Apoproteins of high density lipoproteins in the urine of normal subjects

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Abstract Apoproteins of high density lipoproteins were detected in the urine of normal subjects after the urinary proteins were highly concentrated. By immunoelectrophoresis, all of the urinary apoproteins gave precipitin lines with similar electrophoretic mobility. This suggests that the various apoproteins are present in the same particle. The apoproteins were present only in the ultracentrifugal fraction of density greater than 1.24 g/ml. Neither apoprotein B nor apoprotein E were detected in the urine, suggesting that very low density and low density lipoproteins are not excreted in the urine of normal subjects.—**Segal, P., L. I. Gidez, G. L. Vega, D. Edelstein, H. A. Eder, and P. S. Roheim.** Apoproteins of high density lipoproteins in the urine of normal subjects. *J. Lipid Res.* 1979. **20:** 784–788.

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Normal subjects excrete between 40 and 100 mg of protein in their urine per 24 hr (1–3). The proteins concentrated from the urine of normal subjects contain 14–20 components that react with antisera against human plasma proteins (1). Among these was α_1 lipoprotein, which was identified by a modified indirect method of immunoelectrophoresis. Beta-lipoproteins were not found. In patients with nephrotic syndrome and in rats with aminonucleoside nephrosis, HDL has been identified after ultracentrifugal flotation (4–6).

Recent studies from this laboratory (7) have suggested that the kidney might play a role in the metabolism of lipoproteins. Renal lymph obtained from rats contained only apoE and apoA-IV; it did not contain significant amounts of apoB or apoA-I. On the other hand, the d > 1.21 g/ml fraction of rat serum contains abundant amounts of apoE and apoA-IV and this suggested that these apoproteins enter the lymph as free apoproteins rather than as intact lipoproteins. However, the relative amounts of apoE and apoA-IV in lymph and in the d > 1.21 g/ml fraction differ in that there is a higher proportion of apoA-IV in renal lymph. (ApoA-IV has a molecular weight of 47,000 while that of apoE is 33,000.) This suggests that these apoproteins appear in lymph by a process other than filtration and diffusion. To further investigate the role of the kidney in lipoprotein metabolism, it therefore seemed relevant to study the lipoproteins and apoproteins in urine from normal subjects.

METHODS

Twenty-four-hour urine collections were obtained from nine normal subjects, seven men and two women. Sodium azide was added to the collection flasks, and the specimens were kept at 4°C until they were processed (within 48 hr). The samples were free of proteins, as judged by the sulfosalicylic acid method. The urines were centrifuged to remove cells and debris, and proteins were concentrated either by repeated precipitation with ammonium sulfate at 90% saturation, or by ultrafiltration through a UM-2 Amicon Diaflo membrane. For the latter procedure the urine samples were dialyzed against 0.01% EDTA–0.01% sodium azide prior to concentration. The concentrated urinary proteins obtained by both procedures were then dialyzed against 0.15 M NaCl containing 0.01% EDTA and 0.01% sodium azide prior to further studies. Spectrophor dialysis tubing (3500 dalton exclusion, Spectrum Medical Industries, Los Angeles, CA) was used for all dialyses.

Immunochemical methods

The following antigens were prepared: apo-VLDL, apoHDL, apoLDL, apoA-I, apoA-II, apoC-II, apoC-III, and apoE. ApoVLDL and apoHDL were prepared

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

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by delipidation of d > 1.006 g/ml and d 1.063–1.21 g/ml lipoprotein fractions, respectively, by the method of Brown, Levy, and Fredrickson (8). LDL was prepared by ultracentrifugation of plasma between d 1.03 and 1.05 g/ml. ApoA-I and apoA-II were prepared from apoHDL by gel filtration on Sephadex G-200 (7). ApoC-II and apoC-III were prepared from apoVLDL by gel filtration on Sephadex G-200 followed by chromatography on DEAE (8). The apoE was obtained by preparative SDS–gel electrophoresis (9) with elution of the protein from the gel by the method of Stevens (10).

