Analysis of rat serum apolipoproteins by isoelectric focusing. II. Studies on the low molecular weight subunits

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Abstract The low molecular weight proteins of rat apo HDL and apo VLDL have been isolated and analyzed by the technique of isoelectric focusing. Sephadex fractions from apo HDL (HS-3) and apo VLDL (VS-3) that contain these proteins reveal three major bands with apparent isoelectric points of pH 4.50, 4.67, and 4.74, as well as three minor bands at pH 4.43, 4.57, and 4.61. In addition, apo HDL has a major band at pI of 4.83. DEAE-Cellulose chromatography was used to prepare purified fractions of these components that were characterized by *N*-terminal analyses and molecular weight determinations by SDS gel electrophoresis. The major low molecular weight components of apo HDL were focused on a slab gel and the bands were identified as A-II (pI 4.83), C-II (pI 4.74), C-III-0 (pI 4.67), and C-III-3 (pI 4.50). Neuraminidase treatment of apo HDL, followed by isoelectric focusing, suggested that the other bands, which have not previously been reported, may be additional forms of the C-III protein, differing only in their content of sialic acid.

Supplementary key words high density lipoprotein · very low density lipoprotein · SDS electrophoresis · C apoproteins · sialic acid · DEAE-cellulose chromatography · Sephadex chromatography

The technique of isoelectric focusing (1) has been applied to the analysis of many proteins, including the apolipoproteins of the human (2–8) and rhesus monkey (9). The preceeding paper (10) deals with the application of this method to the study of the middle molecular weight range apolipoproteins.

In this report are presented studies of the low molecular weight polypeptides of rat plasma high density and very low density lipoproteins. The low molecular weight proteins of rat HDL have been isolated and studied by Herbert et al. (11), and we have confirmed and extended these studies by applying the technique of isoelectric focusing (IEF) for resolving and determining relative amounts of these protein components. By this technique, studies of the relative amounts of these individual components in hyperlipidemic rats have been carried out (12).

MATERIAL AND METHODS

Chemicals

Acrylamide (Eastman Organic Chemicals, Rochester, NY) and *N,N'*-methylenebisacrylamide (Canalco, Rockville, MD) were recrystallized from redistilled chloroform. *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate were also obtained from Canalco. Ultra pure Tris (trishydroxymethylaminomethane) and urea were from Schwarz/Mann, Orangeburg, NY. Aqueous solutions of urea were deionized immediately before use by passage through Rexyn I-300 (Fisher Scientific Co., Fairlawn, NJ). Ampholine carrier ampholytes were obtained from LKB Instruments, Inc., Rockville, MD. Tetramethyl urea (Lot 3110) was from Burdick and Jackson Laboratories, Inc., Muskegon, MI. All other chemicals were reagent grade.

Lipoproteins and apolipoproteins

Lipoproteins were isolated by ultracentrifugation (13); apolipoproteins were obtained by delipidation of lyophilized lipoproteins with ethanol–diethyl ether 3:1 (14). Both procedures are described in the preceeding report (10).

Isolation of purified apolipoproteins

Apo HDL and apo VLDL were chromatographed by the method of Bersot et al. (15) on 2.5 × 90 cm columns containing Sephadex G-150. The apolipoproteins were eluted with buffer containing 0.2 M Tris-HCl (pH 8.2), 7 M urea, and 2mM decyl sulfate. The elution patterns were similar to those of Bersot et al. (15). Three peaks were found for both apo

Abbreviations: IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; VLDL, very low density lipoproteins; HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; ARP, arginine-rich protein.

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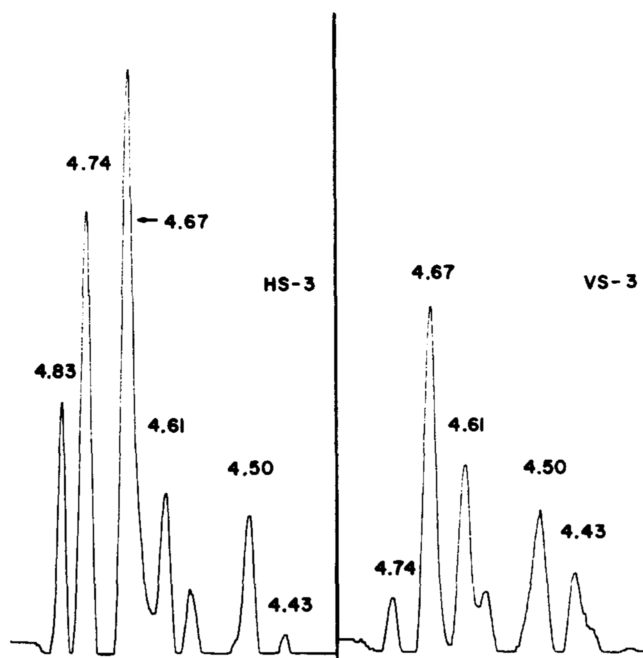

Fig. 1. Scans of 0.3 × 9 cm IEF gels of HS-3 and VS-3 Sephadex fractions. The gels were stained with modified Coomassie blue (10) and scanned at 567 nm. The pI values assigned to the various peaks correspond to those in Fig. 1 of reference 10.

