

# Analysis of rat serum apolipoproteins by isoelectric focusing. I. Studies on the middle molecular weight subunits<sup>1</sup>

Lewis I. Gidez, John B. Swaney, and Sharon Murnane

Departments of Biochemistry and Medicine, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY 10461

**Abstract** Analytical isoelectric focusing (IEF) has been applied to the study of the apolipoprotein components of rat serum high density and very low density lipoproteins. The apolipoproteins were separated on 7.5% polyacrylamide gels containing 6.8% urea, with a pH gradient of 4–6. The middle molecular weight range apolipoproteins were identified on IEF gels by the use of apolipoproteins purified by electrophoresis on gels containing sodium dodecyl sulfate (SDS). The A-I protein focused as 4 to 5 bands from pH 5.46 to 5.82; the A-IV protein and the arginine-rich protein each focused as 4 to 6 bands from pH 5.31 to 5.46. The low molecular weight proteins focused from pH. 4.43 to 4.83 and are the subject of a separate communication. Comparisons of the IEF method with SDS gel electrophoresis, polyacrylamide gel electrophoresis in urea, and Sephadex chromatography are also reported. Additional studies were also carried out that tend to rule out carbamylation or incomplete unfolding of the proteins in the presence of urea as the causes of the observed heterogeneity.

**Supplementary key words** high density lipoprotein · very low density lipoprotein · SDS electrophoresis · arginine-rich protein · A-I apoprotein · A-IV apoprotein · carbamylation · Sephadex chromatography

Studies on the serum lipoproteins have been limited by the fact that no single analytical or preparative technique has sufficient resolving power to adequately describe the various apolipoproteins. Because of its potential for high resolution, we have made a detailed study of the technique of isoelectric focusing (1) for the analysis of rat apolipoproteins. Some isoelectric focusing studies have been carried out on apolipoproteins of human (2–9) and rhesus monkey (10), but there have been no reported studies with the rat.

In this report, we describe the general technique of gel isoelectric focusing of apolipoproteins and, in particular, the results of isoelectric focusing of the middle molecular weight polypeptides of rat serum high density and very low density lipoproteins. By this technique it was found that there is considerable polymorphism in ARP of apo VLDL and in each of

the three major polypeptides in apo HDL (A-I, A-IV, and ARP). The polymorphic forms of A-IV are not separated from those of ARP. Because the separation and analysis of rat apolipoproteins have been carried out with different techniques by various investigators (e.g., 11–15), we have correlated the results of Sephadex gel filtration, polyacrylamide gel electrophoresis in urea, and SDS gel electrophoresis with those obtained by isoelectric focusing.

Isoelectric focusing of the low molecular weight subunits is the subject of a separate report (16).

## MATERIALS 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 (trihydroxymethylamino-methane) and urea were from Schwarz/Mann, Orangeburg, NY. Aqueous solutions of urea were deionized immediately before use by passage through Rexyn 1-300 (Fisher Scientific Co., Fairlawn, NJ). Ampholine carrier ampholytes were obtained from LKB Instruments, Inc., Rockville, MD. Tetramethyl urea (Lot 3110) was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. All other chemicals were reagent grade.

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.

<sup>1</sup> In the context of this study, middle molecular weight apolipoproteins are those in the 27,000–46,000 dalton range.

## Lipoprotein separation

Rat serum was obtained from Sprague-Dawley rats maintained on a normal rat chow diet or on a semi-purified high sucrose diet for 15–22 days. Serum from animals fed the latter diet contains elevated levels of VLDL and HDL (17), and most of the studies were carried out on apolipoproteins from such serum. There were no observable qualitative differences among apolipoproteins isolated from rats fed the two diets, as revealed by various gel electrophoretic and isoelectric focusing techniques. Ethylenediamine tetraacetate (EDTA) disodium salt was added to the serum (1 mg/ml) before ultracentrifugation or directly to the blood after the animals were bled (1–2 drops of 20% EDTA/30 ml). The serum was first centrifuged at 10,000 rpm for 30 min to remove any chylomicrons. Lipoproteins were then isolated by ultracentrifugation, based on the method of Havel, Eder, and Bragdon (18).

The lipoprotein fractions ( $d < 1.006$  and  $d 1.063$ – $1.21$ ) were washed by resuspending them in the appropriate salt solution and repeating the centrifugation. The washed lipoprotein solutions were then dialyzed against 0.05% EDTA pH 7.0, or 0.04% EDTA pH 7.0. The solutions were stored in the cold until used, or lyophilized and stored at 2–4°C.

## Apolipoproteins

The lyophilized lipoproteins were delipidated by extraction with cold ethanol–diethyl ether 3:1 for 20 hr, followed by two additional 4-hr extractions with ethanol–ether 3:1 (19). The apolipoproteins were then washed with ether and dried under  $N_2$ . The apolipoproteins were dissolved in 0.2 M Tris-HCl pH 8.2, or in the same buffer containing 8 M urea, and left overnight at 4°C. In some of the early experiments, the solution also contained 0.06 M decylsulfate. In some studies, the lipoproteins were delipidated with tetramethyl urea according to the method of Kane (20).

## Isolation of purified apolipoproteins

Apo HDL and apo VLDL were chromatographed by the method of Bersot et al. (11) on  $2.5 \times 90$  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 2 mM decylsulfate. The elution patterns were similar to those of Bersot et al. (11). Three peaks were found for both apo 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.

## Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed at pH 8.9 in 8 M urea using 7.5 or 10% acrylamide,

as described by Roheim et al. (13). Electrophoresis on acrylamide gradient SDS slab gels was performed according to the method of Swaney and Kuehl (21). 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

To prepare the 7.5% acrylamide gels used in the focusing studies, the following solutions were mixed in an Erlenmeyer flask: 8 M urea (7.5 ml), glycerol (1.2 ml), 37.5% acrylamide–0.8% *N,N'* methylene-bisacrylamide in 8 M urea (prepared weekly) (2.4 ml), *N,N,N',N'*-tetramethylethylenediamine (0.05 ml), and 40% Ampholine (usually pH 4–6)<sup>2</sup> (0.6 ml). The gel solution was degassed by house vacuum and 0.2 ml of 1.07% ammonium persulfate in 8 M urea (prepared weekly) was added. The final urea concentration was 6.8 M. Proportionately larger volumes were used when necessary. The gel solution was added to the gel tubes ( $0.3 \times 10$  cm, or  $0.5 \times 15$  cm) that had been coated with a 1% solution of the tube rinse concentrate (RDS-K, Canalco) and sealed at the bottom with Parafilm; distilled water was layered on top of the gel solution. The final height of the gel in the short tubes was 9 or 10 cm (depending on how much sample was to be added); the gel height in the 15-cm tubes was 14–14.5 cm. After polymerization, the tubes were sealed with Parafilm and stored in the cold room. They were usually used on the following day.

