papers and notes on methodology

Preparative isotachophoresis of human plasma high density lipoproteins HDL_2 and HDL_3

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Abstract HDL_2 and HDL_3 subclasses of human serum HDL were isolated by preparative ultracentrifugation and further analyzed by isotachophoresis on polyacrylamide gel.The HDL_2 divided into six subfractions and the HDL_3 into ten subfractions differing in chemical composition and in apolipoprotein content. The apoA-I/apoA-II ratios differed widely among the various subfractions. The subfractions with the highest apoA-I/apoA-II ratio appeared to have the greatest affinity for cholesterol. The preparative isotachophoresis, used for the first time in this type of investigation, has high resolving power and is reproducible and thus suitable for use in the study of the structure and metabolism of the lipoproteins.—**Bittolo Bon, G., G. Cazzolato, and P. Avogaro.** Preparative isotachophoresis of human plasma high density lipoproteins HDL_2 and HDL_3. *J. Lipid Res.* 1981. **22:** 998–1002.

Supplementary key word apolipoproteins

The serum lipoproteins constitute a continuum of macromolecules differing from one another in chemical, physicochemical, and immunochemical make-up. The lipoproteins are classified chiefly according to hydrated density and on this basis the serum high density lipoproteins can be divided further into two subclasses: HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.210 g/ml). It was shown as long as 15 years ago that the HDL is heterogeneous both in electrophoretic mobility and in immunochemical behavior (1). Human HDL can be fractionated into the lipoprotein families Lipoprotein-A, -B, and -C by means of a combined procedure based on specific immunoadsorbers, chromatography on hydroxylapatite, and precipitation on polyethylene glycols (2). By chromatographing the HDL on hydroxylapatite columns, Kostner and Holasek (3) identified the subfractions differing in chemical composition and apolipoprotein pattern. Preparative isoelectric focusing has shown that human HDL is even more heterogeneous (4–6).

In this study we show that preparative isotachophoresis, used for the first time in this type of investigation, makes possible the reproducible separation of the HDL into 16 subfractions differing in chemical composition and apolipoprotein pattern. Preparative isotachophoresis is characterized by the use of a discontinous buffer system; the sample is then separated into clearly defined compartments formed between a leading electrolyte having a high mobility and a terminating electrolyte having a low mobility. These compartments appear contiguous to each other and are separated by spacer ions having an intermediate mobility. Ampholines are employed as spacers (7).

MATERIALS AND METHODS

Reagents

Acrylamide, N,N-diallyltartardiamide, riboflavin-5-phosphoric acid (monosodium), ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine (TEMED), morpholinoethane sulfonic acid (MES), and agarose were supplied by Serva (Heidelberg). Tris-(hydroxymethyl)-aminomethane (Tris-HCl) was from Biochemia (Milan); ϵ-aminocaproic acid was from Cal-

Abbreviations: HDL, high density lipoprotein; apo, apolipoprotein; C, cholesterol; P, protein; EC, esterified cholesterol; FC, free cholesterol; PL, phospholipids; TG, triglycerides.

biochem Hoechst (Frankfurt); and Ampholine pI 4–6 and Ampholine pI 5–8 were from LKB (Bromma).

Samples

The HDL was isolated from the plasma of four normolipemic male volunteers. The venous blood, drawn after a 12-hr fast, was collected in test tubes containing EDTA (1 mg/ml) and was immediately centrifuged at low speed. The plasma thus obtained was adjusted to d 1.063 g/ml by adding NaCl. The density measurements were done with a Gibertini (Milano) hydrostatic balance. The VLDL and LDL were then separated by ultracentrifugation at 105,000 *g* at 5°C for 24 hr using a Beckmann L5-65 ultracentrifuge and a Ti 70 rotor. The floating lipoproteins were removed by the tube-slicing technique. The infranatant fraction was then adjusted to d 1.22 g/ml by the addition of solid NaBr and further ultracentrifugation at 105,000 *g* at 5°C was carried out for 24 hr. The floating material was brought to the original volume with NaBr solution d 1.121 g/ml (containing 1 g/l of NaN_3 and 1 g/l of Na_2 EDTA) and was recentrifuged under identical conditions. The tubes were then cut 5.5 cm from the top and the HDL thus obtained was diluted to a density of 1.125 g/ml. This solution was ultracentrifuged at 150,000 *g* at 5°C for 24 hr. The tubes were cut one-third from the top to separate the HDL_2 and HDL_3. Immediately after ultracentrifugation the lipoprotein solutions were dialyzed against repeated changes of 0.15 M NaCl solution containing NaN_3(1 g/l) and Na_2EDTA (1 g/l). Samples (8 ml) of each solution of HDL_2 and HDL_3 were subjected to preparative isotachophoresis. The concentration of HDL_2 was between 1.25 and 1.4 mg/ml while concentration of HDL_3 was between 2.8 and 3.15 mg/ml.