HDL and apo VLDL. In the order of elution, they were termed HS-1, HS-2, and HS-3 from apo HDL, and VS-1, VS-2, and VS-3 from apo VLDL. Sephadex G-150 fractions HS-3 and VS-3 were used as such in isoelectric focusing studies.

HS-3 from the Sephadex G-150 column was fractionated on DEAE–cellulose to isolate the low molecular weight polypeptides. This fraction was concentrated by placing the sample in dialysis tubing (with a molecular weight cutoff of 3500, Science Essentials Co.) and packing the tubing in dry Sephadex G-150. After concentration, the sample was dialyzed exhaustively against 0.005 M Tris-HCl, pH 8.2, and 7 M urea, and then applied to a 0.9 × 100 cm column of DEAE–cellulose (Whatman DE 52, Reeve Angel, Clifton, NJ). This column was eluted at 4°C with a linear gradient of NaCl solution (between 0 and 0.1 M NaCl) containing 0.005 M Tris-HCl, pH 8.2, and 7 M urea. In experiment 1, this gradient was accomplished in a volume of 1000 ml, while in experiment 2, the gradient was expanded to 2100 ml.

Dansylation

Purified proteins from the DEAE–cellulose column were reacted with dansyl chloride in the presence of 8 M urea and 1% SDS for 3 hr at 37°C. The dansylated proteins were separated from low molecular weight reactants and products by passage through Sephadex G-25 columns constructed from Pasteur pipettes. After lyophilization, the proteins were hydrolyzed in 5.6 N HCl and the dansylated amino acids were identified after chromatography on polyamide thin-layer plates by the method of Weiner, Platt, and Weber (16).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed at pH 8.9 in 8 M urea using 7.5 or 10% acrylamide (10). Electrophoresis on acrylamide gradient SDS slab gels was performed according to the method of Swaney and Kuehl (17). In this method, gels with a gradient of acrylamide concentration ranging from 3% (at the top) to 27% (at the bottom) are employed in order to obtain maximum resolution of both low and high molecular weight proteins.

Gel isoelectric focusing

Gel isoelectric focusing and gel staining were carried out by the procedures outlined in the first paper of this series (10). This reference also describes how the pI values used here were obtained.

Two-dimensional separation of apolipoproteins

Duplicate samples of apo HDL and apo VLDL were separated by isoelectric focusing on 9-cm gels, as described above. One gel was stained and a replicate was placed longitudinally in a vertical slab gel apparatus and embedded in a 15% acrylamide gel containing 0.1% SDS. SDS gel electrophoresis was carried out by the method of Shapiro, Vinuela, and Maizel (18), except that the gel was run overnight at 40 volts. The slab was stained for 2 hr in a solution of 0.1% Coomassie brilliant blue in 50% methanol–9% acetic acid, and the gel was destained by diffusion in 5% methanol–7% acetic acid.

Neuraminidase studies

Aliquots of apo HDL solution (1–2 ml containing 2–4 mg of protein) were mixed with an equal volume of 0.2 M acetate buffer, pH 5.0, and 1 ml of neuraminidase solution (1 mg of enzyme per ml of water, diluted 50-fold) per mg of protein was added. The enzyme (from *Cl. perfringens*) was obtained from Worthington Biochemical Corp., Freehold, N. J. The mixture was incubated for 2 hr at 37°C, and was then transferred to dialysis tubing and dialyzed against distilled water for 16–18 hr at 4°C. The solution in the bag was concentrated by covering the bag with Sephadex G-100 for 18 hr, and then by further dialysis against 0.02 M Tris-HCl, pH 8.2, containing 6 M urea. In control experiments, the

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apo HDL was treated in the same manner, except that neuraminidase was not added. Small volumes (100–200 μl containing 200–300 μg of protein) were used for the focusing studies.