Antisera were prepared in goats by intramuscular and intradermal injections of equal volumes of antigen (0.05–5 mg of protein) and complete Freund's adjuvant (Difco Laboratories, Detroit, MI) (11). In addition, 2–5 ml of pertussis vaccine (Eli Lilly and Co., Indianapolis, IN) was administered intramuscularly. Antigens were injected either two or three times at 3–4-week intervals. The antisera were tested against whole serum and against the various isolated antigens by immunoelectrophoresis and by the double diffusion method (12). The antisera to the isolated apoproteins did not exhibit cross reactivity and reaction with the corresponding antigens produced single lines.

The concentrated urinary proteins were tested against the various antisera by the double diffusion technique and by immunoelectrophoresis by the method of Grabar and Williams (13).

Ultracentrifugation

Ten ml of the solution of concentrated urinary proteins was subjected to ultracentrifugation at d 1.24 g/ml at 205,000 *g* for 48 hr. The tubes were cut and the top fraction (d < 1.24 g/ml) and bottom fraction (d > 1.24 g/ml) were collected and dialyzed. A d > 1.24 g/ml fraction of plasma was also prepared by ultracentrifugation at d 1.24 g/ml according to the method of Havel, Eder, and Bradgon (14). The higher density (d 1.24 g/ml) was used in these studies in order to exclude a VHDL from the bottom (d > 1.24 g/ml) fractions.

Lipids

Lipids from seven of the d > 1.24 g/ml fractions prepared from the concentrated urinary proteins were extracted and washed by the method of Folch, Lees, and Sloane Stanley (15). These lipids were then separated by thin-layer chromatography on silica gel G plates developed in petroleum ether (bp 30–60°C)–diethylether–glacial acetic acid 65:35:2 (v/v/v). Two additional urine samples (one from a normal male and one from a normal female) were collected, dialyzed, and concentrated by filtration as previously described. Cholesterol (16) and phosphorus (17) were measured in the concentrated urine samples. These concentrated urine samples were ultracentrifuged at d 1.24 g/ml, and protein (18) was determined on the d < 1.24 and d > 1.24 g/ml fractions. Lipids from the latter fraction were extracted (15) and separated on HPTLC plates (Merck) developed in chloroform–methanol–water 65:35:5 or chloroform–methanol–2.5 N NH_4OH 70:30:5, or on Silica Gel 60 plates (Merck) developed in hexane–diethylether–glacial acetic acid 90:10:1. Lipids were visualized by exposing the plates to iodine. The plates were also sprayed with 50% H_2SO_4 (after most of the iodine had disappeared) and heated at 180°C for 1 hr to char the lipids. Lipids were identified by TLC of known standards.

RESULTS

The concentrated urinary proteins gave precipitin lines when reacted with antisera to apoHDL, apoVLDL, apoA-I, apoA-II, apoC-II, and apoC-III. They did not react to antisera to apoLDL and apoE.

Immunoelectrophoresis against antisera to apoHDL and apoVLDL resulted in the formation of single precipitin lines with identical electrophoretic mobilities. By immunoelectrophoresis against antisera to apoA-I and apoC-II and to apoA-II and apoC-III, precipitin lines similar to those with anti-apoHDL were obtained (**Fig. 1*A***).

The upper and lower fractions obtained after ultracentrifugation at d 1.24 g/ml were tested against these antisera. The d < 1.24 g/ml fractions did not react with any of the antisera as judged by immunodiffusion. The d > 1.24 g/ml fraction gave the same reactions as did the proteins not subjected to ultracentrifugation (Fig. 1*B*).

A second experiment was carried out to determine whether the apoproteins present in the urine d > 1.24 g/ml fraction were present as free apoproteins or were associated in a macromolecular particle. ApoA-I, apoA-II, and a mixture of C apoproteins were combined and subjected to immunoelectrophoresis. The results are shown in **Fig. 2.** On slide A of Fig. 2, one trough contained a mixture of anti-apoA-I and anti-apoC-III, and the second trough contained a mixture of anti-apoA-II and anti-apoC-II. On slide *B*, anti-apoHDL was in one trough and anti-apoVLDL was in the other. Multiple precipitin lines were obtained in contrast to the single lines present with the urinary protein (Fig. 1). Furthermore, the mobility of free apoA-I (Fig. 2) was considerably greater than the mobility of this apoprotein when present in the particle in the urine.

