αII = 15%, αIII = 31%, and αIV = 3%. On the basis of observed frequencies of the apo-E subclasses, the gene frequencies were calculated. The calculated frequencies are as follows: ϵII = 11%, ϵIII = 72%, and ϵIV = 17%. Assuming a Hardy–Weinberg distribution of the apo-E alleles in the general population, the expected frequencies of the apo-E subclasses would be the following: βII = 1%, βIII = 52%, βIV = 3%, αII = 16%, αIII = 25%, and αIV = 4%.

Discussion

In a recent communication, we described an extensive polymorphism of human VLDL apo-E (Zanis & Breslow, 1980). We also showed that VLDL apo-E from type III hyperlipoproteinemia patients was not missing any of the apo-E isoproteins, as had been previously suggested (Utermann et al., 1975, 1977a,b), but rather that the entire group of apo-E isoproteins in these individuals was shifted to more acidic isoelectric points (Zannis & Breslow, 1980). In this report, we provide insight into the biochemical explanation for the complex array of apo-E isoproteins observed. Our studies show that the different apo-E isoproteins arise partly from post-translational modification of an original apo-E polypeptide with sialic acid and partly from the genetic variability of the apo-E alleles present in the general population.

It had been observed for several years that apo-E appeared as a broad band on one-dimensional $NaDodSO_4$–polyacrylamide gel analysis (Shelburne & Quarfordt, 1974, 1977; Weisgraber & Mahley, 1978; Utermann et al., 1975). However, this size heterogeneity of apo-E had been overlooked until recently (Zannis & Breslow, 1980; Utermann et al., 1979a). In a recent communication, Utermann has resolved apo-E into higher and lower molecular weight components and reported

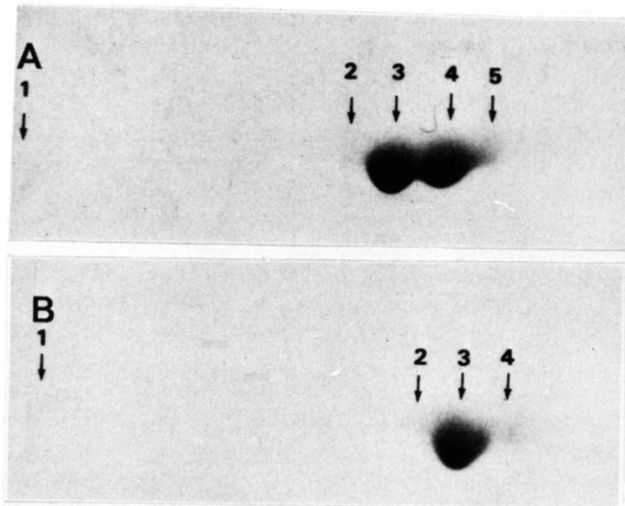

FIGURE 6: Two-dimensional gel electrophoresis of human apo-E after treatment with *C. perfringens* neuraminidase. (Panel A) Class α of apo-E (40 μg). (Panel B) Class β of apo-E (35 μg). Note by comparing panels A and B of this figure with panels A and C or D, respectively, of Figure 2 that the acidic and higher molecular weight isoproteins of both class α and class β are eliminated.