Preparation of the 2% polyacrylamide column

The 2% polyacrylamide column was prepared as suggested by Houghten and Chrambach (8): 3.03 ml of 33% acrylamide; 3.75 ml of N,N-diallyltartardiamide; 5 ml of gel buffer, pH 6.2, (7.3 g MES, 2 g Tris-HCl, 0.3 ml TEMED; diluted to 100 ml with water); 5 ml of 0.008% riboflavin-5-phosphoric acid; 5 ml of 0.1% ammonium persulfate; diluted to 50 ml with water.

Solution was deaerated and transferred to an LKB 7960 plastic column and left to polymerize for 1 h in fluorescent light. The gel thus prepared had a column height of 15 cm.

Preparative isotachophoresis in polyacrylamide gel

The separations were carried out on an LKB Uniphor 7900 apparatus. The LKB plastic column was 30 cm long and had a diameter of 2 cm; it was cooled to 10–12°C with circulating water. The terminator electrolyte consisted of 60.0 g of ϵ-aminocaproic acid and 3.0 g of Tris diluted to 2000 ml with H_2O; The pH was 8.9. The leading and eluting electrolytes consisted of 121 ml 1 M H_2SO_4 and 32.0 g Tris diluted to 4000 ml with H_2O; the pH was 7.1. The material applied to the column was made up of 5 ml of sample (dialyzed against terminator electrolyte), 0.5 ml of ampholine pI 4–6, 0.5 ml of ampholine pI 5–8, and 2 g of sucrose. When the sample had been added, a constant current of 7 mA was supplied by the LKB Power Supply 2103. The voltage, (400 volts at the beginning of the test) was gradually increased to approximately 1200 volts. The elution flow was regulated by an LKB Varioperpex 1200 peristaltic pump at the rate of 12 ml/hr. The eluate was monitored continously with an LKB Uvicord III UV detector at 280 and 250 nm. Fractions of 4-ml were collected with the LKB Ultrorac 7000 collector. The entire separation was carried out over a period of 26 hr.

Chemical and immunochemical assays

Proteins were assayed by the method of Lowry et al. (9) using human albumin as standard. Triacylglycerol and free and esterified cholesterol were determined enzymatically using reagents from Boehringer, Mannheim and Carlo Erba, Milan, respectively. Phospholipids were quantitated with a specific test kit from Poli diagnostics, Milan. For the identification of the polypeptide distribution, radial immunodiffusion in 1% agarose in Tris-HCl buffer, pH 8.2, was performed (10). The monospecific antisera were kindly supplied by Dr. Kostner (Graz). The electroimmunoassay for apoA-I and A-II was carried out as described in a previous paper (11).The lipoprotein subfractions were subjected to electrophoresis on 10% polyacrylamide gel by the method of Kane (12).

RESULTS

Figs. 1 and **2** show the elution patterns and the immunochemical reactions of the various subfractions in two representative experiments.

In the four tests that were carried out, HDL_2 always separated into six subfractions and the HDL_3 into ten subfractions. ApoB was not found in any of these subfractions. All the HDL_2 subfractions were devoid of apoD and albumin. HDL_2 subfractions 2–5 contained apolipoproteins A-I, A-II, E, C-II, and C-III, though in different proportions. Subfraction 1 contained apoA-I and traces of apoA-II and subfraction 6 reacted only against anti-apoA-I.