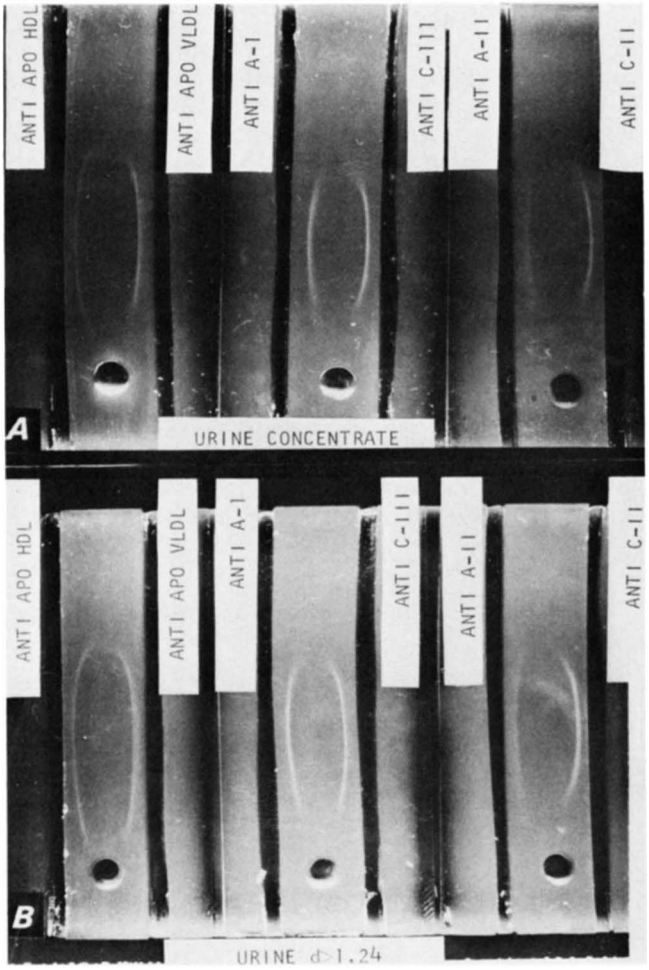

Fig. 1. Immunoelectrophoresis of concentrated urinary apoproteins. *A*, Total urinary proteins; *B*, density >1.24 g/ml fraction of the concentrated urinary proteins. The proteins were placed in the center well and electrophoresis was performed in 0.04 M sodium barbital buffer, pH 8.2, for 2 hr. The antisera designated on the figure were added to the troughs and the slides were placed in a moist chamber overnight.

The immunoreactivity of the d > 1.24 g/ml infranatant of the plasma was determined. No reactivity to antisera against apoA-II, C-III, and C-II was detected either by the Ouchterlony method or by immunoelectrophoresis. However, an intense precipitin line was obtained against anti-A-I, and its mobility was less than that of plasma HDL run against anti-apoA-I. Since the electrophoretic mobility of the urinary particle was less than that of apoA-I, which has a mobility less than that of plasma HDL, the urinary particle must therefore have a mobility less than that of plasma HDL.

Chemical studies

Chemical studies were performed on the two additional 24-hr urine samples collected. The 24-hr protein excretion was 63 mg in the male and 22 mg in the female. The d > 1.24 g/ml fraction contained 99.5% of the total urinary protein. The total cholesterol excretion was 520 μg in the male and 260 μg in the female. No phosphorus could be detected in the aliquot of concentrated urine taken. Because of the limited amount of material, further quantitation of phosphorus was not possible. TLC was performed on the lipid extracts of the d > 1.24 g/ml fractions and cholesterol, free fatty acids, lysolecithin, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were found to be present.