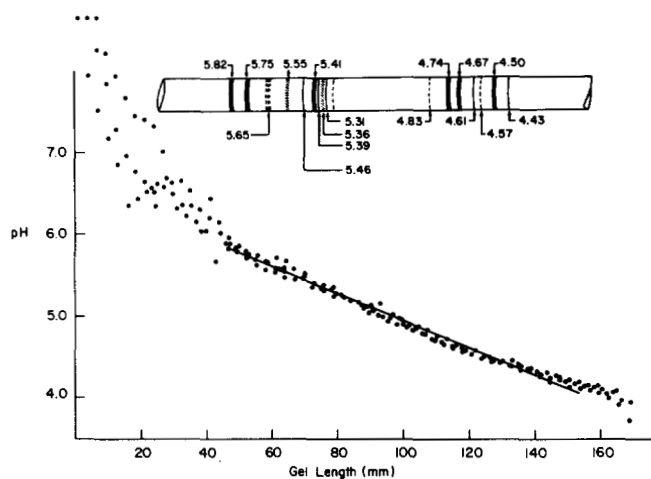
Before use, the Parafilm seal on each gel tube was replaced with a small square of toweling wick (Masslinn non-woven sport towels, Chicopee Mills, Inc., 1450 Broadway, NY) held in place with a rubber sleeve.

Isoelectric focusing was carried out in a Model 137-A (9-cm gel) or Model 137-P (14-cm gel) isoelectric focusing apparatus (MRA Corporation, Boston, MA). The gel tubes were set in place, and cold water (2–4°C) was circulated through the apparatus. The lower chamber was filled with 0.01 M phosphoric acid (anolyte), and 0.02 M sodium hydroxide (catholyte) was put in the upper chamber. The gels were then prefocused for 30 min (1 mA/gel) in order to remove excess persulfate and to partially establish the pH gradient. The aliquot of protein solution to be analyzed (25–100  $\mu$ g in 50  $\mu$ l for the 9-cm gels, and 40–300  $\mu$ g in a maximum of 200  $\mu$ l for the 14-cm gels) was layered under the catholyte and the gels were

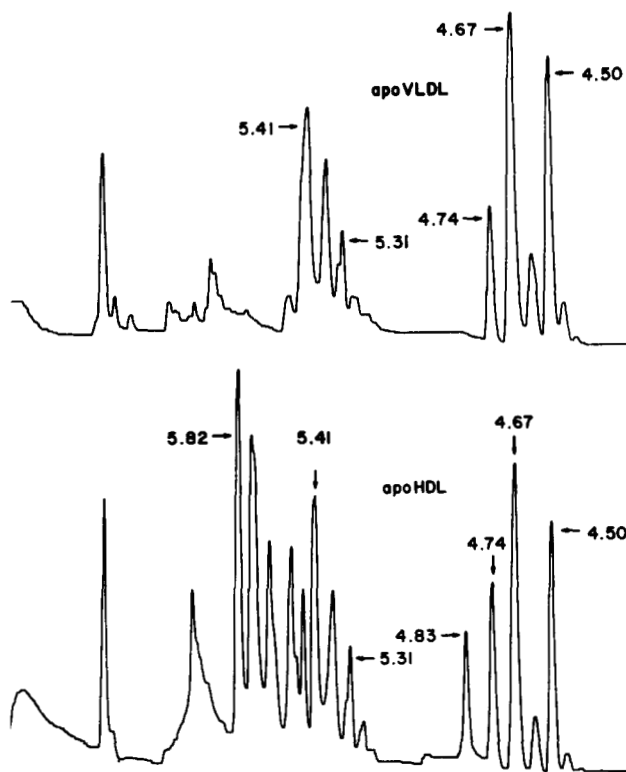
<sup>2</sup> We did not systematically examine pH ranges other than 4–6. However, IEF patterns of apolipoproteins on pH 3–10 or 5–7 gradients were essentially the same as those on pH 4–6 gradients, except for the length of gel occupied by the bands.

focused at 400 volts for 4.5–5 hr until the current stabilized at around 1–1.2 mA (for 12 gels). In the case of the larger gels, the voltage was adjusted to 200 volts and then increased to 400 volts during the first hour. The proteins on these gels were focused for about 20 hr (final current 2.0–2.2 mA). Usually 12 gels were focused in each run. An LKB model 3371E or MRA Model ARP-1 power supply was used in most studies.

Coomassie brilliant blue cannot be used to stain IEF gels unless the Ampholine is first removed, otherwise there is intense background staining. However, this dye can be treated so that it can stain gels without prior removal of the Ampholine, according to the procedure of Malik and Berrie (22). One hundred ml of 2N H<sub>2</sub>SO<sub>4</sub> was added to 100 ml of 2% aqueous Coomassie brilliant blue (R-250, Colab Lab. Inc., Glenwood, IL). The solution was thoroughly mixed and filtered (Whatman No. 1 paper). Ten to fifteen normal KOH was added dropwise to the green filtrate until it turned blue. Twelve g of trichloroacetic acid was then added. If the solution became green, the concentrated KOH was added until the color of the solution was blue. The gels were immersed in this solution for 1–2 hr. Light background staining was removed by allowing the gels to stand in water overnight,



**Fig. 1.** pH Gradient on 0.5 × 16 cm gels (pH 4–6 Ampholine) and apparent isoelectric points of apo HDL peptides. Each point on the pH gradient curve represents a single pH measurement of a 2–3 mm-thick segment cut from four 0.5 × 16 cm gels. A close examination of the individual points shows that the gradient deviates from linearity at the extremes of the pH 4–6 region. Therefore, in order to assign pI values to bands, a linear least mean square analysis of the data, limited to the pH 4.2–5.8 region, was carried out to generate the line shown. The diagram above the curve is a typical pattern of apo HDL. The assigned pI values of the individual bands were calculated using the distance of the band from the top (basic end) of the gel and the least mean squares constants. Appropriate corrections were made to compensate for the swelling of the gel (ca. 10%) as a result of staining and destaining. The dimensions in the figure are for a swollen gel.