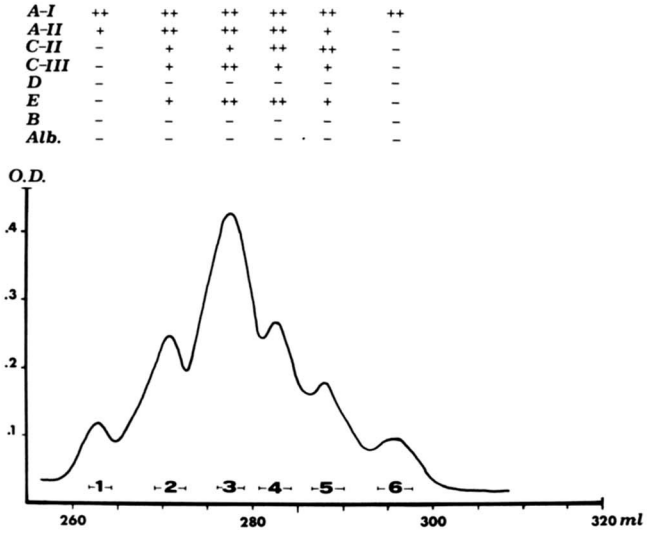

Fig. 1. Elution pattern and immunochemical reaction of different subfractions of HDL_2 separated by preparative isotachophoresis.

As to the HDL_3, it was found that subfraction 1 contained apoA-II and only traces of apoA-I; subfraction 2 contained apoC-II and C-III in addition to the two A apolipoproteins. Subfractions 3 and 5 proved to be immunoreactive also against anti-apoA, anti-apoE and anti-albumin. In subfractions 5 and 7, the reactivity against anti-apoC-III, anti-apoD, and anti-albumin was lost. In subfraction 8, apoE could not be detected either, while subfractions 9 and 10 contained only apoA-I and apoC-III.

These findings were confirmed by analysis of the subfractions by electrophoresis on polyacrylamide

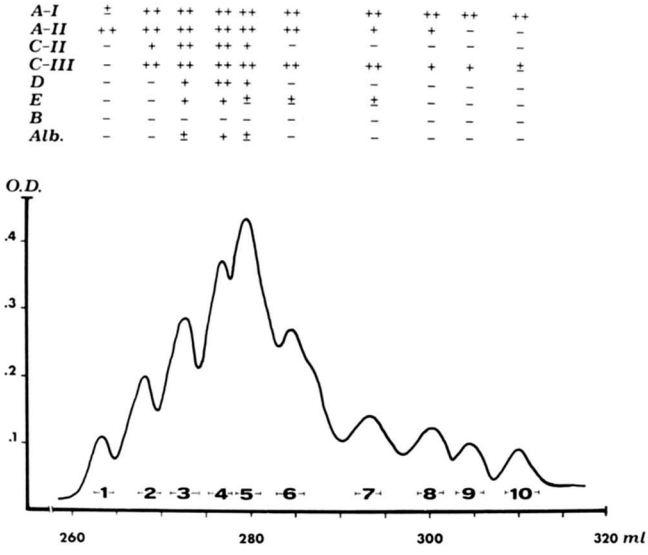

Fig. 2. Elution pattern and immunochemical reaction of different subfractions of HDL_3 separated by preparative isotachophoresis.

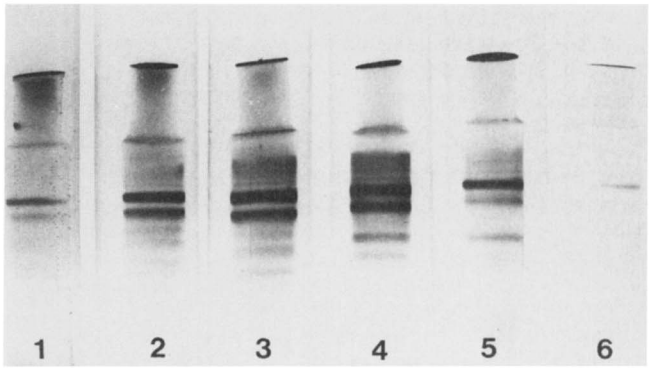

Fig. 3. 8 M Urea-PAGE of isotachophoresis fractions from HDL_2.

gel (**Figs. 3 and 4**). The pattern of the various HDL apolipoproteins obtained by polyacrylamide electrophoresis gel in 8 M urea is shown for comparison (**Fig. 5**).

The composition of the various HDL_2 subfractions is shown in **Table 1**. Ratios of EC/FC, P/C, and apoA-I/apoA-II for each fraction are also shown. The recovery of the subfractions was between 85 and 90%. Most of the material was collected in subfractions 2, 3, and 4; in these fractions the P/C ratio was between 3.5 and 4.3, and the apoA-I/apoA-II ratio was between 1.8 and 3.2. Subfractions 1 and 5 had the highest A-I/A-II ratios. The P/C ratios in subfractions 1, 5, and 6 were the lowest (between 1.2 and 2.4); moreover, in these subfractions the highest values for the EC/FC ratios were observed.