In some experiments, the reaction mixture was lyophilized following dialysis against water (and before concentration) and the residue was dissolved in 3 ml of water. Sialic acid was determined by the method of Warren (19). Sialic acid analyses were not carried out on apolipoproteins that were initially dissolved in urea-containing buffer. Neuraminidase was active in such solutions, but the presence of urea interfered with the color formation in the sialic acid analysis. The sialic acid content of apo HDL was 7–15 μg per mg of protein. These analyses were carried out mainly to make certain that changes in gel patterns occured only with release of sialic acid. The wide range of values was probably due to the different amounts of sialic acid-containing polypeptides in various HDL preparations and to incomplete hydrolysis of all sialic acid residues.

IEF patterns of low molecular weight polypeptides of HDL and VLDL

The general IEF patterns of rat HDL and VLDL proteins have been described in the previous report (10). The individual features of IEF patterns of the low molecular weight polypeptides of HDL and VLDL are shown in **Fig. 1.** This figure shows photographs of two densitometric tracings of scans of 14 cm IEF gels of HS-3 and VS-3 Sephadex fractions (15). For purposes of reference, some of the pH values from Fig. 1 in the preceding paper (10) are included. Of particular interest is the similarity of patterns, the only qualitative difference being the presence of the pH 4.83 component in HS-3. Between the pH 4.50 and 4.67 peaks there are two components; however, in most gels of apo HDL and apo VLDL only one band was seen in this area.

Identification of pH 4.50 to 4.83 peptides of apo HDL

In order to identify each of the polypeptides in the pH 4.50–4.83 region, Sephadex fraction HS-3 (low mol wt proteins) was chromatographed on columns of DEAE–cellulose in experiments with two types of gradients (**Fig. 2**). In experiment 1, using a gradient similar to that used by Herbert et al. (11), results were obtained that were virtually identical to those of these authors and fractions designated 1–6 were pooled as indicated. In experiment 2, a shallower gradient was employed to improve the resolution of the fused peaks, and fractions from this experiment are designated at I–VI. The corresponding fractions can be seen to be 1 and I, 2 and II, 3 and III, 5 and IV, 6 and VI. Herbert et al. (11) identified five proteins in these fractions and designated them as C–I, A–II, C–III–0, C–II, and C–III–3[1], respectively; we have assigned the same nomenclature to our purified proteins on the basis of the chromatographic profile. To confirm this assignment, the *N*-terminal amino acids were determined by dansylation on fractions obtained in experiment 2. The same *N*-terminal amino acids as reported by Herbert et al. (11) were found: aspartic acid for I, III, and VI; threonine for IV; and no detectable *N*-terminal acid for II. Fraction IV proved to contain a minor spot corresponding to aspartic acid, indicating slight contamination. Fraction V was not analyzed because of the paucity of material.

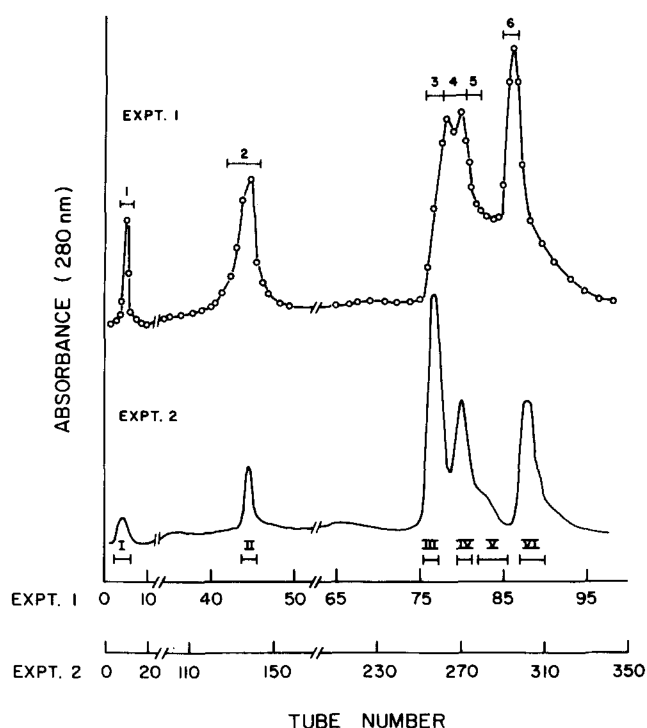

Fig. 2. DEAE–cellulose chromatography of HS-3. In experiment 1 (O- - - O) the low molecular weight apolipoproteins from rat HDL were chromatographed on a DEAE–cellulose column (0.9 × 100 cm) in 7 M urea with a NaCl gradient of 0–0.1 M extending over a volume of 1000 ml; each tube contained 10 ml. Pooled fractions for this experiment are designated by Arabic numerals. In experiment 2 (solid curve) the gradient extended over a volume of 21,000 ml; each tube contained 5.5 ml. Pooled fractions for this experiment are designated by Roman numerals.