**Fig. 2.** Scans of 0.3 × 9 cm IEF gels of apo VLDL and apo HDL. The gels were stained with modified Coomassie blue and scanned at 567 nm. The pI values assigned to the various peaks correspond to those in Fig. 1.

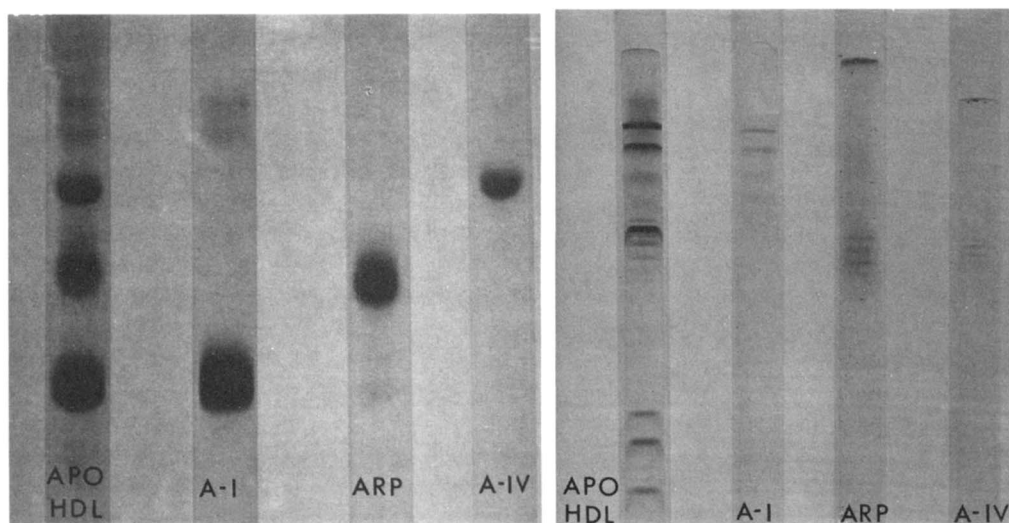
and the gels were stored in water or in 5% methanol–7% acetic acid in the dark. Studies in which different amounts of serum albumin were applied to 9-cm gels showed that as little as 1–2 μg of protein per band could be detected.

The stained gels were scanned at 567 nm in a Gilford Model 240 (Gilford Instrument Labs, Inc., Oberlin, OH) spectrophotometer system (with a Beckman DU Model 2400 Monochromator, Beckman Instruments, Inc., Fullerton, CA) equipped with a Gilford Model 2410-S linear transport and a Model 6040 recorder.

### Measurement of pH

The 9-cm diameter gels (unfixed and unstained) were placed in a repeating dispenser device (MRA, Inc., Boston, MA) and 65–70 aliquots were obtained from each 9-cm gel. The 14-cm gels were sliced with a razor blade into 2-mm segments. Comparable aliquots from each of two 9-cm gels or single segments of 14-cm gels were transferred to small test tubes and 1 ml of deionized water was added. The tubes were then stoppered and left in the cold room overnight. The pH was measured at room temperature on a Model 26 Radiometer pH meter with a GK 2322 C electrode.





**Fig. 3.** Left, SDS electrophoresis patterns and right, IEF patterns of rat apo HDL, A-I, ARP, and A-IV. Details of preparation of A-I, ARP, and A-IV are given in the text.

### Amino acid analysis

Proteins were hydrolyzed in 5.6 N HCl for 21 hr, followed by analysis on a Beckman 120C amino acid analyzer.

## RESULTS

### General description of IEF pattern of apo HDL and apo VLDL

**Fig. 1** shows a diagrammatic representation of an IEF pattern of apo HDL on a  $0.5 \times 14$  cm gel. Beneath the gel is a typical pH 4–6 gradient curve. Individual values were obtained from pH measurements on four blank gels that were focused in four different experiments. The pI values in this report and in a companion study (16) were obtained from a typical long gel. This gel was selected at random from six gels that were focused at the same time. By visual inspection, the pattern of bands on the six gels appeared identical. We often found different pI values for comparable bands on gels from different experiments. However, on long (14 cm) gels, the range of values was 0.08 pH units; on short (9 cm) gels, the range was 0.14 pH units. The pI values given are intended as frames of reference and should be regarded as apparent isoelectric points. The bands in the pH range of 4.43–4.83 were found to consist of the low molecular weight subunits, and isoelectric focusing studies on these proteins are described in a separate report (16). The middle molecular weight subunits to be described here are found in the pH range 5.31–5.82.

The individual features of IEF patterns of apo HDL and apo VLDL are seen in **Fig. 2**. This figure

shows densitometric tracings obtained by scanning 9-cm IEF gels. For purposes of reference, some of the pH values from **Fig. 1** are included.

The assignment of pI values of apo VLDL bands was based on measurements of apo HDL bands. Estimated pI values of VLDL bands obtained by relating distance to pH on blank gels were comparable to similarly located apo HDL bands and were within the ranges noted above. Apo VLDL lacks bands at pH 5.55–5.82, but has a group of bands at pH 5.31–5.46, similar to those seen in apo HDL. Apo VLDL also has a group of bands from 4.43 to 4.74; however, there is no pH 4.83 band in apo VLDL.

In addition to the peaks between pH 4.43 and 5.82, some gels had bands above pH 5.82. At, or near, the tops of the gels there were often bands (not shown in **Fig. 2**) that may represent the high molecular weight proteins of VLDL, or possibly aggregates in apo HDL that did not penetrate the 7.5% gels. On some gels (e.g., **Fig. 2**), other bands were seen. One of these (the narrow peak to the left in **Fig. 2**) was often seen on the gels that contained no protein and is probably artifactual. The broad peak to the immediate left of the pH 5.82 peak in apo HDL was seen in many, but not all, preparations of apo HDL and may represent albumin. In separate experiments (gels not shown), we observed that rat serum albumin focused as one major and 2–3 minor closely-spaced bands just above the band designated as pI 5.82. Bovine albumin has been found by Salamon and Williamson (23) to focus around pH 6 in the presence of 6 M urea, but below pH 5 in the absence of urea.