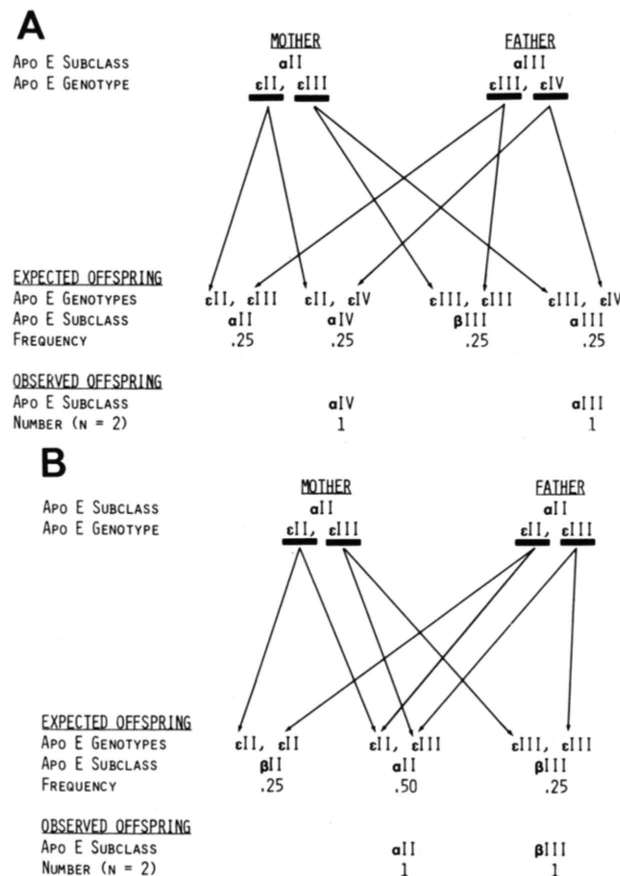

FIGURE 7: Schematic presentation of the inheritance of apo-E subclasses assuming the existence of three apo-E alleles (ϵII, ϵIII, and ϵIV) at a single genetic locus.

that the higher molecular weight form of apo-E was seen only in certain individuals (Utermann et al., 1979a). Our results utilizing both one- and two-dimensional gel electrophoresis show that the major apo-E isoproteins are of mol wt 38 000 with several minor apo-E isoproteins of slightly higher molecular weight (38 500–39 500). These higher molecular weight apo-E species were present in VLDL specimens from each of over 150 individuals studied. Our two-dimensional gel electrophoretic analysis which separates isoproteins by both size

Apo E Subclasses (Frequency)**	Apo E Alleles (Frequency): εII (0.11)	εIII (0.72)	εIV (0.17)
βII (0.01)	●		
βIII (0.52)		●	
βIV (0.03)			●*
αII (0.16)	●	●	
αIII (0.25)		●	●
αIV (0.04)	●		●

* The βIV subclass is associated with type III hyperlipoproteinemia.

** The frequencies were calculated assuming a Hardy-Weinberg distribution of the apo E alleles.

FIGURE 8: Schematic presentation of the relationship of apo-E genotypes to phenotypes and estimated frequencies of genotypes and phenotypes in the population.

and charge has revealed that the apo-E isoproteins of mol wt 38 500–39 500 result from posttranslational glycosylation with sialic acid. This result, although obvious with the two-dimensional gel electrophoresis technique, was not previously appreciated when apo-E isoproteins were analyzed only by one-dimensional methods that separated by either size of charge (Shelburne & Quarfordt, 1974; Weisgraber & Mahley, 1978; Utermann et al., 1975, 1977a,b, and 1979a; Pagnan et al., 1977; Marcel et al., 1979; Warnick et al., 1979; Weidman et al., 1979). Another reason that our results were previously missed was that some commercial sources of neuraminidase, other than *C. perfringens*, were used, and we have shown that these are less effective in desialating apo-E (Utermann et al., 1979a; Jain & Quarfordt, 1979). Our results also showed that both the molecular weight and the charge of the major apo-E isoproteins are unaffected by the neuraminidase treatment.

The carbohydrate content of human apo-E has been recently reported and consists of galactose, *N*-acetylglucosamine, glucosamine, *N*-acetylgalactosamine, galactosamine, and sialic acid (Jain & Quarfordt, 1979). The sialic acid content ranged from 0.79 to 1.69 mol/mol of apo-E in different preparations. Since sialo apo-E isoproteins in our analysis comprise approximately 20% of the total apo-E, such molar ratios of sialic acid to apo-E imply either that more than one apo-E sugar linkage containing sialic acid exists per apo-E monomer or that, if only one such linkage exists, it contains several sialic acid residues. Sialic acid could explain the molecular weight and isoelectric point differences of the group of minor acidic apo-E isoproteins affected by neuraminidase treatment.

The physiological significance of sialo apo-E isoproteins is not known. Literature reports indicate that asialo glycoproteins are taken up by the liver at a considerable faster rate than corresponding native glycoproteins (Hickmann & Ashwell, 1971). There are conflicting results reported for whether desialation of another cholesterol carrying lipoprotein LDL (which has as its major apoprotein the glycoprotein apo-B) affects the uptake and catabolism of LDL by the liver (Filipovic et al., 1979; Attie et al., 1979). It remains to be determined whether desialation of apo-E affects cellular recognition and uptake of apo-E containing lipoproteins.