[1] Herbert et al. (11) demonstrated that A–II is a monomeric equivalent of human A–II, that C–II is an activator of lipoprotein lipase, like the human C–II, and that C–III–0 and C–III–3 are identical, except that the latter polypeptide has three sialic acid residues and one galactosamine.

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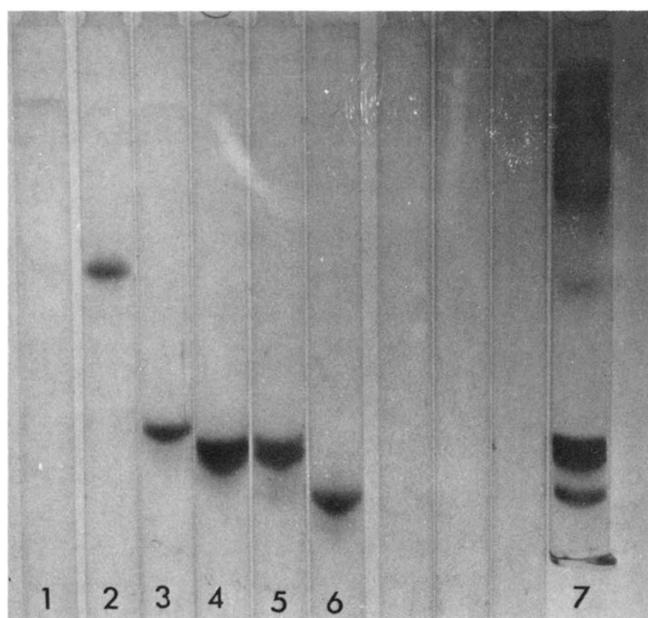

Fig. 3. Polyacrylamide gel electrophoresis (pH 8.9) in 8 M urea of DEAE fractions. Fractions 1–6 from DEAE–cellulose chromatography, experiment 1 (see Fig. 2), were applied in gels 1–6, respectively, in the downward direction. Gel 7 contains apo HDL (this gel was heavily loaded, as can be seen by the dense bands in the upper portion of the gel, which contain the middle molecular weight HDL apolipoproteins). The gels located between gel 6 and gel 7 had no protein and were DEAE fractions pooled after fraction 6. The approximate amount of protein applied to each gel was: Gel 2, 5–10 μg; Gel 3, 15 μg; Gel 4, 25 μg; Gel 5, 20 μg; Gel 6, 15 μg; Gel 7, 100–150 μg.

Polyacrylamide gel electrophoresis of DEAE fractions

As an additional correlation of our results with those of Herbert et al. (11) and because the method is widely used for apolipoprotein studies, polyacrylamide gel electrophoresis in urea was performed on the DEAE fractions from experiment 1 (**Fig. 3**). Fraction 1 (C–I) did not give a recognizable band under the conditions employed and further studies were not carried out. The remaining fractions can be seen to correspond to bands below the high molecular weight bands shown in apo HDL (gel 7). Thus, A–II (fraction 2, Fig. 2; gel 2, Fig. 3) is seen to be a very lightly stained zone in apo HDL. Gel 3 contains essentially pure C–III–0 (fraction 3); gel 5 contains C–II (fraction 5) somewhat contaminated with C–III–0; and gel 4 contains a mixture of these two proteins (fraction 4). These proteins are not well-resolved in this system; the apparent lower mobility of C–III–0 in gel 3 compared to gel 4 was not reproducible and was only a peculiarity of this run. The remaining protein, C–III–3 (fraction 6, Fig. 2; gel 6, Fig. 3) clearly corresponds to the lowest band in apo HDL and also appears reasonably pure.