HDL or VLDL could be focused without prior solvent delipidation by pretreatment of the lipopro-

tein solution with an equal volume of tetramethyl urea (20). Patterns identical to those of solvent-delipidated apolipoproteins were obtained.

### Correlation of SDS electrophoresis and IEF gel patterns

Apo HDL was fractionated by SDS gel electrophoresis on several gels. Using one stained gel as a template, fractions containing A-I (mol wt 27,000), ARP (mol wt 35,000), and A-IV (mol wt 46,000) were isolated from unfixed and unstained gels (15). The gel segments were placed in the barrel of a 5 ml syringe and extruded into a 50 ml plastic centrifuge tube. The crushed gel was then treated three times with approximately 6 volumes of 0.1 M phosphate buffer pH 7.0, containing 0.1% SDS to extract the protein. The total extraction time was ca. 30 hr. The extracts were placed in Spectrapor 1 dialysis tubing and concentrated by imbedding the tube in Sephadex G-200. Aliquots were then subjected to SDS gel electrophoresis (Fig. 3A) and gel isoelectric focusing (Fig. 3B). The patterns showed that A-I focused as four bands (pH 5.55–5.82) and that ARP and A-IV focused as multiple bands (pH 5.31–5.46) below A-I. The staining at the top of some of the gels (especially the ARP gel) was probably due to aggregated material. The coincident focusing of ARP and A-IV was also shown by a two-dimensional electrofocusing–SDS electrophoretic technique (16). On the basis of these studies, we have concluded that A-IV and ARP have similar isoelectric points that preclude their separation by isoelectric focusing.

### Comparison of polyacrylamide electrophoresis and IEF gel patterns

In view of the fact that many investigators have utilized polyacrylamide gel electrophoresis (PAGE) in urea for studies of metabolism and identification of apolipoproteins (e.g., 13, 14), we compared apolipoprotein gel patterns obtained by this method and IEF. Samples of apo HDL and apo VLDL were subjected to electrophoresis on 7.5% polyacrylamide gels containing urea, and sections of unfixed, unstained gels were cut out, using stained gels as a guide. These sections were then placed on the top of IEF gels, and acrylamide solution was added. After polymerization, IEF was carried out.

Fig. 4 shows results of some representative experiments. The gel designated by Roman numeral I is a PAGE gel of apo HDL; gels designated by letters (A–G) are IEF gels.

Segments (indicated by letters A–F) were cut from other unstained PAGE gels of apo HDL and subjected to IEF (gels A–F). Gel G (on the right) is an IEF

pattern of apo HDL. For purposes of reference, bands on gel G are designated by pI values, as in Fig. 1, and by the nomenclature of Alaupovic (24) (See also Fig. 3).

Considering the IEF bands in order of decreasing pI, the A-I components of pI 5.55–5.82 are located predominantly in sections A and C. The A-IV and ARP components, pI 5.31–5.41, are found mainly in section B. In the middle of the PAGE gels, there is a single well-resolved band which has been designated A-II by Herbert et al. (25). Section C contains a portion of this band, which appears to focus at pH 4.83, as well as components corresponding to sections A and B. The lower bands of pI 4.57–4.74 appear to be found mainly in section F, while section E contains most of the 4.50 bands. Section D is comprised mainly of proteins that focus above pH 5.82 and perhaps aggregated material, with only traces of the other protein components of apo HDL. A minor band at approximately pH 5.46 was found in both HS-1 and HS-2.

### IEF of Sephadex G-150 fractions of apo HDL and apo VLDL

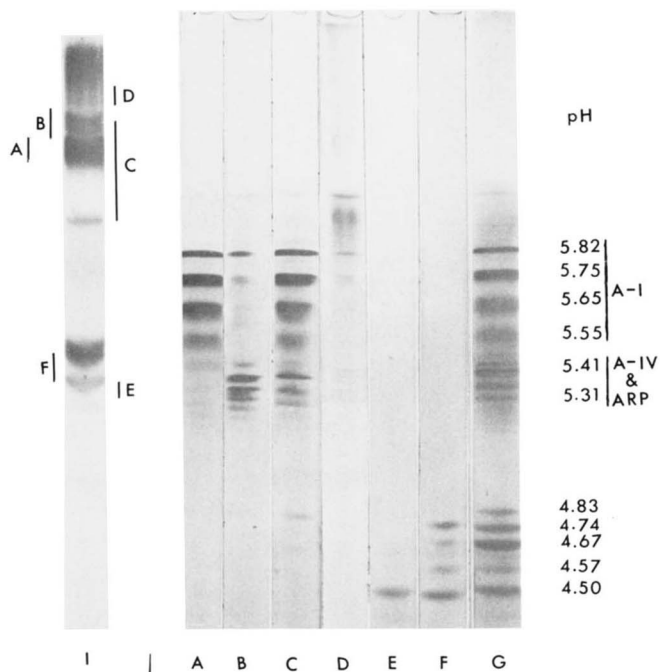
Fig. 5A shows IEF patterns of Sephadex G-150 fractions HS-1, HS-2, and HS-3 of apo HDL (11). The first Sephadex peak (HS-1) shows multiple bands in the pH 5.31–5.46 region (A-IV and ARP), as well as some lighter staining bands from pH 5.46–5.82. A thin band above pH 5.82 is unidentified. This band is not seen on the apo HDL gel and its presence in HS-1 may be artifactual as a result of the gel filtration procedure, or the band may represent a trace component (albumin contaminant or an aggregated protein). HS-2 focused as five bands in the pH 5.46–5.82 range; these bands correspond to A-I. HS-3 focused in the pH 4.43–4.83 range; the bands comprised A-II (the top band in this pH range) and the various C proteins (16).

Patterns of Sephadex G-150 fractions of apo VLDL are shown in Fig. 5B. VS-2 focused as multiple bands in the pH 5.31–5.46 region. VS-3 (the C proteins) focused from pH 4.43 to 4.75. A VS-1 fraction was focused, but the protein failed to enter the gel, and this gel pattern was not included in the figure.