The composition of the HDL_3 subfractions is shown in **Table 2** with the values for the EC/FC, P/C, and apoA-I/apoA-II ratios. The recovery was approximately 90%. Most of the material was collected in subfractions 3–6. In the first five subfractions, the P/C ratio was between 3.7 and 4.1, while apoA-I/apoA-II ratios were always below 1.5. In subfractions 6–10, apoA-I was the major apolipoprotein and the P/C ratios were lower, and in subfractions 9 and 10 they were less than 1. In subfractions 6–10, EC/FC ratios were increased.

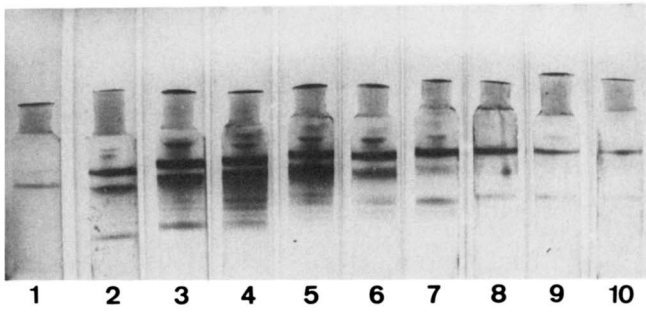

Fig. 4. 8 M Urea-PAGE of isotachophoresis fractions from HDL_3.

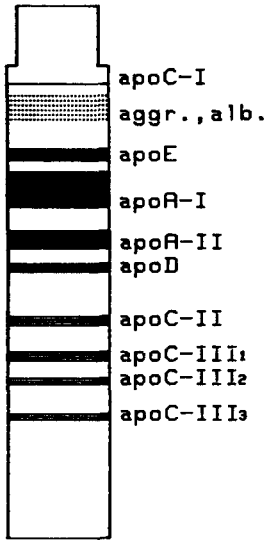

Fig. 5. Distribution of HDL apolipoproteins in 8 M Urea-PAGE.

DISCUSSION

Human HDL can be separated into several subfractions by zonal ultracentrifugation (13), isoelectric focusing (4–6), and column chromatography (3). On the strength of these findings, we assessed preparative isotachophoresis as a method for obtaining HDL subfractions. Our results confirm that the HDL_2 and HDL_3 are made up of a number of subfractions that differ in chemical composition and apolipoprotein pattern. It is highly unlikely that our findings are due to artefacts. In experiments in which single subfractions were subjected to isotachophoresis, we consistently found a single peak with the same mobility, chemical composition, and apolipoprotein fractions. This proved the genuine heterogeneity of the HDL_2 and HDL_3. Furthermore, when two or more subfractions were reconstituted and newly submitted to isotachophoresis, they always reproduced the same pattern.

Isotachophoresis of whole HDL produced 12 subfractions. They appeared to be overlapping subfractions of HDL_2 and HDL_3. Only the subfractions 1, 9, and 10 of HDL_3 were clearly separated (the first contained apoA-II and traces of apoA-I and the others (9 and 10) contained only apoA-I and C-III).

Our results do not agree completely with those obtained with the other methods cited above. Not only did we obtain a different number of subfractions, but the distribution of apoprotein peptides differed. Sodhi, Sundaran, and MacKenzie (6) found six HDL_2 subfractions with preparative isoelectric focusing but did not find any with only apoA-I. On the other hand, Kostner and Holasek (3) noted two HDL_2 subfractions containing only apoA-I in some cases. With chromatography on hydroxylapatite the HDL_2 separated into only four subfractions.

With regard to HDL_3, the number of subfractions obtained from the previous methods ranged from four (4) to six (3, 5). In agreement with Kostner and Holasek (3), we found that not all the subfractions containing C peptides contained apoC-II. In fact, in HDL_3 subfractions 6–10, apart from apoA-I, we detected only one band of apoC-III; it probably consisted of only apoC-III_1. On the other hand, with preparative isotachophoresis, we did not detect subfractions containing apoC only, which was noted by chromatography on hydroxylapatite.