Slab isoelectric focusing of DEAE fractions

In order to relate the bands observed on IEF gels with the DEAE fractions, a slab IEF procedure was employed since the alignment of cylindrical gels is equivocal with such tightly spaced bands. **Fig. 4** shows the results of slab IEF on a gel containing a pH 4–6 gradient. Although the pH 5.31–5.82 bands (middle mol wt proteins) are not as well resolved on this particular slab, the lower bands are quite clearly separated, as indicated by the apo HDL samples applied in positions 1, 3, 6, 9, and 11. C–I (position 2) focused at a very high pH, probably in the 7–9 region. This polypeptide was never definitively located on cylindrical gels, even when pH 7–9 Ampholines were employed. A–II (position 4) corresponded to the pI 4.83 peptide; C–III–0 (position 5) to the pI 4.67 peptide; C–II (position 7) to the pI 4.74 peptide; and C–III–3 (position 10) to the pI 4.50 peptide. Fraction V (Fig. 2), which eluted from DEAE–cellulose between C–II and C–III–3 and which is contaminated with these proteins, was focused at position 8 on the gel. This gave a faint band between C–III–3 and C–III–0 at a position that corresponds to about pH 4.57 (indicated by the arrow); although visible in the gel, this band is not apparent in the photograph. Just below that band a similar band is seen as a contaminant of C–III–0 (position 5), and two bands are seen in this area in Fig. 1. It should be noted that C–II (position 7) contains a small amount of C–III–0.

SDS gel electrophoresis of DEAE–cellulose fractions

Fractions obtained by DEAE–cellulose chromatography were electrophoresed on SDS slab gels employing a 3–27% acrylamide gradient (17) to obtain molecular weight estimates and identification of bands. **Fig. 5** shows the results obtained with these fractions. Positions 3–7 contained C–I, A–II, C–III–0, and C–III–3, respectively, as identified by Herbert et al. (11), and can be compared with the apo HDL in position 8. Apo HDL also shows heavily stained bands in the middle of the gel corresponding to the proteins A–IV, ARP, and A–I (15). In addition, position 1 contains the DEAE fraction V (Fig. 2) obtained by pooling tubes between the C–II and C–III–3 fractions which, although grossly contaminated with these proteins, contained principally a component with mobility similar to that of C–III–0, but which corresponds to the IEF band with a pI of 4.57–4.61 (see above) and may represent C–III–I or C–III–2. Proteins of known molecular weights and human apolipoproteins were electrophoresed in adjacent slots (not shown); from the results we estimate the following molecular weights

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for the rat apolipoproteins: A–II, 8,000; C–I, 7,000; C–II, 8,000; C–III–0, 10,000; and C–III–3, 11,000. There may be some uncertainty in these values due to the known anomalous electrophoretic behavior of glycoproteins on SDS gels (20).

Two-dimensional electrophoresis

The major apoproteins of rat HDL have been partially characterized and identified by Swaney, Reese, and Eder (21) on the basis of SDS gel electrophoresis. In order to correlate those results with the present IEF studies, a two-dimensional technique was devised. **Fig. 6** shows results of a study in which apo HDL (ca. 150 μg) was focused on a cylindrical gel and then imbedded on an SDS slab gel for electrophoresis in a second dimension. For purposes of orientation, a stained IEF gel of apo HDL (ca. 100 μg) is shown at the top. The two uppermost groups of spots on the slab correspond to the A–IV and the arginine-rich proteins, with molecular weights of 46,000 and 35,000, respectively. These correspond to the pH 5.31–5.46 bands on the IEF gel. The next lower group of bands corresponds to the pH 5.46–5.82 bands on the IEF gel, and as a group have been designated A–I, with a molecular weight of 27,000 (21). The spots at the bottom of the slab (to the right) are lower molecular weight peptides in approximately the 8,000–11,000 range. The uppermost of these spots corresponds to C–III–3 (extreme right spot). The peptide with the next highest molecular weight corresponds to C–III–0. The vertical difference between these spots is roughly equivalent to 1000 daltons; the difference in molecular weight between C–III–3 and C–III–0 is on the order of 900, i.e., three sialic acid residues, if one assumes identical primary structures. The two components with the lowest molecular weights correspond to C–II and A–II. The C–I peptide was not seen.