### Studies on the nature of the heterogeneity observed by IEF

1. *Effect of urea concentration.* In order to evaluate whether incomplete unfolding by urea caused the multiple bands observed on IEF, gels were prepared that contained 2.2 M, 5.6 M, and 8.5 M urea. Identical samples of rat apo HDL were focused on these gels, as well as on a gel containing the usual (6.8 M)





**Fig. 4.** Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) patterns of apo HDL. Gel 1 is a PAGE pattern of apo HDL showing sections A-F (indicated by the vertical lines) that were cut out and subjected to IEF (gels A-G, 0.5 × 15 cm).

concentration of urea (**Fig. 6**). Lower concentrations of urea produced less distinct patterns, but the multiplicity of bands in the A-I region was observed at all urea concentrations.

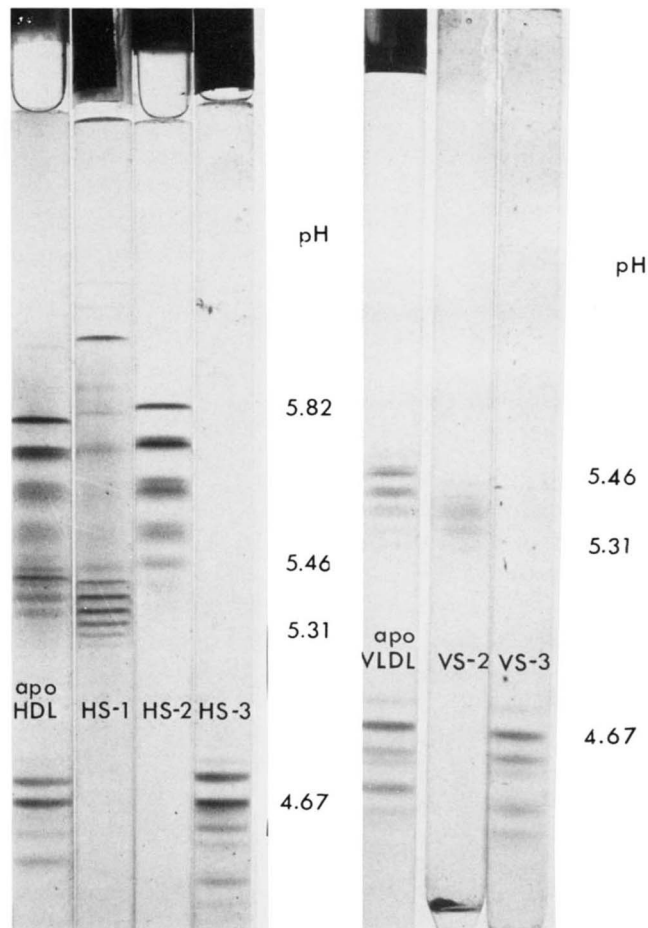
**2. Effect of carbamylation.** The possibility that multiple bands are created by carbamylation of proteins by urea breakdown products was investigated. In one experiment, rat apo HDL was incubated in a solution of freshly deionized 8 M urea at 4°C for 1, 2, and 5 days (**Fig. 7**, gels 1, 2, 3).<sup>3</sup> At the same time, other samples were incubated at 4°C for the same periods in a solution of 8 M urea that had been previously boiled (**Fig. 7**, gels 4, 5, 6). It can be seen that apo HDL incubated in freshly deionized urea did not undergo any changes upon standing up to 5 days, and in fact, showed banding patterns identical to those of samples applied immediately to the IEF gels after dissolution in urea (**Fig. 2**). However, samples incubated in urea that had been boiled to produce cyanate showed noticeable changes after incubation for one day, with severe changes by 5 days.

**3. Isolation of A-I subfractions.** In order to examine the amino acid composition of individual IEF bands, the isolation of these bands on large gels was attempted.

<sup>3</sup> Our routine procedure was to perform the first focusing study of a given sample 1-3 days after solubilization of the apolipoprotein.

A-I was chosen for these studies, since the individual subfractions on IEF gels are better separated for this protein than the others. Five mg of rat apo HDL was focused (pH 4-6) for 20 hr at 400 volts at 2°C on gels that had been prefocused for 30 min at 200 volts. One gel was stained for a brief period and was used as a template to cut four A-I subfractions from the unstained gel. Each gel segment was crushed and inserted into a 9 × 0.9 cm tube. Acrylamide solution (8%) containing 0.01 M Na<sub>2</sub>HPO<sub>4</sub> pH 8.6, and 0.1% SDS was then added. After polymerization occurred, the A-I subfractions were electrophoresed for 20 hr at 300 volts into Visking tubing (which contained the phosphate buffer and SDS solution) attached to the bottom of the gel tube. The samples were then dialyzed against water for 48 hr to remove the SDS. Aliquots of the solution (final volumes 0.7-20.0 ml) were removed for amino acid analyses (**Table I**) and IEF (**Fig. 8**).

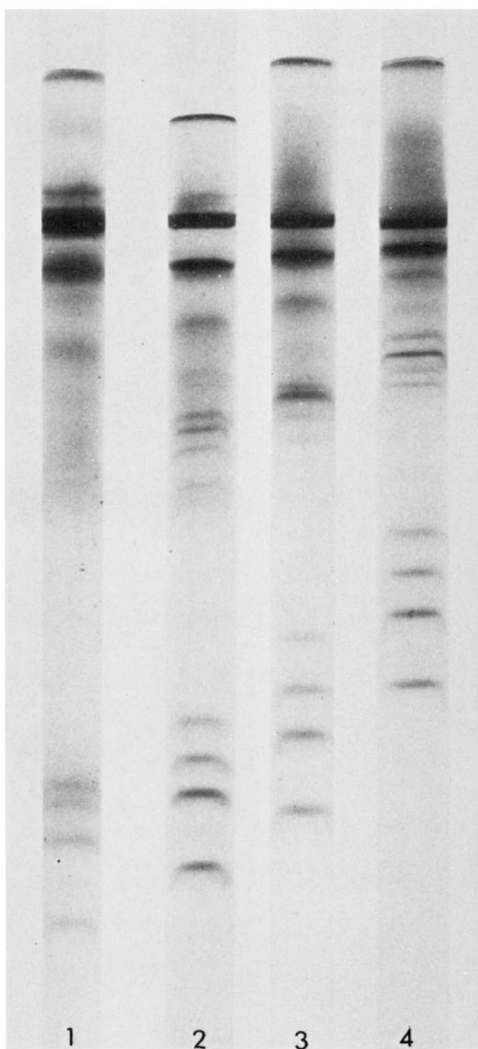
It can be seen in **Fig. 8** that the subfractions obtained