Our data can be summarized as follows: *1*. Subclasses HDL_2 and HDL_3 can be separated by isotachophoresis into 16 subfractions differing in chemical composition and apolipoprotein pattern. *2*. Subfractions characterized by a different hydrated density may have the same electrophoretic mobility. *3*. The apoA-I/apoA-II ratios vary widely among the various subfractions. *4*. The subfractions with the highest apoA-I/apoA-II ratio appear to have the greatest affinity for cholesterol, particularly EC. This last observation is very interesting in the light of some clinical findings. Numerous studies have shown reduced levels of apoA-I in coronary atherosclerosis (14–16). However, we recently found that the same

TABLE 1. Composition of subfractions obtained from HDL_2[a]

Subfraction	% of HDL_2	EC	FC	TG	PL	P	EC/FC	P/C	A-I/A-II
				% (w/w)					
1	5.1 ± 1.3[b]	17.8 ± 2.5	4.1 ± 0.8	n.d.[c]	34.1 ± 9.2	44.0 ± 7.0	4.3 ± 0.9	2.0 ± 0.2	8.3 ± 2.3
2	21.8 ± 7.4	10.7 ± 2.0	5.4 ± 1.4	7.1 ± 0.6	21.8 ± 5.2	55.0 ± 2.8	2.0 ± 0.8	3.5 ± 0.6	3.2 ± 1.6
3	34.3 ± 6.9	7.9 ± 1.0	4.8 ± 1.2	7.2 ± 0.9	27.4 ± 4.8	52.7 ± 6.1	1.8 ± 0.5	4.3 ± 0.9	1.8 ± 1.6
4	23.4 ± 4.3	7.5 ± 0.9	4.6 ± 1.3	8.1 ± 2.1	28.1 ± 4.5	51.7 ± 2.2	1.8 ± 0.8	4.3 ± 0.4	2.5 ± 1.5
5	10.1 ± 3.9	16.0 ± 2.3	4.8 ± 0.8	3.6 ± 0.5	26.1 ± 3.7	49.5 ± 3.5	3.4 ± 0.5	2.4 ± 0.5	10.3 ± 1.5
6	5.3 ± 1.6	28.6 ± 3.3	8.5 ± 0.6	n.d.	20.0 ± 9.4	42.9 ± 13.9	3.4 ± 0.5	1.2 ± 0.5	

[a] All values are means ± SD.
[b] Percentage of HDL_2 added to column.
[c] Not determined.

TABLE 2. Composition of subfractions obtained from HDL_3[a]

Subfraction	% of HDL_3	EC	FC	TG	PL	P	EC/FC	P/C	A-I/A-II
				% (w/w)					
1	2.9 ± 0.6[b]	5.6 ± 0.8	4.4 ± 1.0	n.d.[c]	47.6 ± 0.9	42.4 ± 1.3	1.3 ± 0.5	4.4 ± 0.6	0.11 ± 0.03
2	5.8 ± 1.6	7.1 ± 1.5	3.8 ± 0.3	3.5 ± 0.8	33.6 ± 7.1	52.0 ± 6.3	1.9 ± 0.5	4.8 ± 0.2	1.4 ± 0.6
3	14.2 ± 3.0	8.7 ± 2.1	4.2 ± 1.8	3.4 ± 0.5	24.1 ± 7.4	59.6 ± 4.9	2.1 ± 0.9	4.7 ± 0.6	1.0 ± 0.3
4	16.9 ± 2.3	8.3 ± 1.4	3.8 ± 0.3	3.7 ± 0.9	26.2 ± 7.0	58.0 ± 4.9	2.2 ± 0.4	4.8 ± 0.2	0.5 ± 0.2
5	22.3 ± 5.0	11.6 ± 1.7	5.6 ± 1.2	3.5 ± 0.7	17.3 ± 6.6	62.0 ± 4.7	2.1 ± 0.2	3.7 ± 0.6	0.8 ± 0.2
6	12.1 ± 2.5	15.5 ± 3.7	4.6 ± 1.3	3.5 ± 1.4	17.1 ± 7.3	59.4 ± 8.5	3.4 ± 0.4	3.1 ± 0.7	2.1 ± 1.0
7	9.5 ± 1.7	18.2 ± 3.5	4.1 ± 0.7	5.2 ± 0.9	28.9 ± 2.2	43.6 ± 4.1	4.4 ± 0.2	2.0 ± 0.5	12.3 ± 3.8
8	6.2 ± 1.1	24.1 ± 3.7	6.3 ± 1.5	6.4 ± 1.4	19.9 ± 6.9	41.6 ± 3.2	3.9 ± 0.6	1.4 ± 0.3	20.0 ± 3.9
9	5.7 ± 1.2	33.1 ± 2.8	10.7 ± 1.5	2.1 ± 0.4	18.9 ± 7.3	35.2 ± 10.1	3.3 ± 0.3	0.9 ± 0.3	
10	4.4 ± 0.9	36.7 ± 5.3	8.7 ± 1.4	n.d.	22.7 ± 8.6	31.9 ± 6.2	3.8 ± 0.8	0.6 ± 0.2	