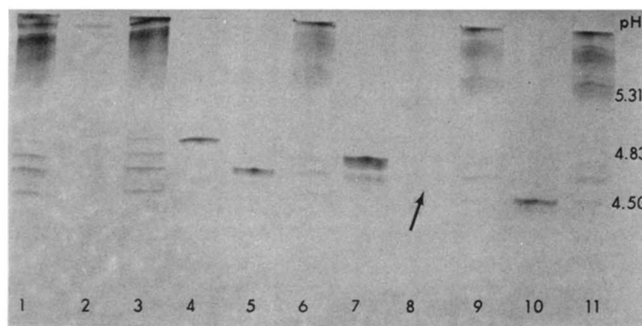

Fig. 4. Slab isoelectric focusing of DEAE–cellulose fractions of HS-3. An MRA M-150 slab electrofocusing apparatus (MRA Corp., Boston, MA) was used in this experiment. A 1.5-mm thick slab (same gel formulation as cylindrical gels; pH 4–6) was prefocused at 100 volts for 30 min. A 10 μl portion of apoprotein solution (in 0.005 M Tris, pH 8.2, 8 M urea) was added to each well, and the samples were focused for 3.5 hr as follows: 100 volts for 30 min, 200 volts for 30 min, 300 volts for 60 min, and 400 volts for 90 min, using a MRA automatic regulated pulse power supply ARP-1. The DEAE–cellulose fractions used were from DEAE experiment 2 (see Fig. 2). Positions 1, 3, 6, 9, and 11, 30 μg of apo HDL; Position 2, 10 μg of Fraction I (C–I); Position 4, 15 μg of Fraction II (A–II); Position 5, 10 μg of Fraction III (C–III–0); Position 7, 15 μg of Fraction IV (C–II); Position 8, 1 μg of Fraction V; Position 10, 5 μg of Fraction VI (C–III–3). The slab was stained overnight in modified Coomassie blue (10), and destained in water for 48 hr.

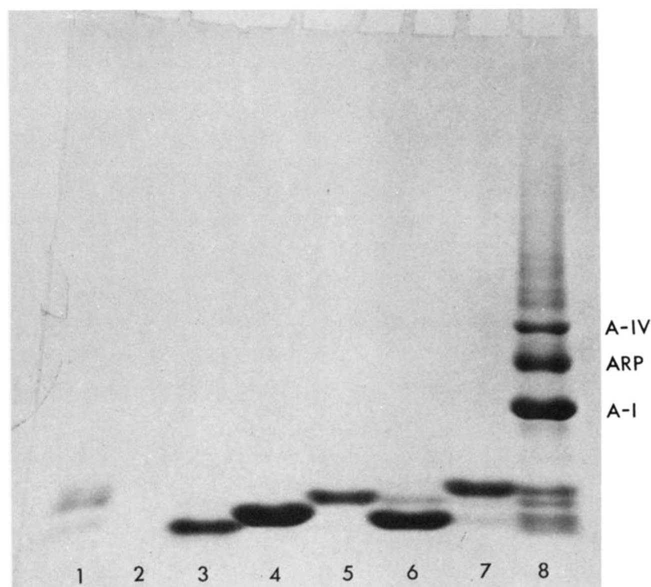

Fig. 5. SDS gel electrophoresis of DEAE–cellulose chromatography fractions (Exp. 2, Fig. 2) on a slab containing a linear gradient of acrylamide concentration ranging from 3% (top) to 27%. Position 1, Fraction V; Position 2, blank; Position 3, Fraction I (C–I); Position 4, Fraction II (A–II); Position 5, Fraction III (C–III–0); Position 6, Fraction IV (C–II); Position, 7, Fraction VI (C–III–3); Position 8, apo HDL. Samples applied to end slots of slab gels tend to show downward curvature of the dye front; consequently, bands in position 1 are displaced slightly downward relative to other positions of the slab. Each slab has 13 sample positions.

Effect of neuraminidase on apo HDL

In order to obtain further verification of the identity of the sialic acid-containing apoproteins on IEF gels, apo HDL was treated with neuraminidase and then focused. **Fig. 7** shows the changes in the IEF pattern. C–III–3 decreased markedly, and there was an increase in the band(s) between C–III–3 and C–III–0. There was also a thickening of the C–III–0 band. Below C–III–3, on the control gel, there was at least one very faint band that was not evident in the gel of the enzyme-treated apo HDL. There may also have been some changes in some of the minor components with pI values 5.31–5.39. The pattern shown in Fig. 7 was observed in several other experiments in which apo HDL was treated with

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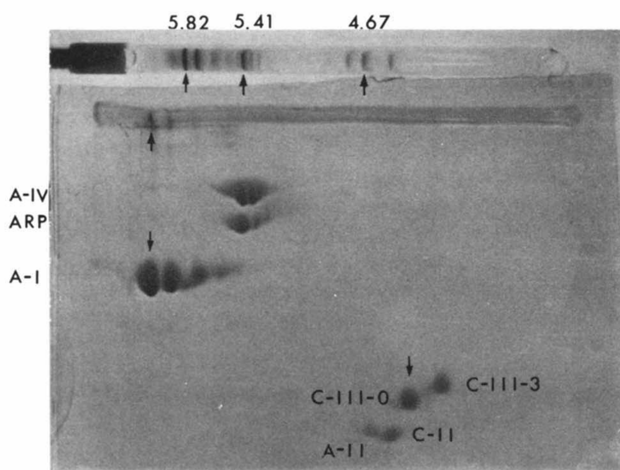