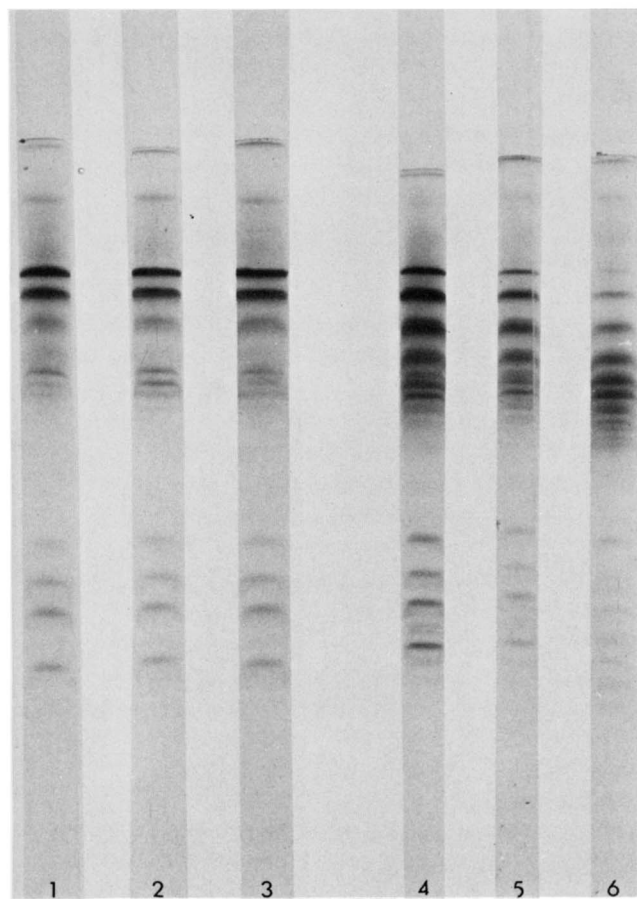
**Fig. 5.** IEF patterns of Sephadex G-150 fraction of: A, apo HDL and B, apo VLDL. For purposes of reference, apparent pI values are listed to the right of each group of gels.

in this way were contaminated with adjacent bands. It was also observed, by SDS gel electrophoresis, that the fourth fraction (Fig. 8, gel 5) was contaminated with A-IV and ARP proteins; this fraction was not used for amino acid analysis. The fractions did, however, generally retain their relative isoelectric focusing properties, indicating that these species are not in equilibrium and may be distinct chemical entities.

Amino acid analysis of the A-I subfractions (Table I) indicates that these fractions have similar, if not identical, amino acid compositions, within experimental error. The most significant result was the finding of no homocitrulline peak in these samples, indicating that no carbamylation had occurred. Homocitrulline standard (ICN Pharmaceuticals, Inc.,



**Fig. 6.** IEF of apo HDL on gels containing different concentrations of urea. 100  $\mu$ g of apo HDL was focused on each gel (15  $\times$  0.5 cm gels, pH 4–6, IEF for 18 hr). Urea concentration: gel 1, 2.2 M; gel 2, 5.6 M; gel 3, 6.8 M; gel 4, 8.5 M.



**Fig. 7.** IEF patterns of apo HDL incubated (room temperature) in freshly deionized 8 M urea for 1, 2, and 5 days (gel 1, 2, and 3, respectively) or in boiled 8 M urea for 1, 2, and 5 days (gels 4, 5, and 6, respectively). The final concentration of urea in the gels was 6.8 M, and 100  $\mu$ g of protein was applied to each gel (15  $\times$  0.5 cm gels, pH 4–6, IEF for 18 hr).

Plainview, NY) was found to elute as well-resolved peak just prior to valine in the two-column methodology.

## DISCUSSION

The purpose of this study was to describe and evaluate the technique of gel isoelectric focusing as applied to rat serum apolipoproteins, and to compare IEF with other techniques commonly used for the resolution of the subunit proteins, such as polyacrylamide gel electrophoresis, SDS gel electrophoresis, and gel filtration.

Isoelectric focusing of rat apo HDL reveals a complex pattern of bands (Fig. 1). Each of the major protein components, A-I, ARP, and A-IV appears to be composed of 4–6 bands. The bands for ARP and A-IV overlap to such a degree that it is difficult to assign identities to specific bands in this region (pH



TABLE 1. Amino acid composition of A-I and IEF subfractions

Amino Acid	A-I <sup>a</sup>	A-I-1	A-I-2	A-I-3
	% by weight $\pm$ SD			
Lysine	9.8 $\pm$ 1.2	10.9 $\pm$ 1.4	11.3 $\pm$ 1.7	8.8 $\pm$ 0.6
Histidine	2.4 $\pm$ 0.3	2.8 $\pm$ 0.7	1.9 $\pm$ 1.0	1.9 $\pm$ 0.2
Arginine	7.0 $\pm$ 1.6	6.1 $\pm$ 1.2	6.3 $\pm$ 0.7	4.5 $\pm$ 0.5
Aspartic acid	13.4 $\pm$ 0.2	14.2 $\pm$ 0.8	14.8 $\pm$ 2.0	14.6 $\pm$ 0.7
Threonine	4.2 $\pm$ 0.1	4.3 $\pm$ 0.4	4.4 $\pm$ 0.4	4.7 $\pm$ 0.2
Serine	3.3 <sup>b</sup>	3.2 $\pm$ 0.6	3.2 $\pm$ 0.4	3.4 $\pm$ 0
Glutamic acid	20.0 $\pm$ 0.1	20.1 $\pm$ 0.7	21.2 $\pm$ 1.4	23.5 $\pm$ 0.1
Proline	2.4 $\pm$ 0.1	2.6 $\pm$ 0.2	2.3 $\pm$ 0.6	2.5 $\pm$ 0.5
Glycine	2.4 $\pm$ 0.2	2.9 $\pm$ 0.4	2.9 $\pm$ 0.2	3.1 $\pm$ 0.4
Alanine	4.8 $\pm$ 0.1	5.0 $\pm$ 0.2	5.3 $\pm$ 0.2	5.0 $\pm$ 0.3
Homocitrulline		<0.1	<0.1	<0.1
Valine	4.6 $\pm$ 0.5	5.1 $\pm$ 0.4	4.5 $\pm$ 0.6	5.3 $\pm$ 0.2
Methionine	2.8	2.8 $\pm$ 0.7	2.2 $\pm$ 0.7	2.5 $\pm$ 0.2
Isoleucine	1.8 $\pm$ 0.2	1.9 $\pm$ 0.1	1.9 $\pm$ 0.2	2.3 $\pm$ 0.2
Leucine	12.6 $\pm$ 0.1	12.9 $\pm$ 0.9	12.4 $\pm$ 0.9	12.8 $\pm$ 0.3
Tyrosine	2.9 $\pm$ 0.3	2.3 $\pm$ 1.2	2.1 $\pm$ 0.2	1.8 $\pm$ 0.3
Phenylalanine	3.7 $\pm$ 0.2	3.6 $\pm$ 0.2	3.3 $\pm$ 0.3	3.2 $\pm$ 0.7
Tryptophan	1.5	ND <sup>c</sup>	ND	ND
Cys/2	ND	ND	ND	ND
No. of determinations	6	5	4	3