[a] All values are means ± SD.
[b] Percentage of HDL_3 added to column.
[c] Not determined.

does not apply to apoA-II (17), although in patients with coronary atherosclerosis the HDL_2 and HDL_3 are reduced (18). It could therefore be argued that in atherosclerotic subjects the low HDL values reflect mainly the subfractions with the higher apoA-I/apoA-II ratios. These same subfractions, because of their greater affinity for cholesterol, might play a more specific anti-atherogenic role.

In conclusion, we may state that this study does not warrant the assumption that the subfractions as separated actually reflect an in vivo situation, inasmuch as dissociation phenomena could occur in the course of ultracentrifugation. However, the method has high resolving power and is reproducible and thus is suitable for use in the study of the structure and metabolism of the lipoproteins in various normal and pathological conditions.

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REFERENCES

1. Levy, R. I., and D. S. Fredrickson. 1965. Heterogeneity of plasma high density lipoproteins. *J. Clin. Invest.* **44:** 426–441.
2. Kostner, G., and P. Alaupovic. 1972. Studies of the composition and structure of plasma lipoproteins. Separation and quantification of the lipoprotein families occurring in the high density lipoproteins of human plasma. *Biochemistry.* **11:** 3419–3428.
3. Kostner, G. M., and A. Holasek. 1977. The separation of human serum high density lipoproteins by hydroxylapatite column chromatography. Evidence for the presence of discrete subfractions. *Biochim. Biophys. Acta.* **488:** 417–431.
4. Scanu, A. M., C. Edelstein, and L. Aggerbeck. 1973. Application of the technique of isoelectric focusing to the study of human plasma lipoproteins and their apoproteins. *Ann. NY Acad. Sci.* **209:** 311–327.
5. Sundaram, G. S., S. L. Mackenzie, and H. S. Sodhi. 1974. Preparative isoelectric focusing of human serum high density lipoprotein (HDL_3). *Biochim. Biophys. Acta.* **337:** 196–203.
6. Sodhi, H. S., G. S. Sundaram, and S. L. Mackenzie. 1974. Isoelectric fractionation of plasma high density lipoproteins. *Scand. J. Clin. Lab. Invest.* **33:** Suppl. 71–72.
7. Chrambach, A., G. Kapadia, and M. Cantz. 1972. Isotachophoresis in polyacrylamide gel. *Sep. Sci.* 7: 785–797.
8. Houghten, R., and A. Chrambach. 1977. Preparative isotachophoresis on polyacrylamide gels: resolution as a function of time and wattage. *Anal. Biochem.* **77:** 303–309.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
10. Mancini G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2:** 235–242.
11. Kostner, G. M., P. Avogaro, G. Bittolo Bon, G. Cazzolato, and G. B. Quinci. 1979. Determination of high density lipoproteins: screening methods compared. *Clin. Chem.* **25:** 939–942.
12. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
13. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J. Lipid Res.* **15:** 356–366.
14. Albers, J. J., M. C. Cheung, and W. R. Hazzard. 1978. High density lipoproteins in myocardial infarction survivors. *Metabolism.* **27:** 479–485.
15. Avogaro, P., G. Bittolo Bon, G. Cazzolato, G. B. Quinci, and F. Belussi. 1978. Plasma levels of apolipoprotein A-I and apolipoprotein B in human atherosclerosis. *Artery.* **4:** 385–394.
16. Avogaro, P., G. Bittolo Bon, G. Cazzolato, and G. B. Quinci, 1979. Are apolipoproteins better discriminators than lipids for atherosclerosis? *Lancet.* **1:** 901–903.
17. Avogaro P., G. Bittolo Bon, G. Cazzolato, G. B. Quinci, and F. Belussi. 1980. Plasma levels of some apolipoproteins in human atherosclerosis. Atherosclerosis V. Proc. V. International Symposium. Springer-Verlag, New York. 816.
18. Avogaro, P., G. Cazzolato, G. Bittolo Bon, and F. Belussi. 1979. Levels and chemical composition of HDL_2, HDL_3 and other major lipoprotein classes in survivors of myocardial infarction. *Artery.* **5:** 495–508.