In this paper, we also describe one additional subclass of apo-E (αIV) that had not been seen at the time of our recent communication. We also redefine the charge difference of the apo-E subclass which is associated with type III hyperlipoproteinemia, and this apo-E subclass has been renamed βIV instead of βIVa (Zannis & Breslow, 1980). The apo-E sub-

class βIV is shifted −2 and −1 charge units, respectively, relative to the apo-E subclasses βII and βIII. In addition, we provide evidence concerning the genetic origin of the apo-E subclasses. The first suggestion of the genetic origin of the apo-E subclasses was revealed by analysis of the electrophoretic patterns obtained by mixing different apo-E subclasses. These experiments showed that there exists a defined homology (of isoelectric point and molecular weight) between isoprotein belonging to different subclasses. This homology is as follows: (a) the basic and acidic major isoproteins of apo-E subclass αII have the same isoelectric point and molecular weight as the major isoprotein of subclass βII and βIII, respectively; (b) the basic and acidic major isoproteins of apo-E subclass αIII have the same isoelectric point and molecular weight as the major isoprotein of subclass βIII and βIV, respectively; (c) the basic and acidic major isoproteins of apo-E subclass αIV have the same isoelectric point and molecular weight as the major isoprotein of subclass βII and βIV, respectively. The genetic origin of the apo-E subclasses was confirmed by family studies which allowed us to determine the mode of inheritance of apo-E. All family studies are consistent with the hypothesis that the subclasses of apo-E are determined at a single genetic locus with three common alleles, ϵII, ϵIII, and ϵIV. The class β phenotypes (βII, βIII, and βIV) represent homozygosity for two identical apo-E alleles. In contrast, class α phenotypes (αII, αIII, and αIV) represent heterozygosity for two different apo-E alleles.

The apo-E subclasses may have great clinical significance since they may be associated with, or represent, a genetic predisposition to hyperlipidemia and/or atherosclerosis. The apo-E subclass βIV appears to be associated with type III hyperlipoproteinemia (Hazzard et al., 1972; Mishkel et al., 1975; Fredrickson et al., 1975). This is a condition which leads to hyperlipidemia, xanthomatosis, and premature atherosclerosis with both coronary heart disease and peripheral vascular disease (Fredrickson et al., 1978). It is not yet clear whether the apo-E subclasses βIV alone can produce type III hyperlipoproteinemia or whether factors determined by other genetic loci, as suggested by Utermann et al. (1979b), are also required. We are currently studying the inheritance of type III hyperlipoproteinemia along with the inheritance of the apo-E subclasses in families affected by this disease to resolve this important question. We estimate that the apo-E subclass βIV (homozygosity for the ϵIV apo-E allele) would have a frequency in the population of approximately 3%. This finding suggests that a relatively large number of individuals in the population may be at risk for type III hyperlipoproteinemia and the attendant premature atherosclerosis. Our results indicate that none of the type III hyperlipoproteinemia patients had a deficiency of an apo-E isoprotein (Utermann et al., 1975, 1977a,b) or an apo-E isoprotein of altered molecular weight (Utermann et al., 1979a) compared to the normal controls as suggested by Utermann and colleagues. This discrepancy may have resulted either due to a difference in the type III mutation described by Utermann as opposed to those described by us or due to the interpretation of charge shifts of the apo-E subclasses shown in Figure 4 as a deficiency in a basic apo-E isoprotein.

The data presented in this paper greatly simplify our understanding of the complex array of apo-E isoproteins revealed by two-dimensional polyacrylamide gel electrophoretic analysis in our recent communication (Zannis & Breslow, 1980). In addition, these results lay the foundation for genetic and physiological studies in the future concerning apo-E, an apoprotein which is intimately involved in regulation of plasma cholesterol metabolism (Mahley et al., 1974–1976, 1978; Shore et al., 1974; Rodriguez et al., 1976; Mahley & Holcomb, 1977; Breslow et al., 1977; Kushwaha & Hazzard, 1978; Wong & Rubinstein, 1978; Rudel et al., 1979; Noel et al., 1979; Zilversmit, 1979; Sherrill et al., 1980). Such studies may be helpful in understanding aspects of the atherosclerotic process itself.

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