<sup>a</sup> Swaney, J. B., F. Braithwaite, and H. A. Eder. Data from ref. 29.

<sup>b</sup> Extrapolated value obtained by time course hydrolysis.

<sup>c</sup> ND, not determined.

5.31–5.41) The A-I protein bands have higher pI values (pH 5.55–5.82). Rat apo VLDL presents a less complex IEF pattern (Fig. 2), due to the absence of the A-I, A-IV, and A-II (16) proteins. Gels shown in Figs. 4 and 5 demonstrate how identification of components by IEF can be correlated with separations obtained by polyacrylamide gel electrophoresis in urea and Sephadex chromatography. These studies show the superior resolving power of the IEF method.

The multiplicity of bands observed for the A-I, ARP, and A-IV proteins presents important problems for interpretations of IEF data. It must be resolved whether the multiple bands are artifactually generated or are unique chemical species, which are present in vivo. Utermann, Jaeschke, and Menzel (26) have provided support for the latter hypothesis: by IEF, they found human apo E to consist of three major components, one of which was missing in certain patients.

We have attempted to deal with the question of heterogeneity in a systematic way, concentrating on the multiple bands found for rat apo A-I. Since only the larger proteins produce multiple IEF bands that cannot be attributed to compositional differences,

one hypothesis tested was that these larger proteins were only partially unfolded in the 6.8 M urea milieu of the gel and that the proteins occupied several conformational states differing in their pI values. However, it was found that varying the urea concentration in the gels up to a level of 8.5 M (Fig. 6) had no apparent effect on the observed heterogeneity, although high concentrations of urea were found to produce sharper bands in the IEF gels. Thus, we feel that incomplete unfolding of these proteins is not the cause of the multiple bands.

Carbamylation by cyanate in urea gels is a commonly invoked explanation for multiple bands seen in IEF gels, and intentional carbamylation of apo HDL (Fig. 7, gels 4, 5, 6) did, indeed, appear to generate the bands of lower pI seen beneath the main A-I bands and the lower molecular weight bands. However, several lines of thought argue against this possibility. For example, the multiple bands, of which the two major ones are of almost equal intensity, would have to be formed from one band during the short period of 4 hr during which the focusing run is carried out; yet incubation for 5 days in a deionized urea solution produced no further changes. Secondly, amino acid analysis of semipurified IEF bands gave no indication of any homocitrulline, the product of carbamylation (Table 1). Focusing of apo HDL never exposed to urea, in gels containing only buffer or Triton X-100, also gave patterns with multiple bands (gels not shown).

We believe the data suggest that the two major A-I bands (pI = 5.82 and 5.75) and possibly also the minor bands (pI = 5.56 and 5.55) are chemically different species which could be functionally different. The amino acid compositions of the subfractions

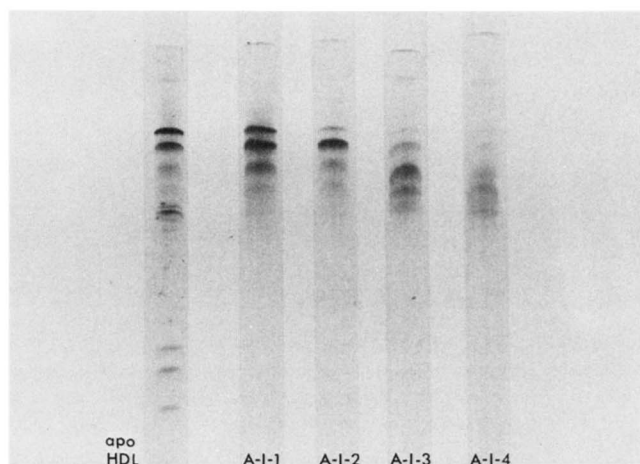


Fig. 8. IEF patterns of apo HDL and subfraction of A-I: A-I-1, A-I-2, A-I-3, and A-I-4 (15  $\times$  0.5 cm gels, 6.8 M urea, pH 4–6, IEF for 18 hr).



(Table 1) were very similar, but precision of the analysis is inadequate for ruling out subtle differences. Differences in the amide content or degrees of deamidation could also account for the heterogeneity.

Another source of difference, which we have not investigated, is the carbohydrate content, or the possibility of trace amounts of residual phospholipid. However, one indication that residual lipid is not the cause of the observed heterogeneity is that the same pattern is obtained whether the sample has been delipidated by organic solvents, tetramethylurea, or sodium dodecyl sulfate.

The findings of polymorphic forms of rat A-I protein, in fact, coincides with similar findings for human A-I. Shore and Shore (27) noted three R-Thr fractions ( $R_1$ -Thr,  $R_2$ -Thr, and  $R_3$ -Thr) by DEAE-cellulose chromatography of HDL<sub>2</sub> apoproteins. These fractions had similar amino acid compositions, except for the lack of isoleucine in  $R_1$ -Thr. Lux and John (28) found five subfractions of A-I by DEAE-cellulose chromatography that were identical by all chemical criteria employed for their study. In addition, Albers, Albers, and Aladjem (4) found five subfractions of human A-I by IEF on sucrose density columns. In each case, identical amino acid compositions were found for the polymorphic forms. ■

This work was supported by Research Grants HL 02965, HL 14236, and AM 13985 from the National Institutes of Health, and by Training Grant HL 5273 from the U.S. Public Health Service.