Fig. 6 Two dimensional separation of apo HDL peptides. One hundred and fifty μg of apo HDL was focused on each of two 0.3 × 9 cm gels. One gel was fixed, stained, and destained (10); the second gel (unfixed) was imbedded in a 15% acrylamide slab gel. The spots on the slab gel are not all in direct alignment with corresponding bands on the IEF gel because of the compression (and thus elongation) of the unfixed IEF gel between the plates of the slab apparatus during electrophoresis and the swelling of the slab gel during staining and destaining. For purposes of orientation, the apparent pI values (from Fig. 1 of reference 10) of some bands are indicated on the figure, and arrows indicating the positions of the pH 5.82 and 4.67 bands in both the slab and reference gel are included.

neuraminidase. In three of these studies, the gels were scanned in the pH 4.43–4.83 segment, and the areas under the peaks were determined by triangulation.

In one of these studies, the HDL had been labeled in vivo as a result of the rats being injected intraveneously with L-[4,5-^{3}H]lysine (1 mCi to each of six rats). After scanning the gels, the stained bands were cut out and combusted in an Intertechnique (Dover, NJ) Oxymat. The ^3H_2O was collected in scintillation fluid (2% naphthalene, 0.7% butyl-PBD in dioxane–toluene 7:3) and counted in an Intertechnique liquid scintillation counter.

Table 1 shows the results of the experiments. Relative amounts of C–II were unchanged; this is consistent with the fact that this protein contains no sialic acid (11). The most striking changes were decreases of C–III–3 and increases of C–III–0. In addition, there appeared an intense band between C–III–3 and C–III–0. We have designated this band as C–III–2(–1). The data suggest that sialic acid may have been completely removed from some C–III–3 molecules, producing C–III–0, or partially removed to form C–III–2 or C–III–1. The fact that the radioactivity data confirm the information from scans of stained gels is important because this technique is independent of factors such as stain uptake or linearity of optical density as a function of concentration of protein.

DISCUSSION

The purpose of this study was to describe and evaluate the technique of gel isoelectric focusing of low molecular weight apolipoproteins of rat serum. The simplicity of gel IEF and the high degree of resolution of the subunit peptides of the apolipoproteins, especially the low molecular weight apolipoproteins, indicate that this technique is a valuable method for identifying polypeptides after their isolation and assessing their purity, as well as for the quantitation of components.

Table 2 shows a summary of the results of the present report and of the preceeding study (10) in tabular form. The middle molecular weight proteins of rat HDL and VLDL (exclusive of the "B" protein) are found in the pH 5.31–5.82 region. Each of these proteins comprises several forms that differ in their isoelectric points; the basis for these differences is not known, nor is it known whether these differences occur in vivo.

By contrast, the low molecular weight components, found in the range of pH 4.43–4.83 (except for C–I), appear to be homogeneous with respect to isoelectric

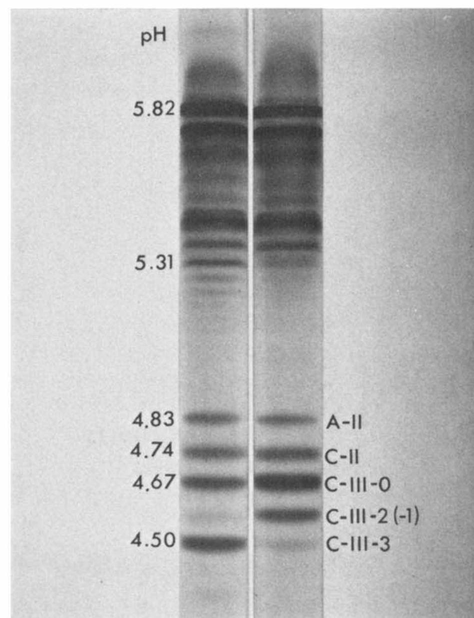

Fig. 7. IEF gels of control and neuraminidase-treated apo HDL. The left-hand gel in this figure shows an IEF gel of apo HDL. pI values corresponding to several bands are indicated for reference. The right-hand IEF gel shows the effects of treatment of apo HDL by neuraminidase. The identification of the pH 4.50–4.83 bands is indicated to the right.