Manuscript received 16 May 1975 and in revised form 12 May 1976; accepted 11 October 1976.

## REFERENCES

1. Haglund, H. 1971. Isoelectric focusing in pH gradients—A technique for fractionation and characterization of ampholytes. *Methods Biochem. Anal.* **19**: 1–104.
2. Albers, J. J., and A. M. Scanu. 1971. Isoelectric fractionation and characterization of polypeptides from human serum very low density lipoproteins. *Biochim. Biophys. Acta* **236**: 29–37.
3. Albers, J. J., and F. Aladjem. 1971. Precipitation of <sup>125</sup>I-labeled lipoprotein with specific antisera. Evidence for two populations with differing polypeptide compositions in human high density lipoproteins. *Biochemistry*. **10**: 3436–3443.
4. Albers, J. J., V. Albers, and F. Aladjem. 1971. Isoelectric heterogeneity of the major polypeptide of human serum high density lipoproteins. *Biochem. Med.* **5**: 48–55.
5. Edelstein, C., C. T. Lim, and A. M. Scanu. 1972. On the subunit structure of the protein of human serum high density lipoprotein. I. A study of its major polypeptide component (Sephadex, fraction III). *J. Biol. Chem.* **247**: 5842–5849.
6. Scanu, A. M., C. T. Lim, and C. Edelstein. 1972. On the subunit structure of the protein of human serum high density lipoprotein. II. A study of Sephadex fraction IV. *J. Biol. Chem.* **247**: 5850–5855.
7. Scanu, A. M., J. Toth, C. Edelstein, S. Koga, and E. Stiller. 1969. Fractionation of human serum high density lipoprotein on urea solutions. Evidence for polypeptide heterogeneity. *Biochemistry*. **8**: 3309–3316.
8. Shelburne, F. A., and S. H. Quarfordt. 1974. A new apoprotein of human plasma very low density lipoprotein. *J. Biol. Chem.* **249**: 1428–1433.
9. Utermann, G. 1975. Isolation and partial characterization of an arginine-rich apolipoprotein from human plasma very low density lipoproteins: Apolipoprotein E. *Hoppe Seyler's Physiol. Chem.* **356**: 1113–1121.
10. Edelstein, C., C. T. Lim, and A. M. Scanu. 1973. The serum high density lipoproteins of *Macacus rhesus*. II. Isolation, purification, and characterization of their two major polypeptides. *J. Biol. Chem.* **248**: 7653–7660.
11. Bersot, T. P., W. V. Brown, R. I. Levy, H. G. Windmueller, D. S. Fredrickson, and V. S. LeQuire. 1970. Further characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry*. **9**: 3427–3433.
12. Koga, S., L. Bolis, and A. M. Scanu. 1971. Isolation and characterization of subunit polypeptides from apoproteins of rat serum lipoproteins. *Biochim. Biophys. Acta.* **236**: 416–430.
13. Roheim, P. S., H. Hirsch, D. Edelstein, and D. Rachmilewitz. 1972. Metabolism of iodinated high density lipoprotein subunits in the rat. III. Comparison of the removal rate of different subunits from the circulation. *Biochim. Biophys. Acta.* **278**: 517–529.
14. Noel, S. P., and D. Rubinstein. 1974. Secretion of apolipoproteins in very low density and high density lipoproteins by perfused rat liver. *J. Lipid Res.* **15**: 301–308.
15. Swaney, J. B., H. Reese, and H. A. Eder. 1974. Polypeptide composition by SDS-gel electrophoresis. *Biochem. Biophys. Res. Comm.* **59**: 513–519.
16. Swaney, J. B., and L. I. Gidez. 1976. Analysis of rat serum apolipoproteins by isoelectric focusing. II. Studies on the low molecular weight subunits. *J. Lipid Res.* **18**: 69–76.
17. Shiff, T., P. S. Roheim, and H. A. Eder, 1971. Effects of high sucrose diets and 4-aminopyrazolopyrimidine on serum lipids and lipoproteins in the rat. *J. Lipid Res.* **12**: 596–603.
18. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345–1353.
19. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244**: 5687–5694.
20. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53**: 350–364.
21. Swaney, J. B., and K. S. Kuehl. 1976. Separation of apolipoproteins by an acrylamide-gradient SDS gel electrophoresis system. *Biochim. Biophys. Acta.* **446**: 561–565.
22. Malik, N., and A. Berrie. 1972. New stain fixative for proteins separated by gel isoelectric focusing based on Coomassie brilliant blue. *Anal. Biochem.* **49**: 173–176.

23. Salamon, M. R., and A. R. Williamson. 1971. Isoelectric focusing of proteins in the native and denatured states. Anomalous behavior of plasma albumin. *Biochem. J.* **122**: 93–99.
24. Alaupovic, P. 1971. Conceptual development of the classification systems of plasma lipoproteins. In *Proteins of the Biological Fluids*. H. Peeters, editor. Pergamon Press, Oxford. 9–19.
25. Herbert, P. N., H. B. Windmueller, T. P. Bersot, and R. S. Shulman. 1974. Characterization of the rat apolipoproteins. I. The low molecular weight proteins of rat plasma high density lipoproteins. *J. Biol. Chem.* **249**: 5718–5724.
26. Utermann, G., M. Taeschke, and J. Menzel. 1975. Familial hyperlipoproteinemia type III: Deficiency of a specific apolipoprotein Apo E-III in the very low density lipoproteins. *FEBS Lett.* **56**: 352–355.
27. Shore, B., and V. Shore. 1969. Isolation and characterization of polypeptides of human serum lipoproteins. *Biochemistry.* **8**: 4510–4516.
28. Lux, S. E., and K. M. John. 1972. Further characterization of the polymorphic forms of a human high density apolipoprotein, apo LP-Gln-1 (apo A-I). *Biochim. Biophys. Acta.* **278**: 266–270.
29. Swaney, J. B., F. Braithwaite, and H. Eder. 1977. Characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry*. In press.