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TABLE 1. Effect of neuraminidase on the composition of apo C-proteins of HDL

Experiment[a]	Apo HDL from Sucrose-Fed Rats 1 Control	Apo HDL from Sucrose-Fed Rats 1 Enzyme-Treated	Apo HDL from Sucrose-Fed Rats 2 Control	Apo HDL from Sucrose-Fed Rats 2 Enzyme-Treated	Apo HDL from Normal Rats 3 Control		Apo HDL from Normal Rats 3 Enzyme-Treated	
				% Composition				
C-II	22.5[b]	25.1[c]	29.9[b]	27.2[d]	24.4[b]	(18.6)[e]	22.2[d]	(15.4)[3]
C-III-0	41.6	53.4	48.6	61.4	33.8	(35.6)	48.9	(51.0)
C-III-2 (1?)	5.1	16.4	2.6	10.8	4.0	(7.0)	24.4	(26.7)
C-III-3	30.8	5.0	19.0	0.7	37.8	(38.8)	4.4	(7.0)

[a] 200–300 μg of protein were applied to each gel. Different groups of rats were used in Experiments 1 and 2.
[b] Results are from a single scan of one gel.
[c] Results are the means from three gels of the same neuraminidase-treated apo HDL.
[d] Results are the means from two gels of the same neuraminidase-treated apo HDL.
[e] The values in parentheses represent the percentage distribution of ^{3}H-labeled apo C-proteins. 1997 dpm and 2717 dpm, respectively, were recovered in the four apoproteins from the control and enzyme-treated apo HDL.

point and each gives a single, distinct band on IEF gels. IEF is clearly a superior method for resolving these components and will be useful in defining changes in apolipoprotein composition resulting from metabolic perturbations. For this purpose, integration of densitometric scans of IEF gels, as is shown in Table 1, can be used to describe quantitatively alterations in the distribution of these proteins. This approach has been used by Bar-On, Roheim, and Eder (12) for the study of apolipoprotein distribution in rats with induced diabetes.

By such procedures, one can see that there are significant differences between HDL and VLDL in the distribution of these proteins. As might be expected, the apo A–II protein appears to be found only in HDL. It is somewhat surprising, however, that apo VLDL is relatively poor in C–II (Fig. 1). Since the low molecular weight proteins are known to exchange readily between HDL and VLDL (23), such differences imply that these proteins interact in a specific and individual manner with the HDL and VLDL, rather than by nonspecific, adsorptive mechanisms.

The high degree of resolution of components in the pH 4.43–4.83 region (Fig. 1) has allowed us to detect three proteins at pH 4.61, 4.57, and 4.43, which have not been described before. We obtained a fraction from DEAE-cellulose chromatography that was enriched in the pH 4.61 and 4.57 components (Fig. 2 and 3). Because of contamination and low quantities obtained, we have not attempted to characterize these proteins. However, treatment of apo HDL by neuraminidase (Table 1, Fig. 7) suggests that the pH 4.57 and 4.61 proteins may be polymorphic forms of the C–III protein, differing primarily in sialic acid content. This is seen by the loss of stain intensity or radioactivity from bands at pH 4.50 with an increase in bands at pH 4.67 and 4.57–4.61. Alternatively, the band(s) between pH 4.50 and 4.67 on the control gel of Fig. 7 could be unrelated to C–III–2 or C–III–1, which coincidentally focus at the same pH. The fact that the band at pH 4.57–4.61, which we believe to be C–III–2 or C–III–1, or both, often is seen to increase in amount is probably due to an inability of the enzyme to completely hydrolyze all sialic acid residues (24).

TABLE 2. Classification of apolipoproteins of HDL and VLDL

Isoelectric Designation	Protein Designation[a]	Estimated Mol. Wt.	Sephadex Fraction
>6.00	C-I	7,000	HS-3 (VS-3)
5.82, 5.75, 5.65, 5.55, 5.46[b]	A-I	27,000	HS-2
5.46[b], 5.41, 5.39, 5.36, 5.31	ARP and A-IV	35,000 / 46,000	HS-1 (VS-2) / HS-1
4.83	A-II	8,000	HS-3
4.74	C-II	8,000	
4.67	C-III-0	10,000	
4.61	C-III-1?		HS-3
4.57	C-III-2?		(VS-3)
4.50	C-III-3	11,000	
4.43	C-III-4?		

[a] References 21 and 22.
[b] The pH 5.46 components are believed to be different polypeptides.

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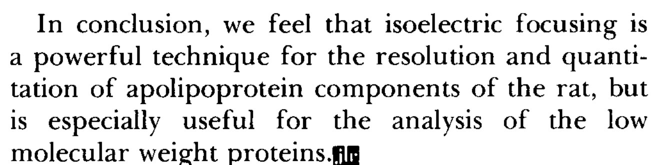

In conclusion, we feel that isoelectric focusing is a powerful technique for the resolution and quantitation of apolipoprotein components of the rat, but is especially useful for the analysis of the low molecular weight proteins.

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