DISCUSSION

The absence of apoB and apoE indicates that LDL and VLDL are not excreted in the urine of normal subjects. The only apoproteins found were those that are constituents of HDL.

It is of importance to know the state of the apoproteins that are present in urine, i.e., whether they are free apoproteins or are associated in a macromolecular particle that resembles HDL. The findings

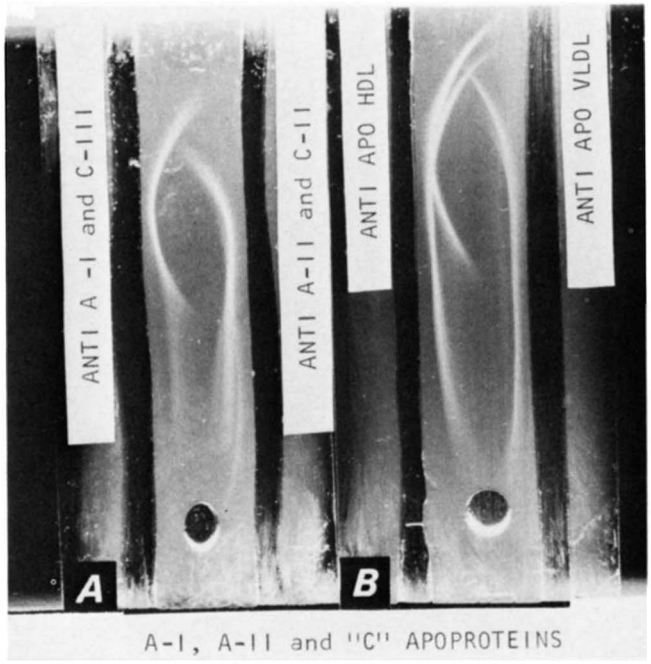

Fig. 2. Immunoelectrophoresis of mixtures of apoproteins. A mixture of apoA-I, A-II, and C apoproteins was placed in the well. Electrophoresis was performed, as described in Fig. 1. *A*, Antisera to apoA-I and apoC-III were placed in the left trough and antisera to apoA-II and apoC-II were placed in the right trough. *B*, Antiserum to apoHDL was placed in the left trough and antiserum to apoVLDL in the right trough and the slides were incubated, as described in the legend of Fig. 1. Multiple precipitin lines produced by the isolated apoproteins should be compared to the single lines produced by the urinary proteins (Fig. 1).

that the apoproteins present in the urine migrate with identical mobilities and yield a single precipitin line, in contrast to the multiple lines obtained with individual apoproteins, suggest that in the urine these apoproteins are present in a single macromolecular particle that resembles HDL. This particle differs from the major HDL species present in plasma in that it has a density >1.24 g/ml. The composition of the particle could not be determined because it was not separated from the remaining urinary proteins. Cholesterol and phospholipids were probably associated with the particle. The free fatty acids and lysolecithin were undoubtedly associated with albumin in the urine, although the presence of lysolecithin in the particle is not unlikely.

Several possible mechanisms could account for the presence of these HDL-like particles in the urine. One is that the individual apoproteins are filtered and then recombined with phospholipids and cholesterol to form the urinary particles. In vitro studies by Assmann et al. (19) have shown that A-I in the presence of A-II combines with phosphatidylcholine. Nichols et al. (20) have shown that the ability of A-I to combine with phosphatidylcholine is markedly increased by the addition of lysophosphatidylcholine, which was present in our preparations. However, this possibility seems unlikely since it would require that all of the apoproteins found in the urine would be present in the plasma d > 1.21 g/ml fraction, and only A-I is found in that fraction in plasma. A second possibility is that these particles constitute a species of HDL normally present in plasma that is selectively excreted by the kidney. Relevant to this hypothesis is the observation by Forte et al. (21) of small particles in the plasma of patients with LCAT deficiency. However, such particles have not as yet been found in plasma from normal subjects.

The most likely explanation is that a very small amount of HDL is excreted by the kidney but is altered either in its passage through the urinary tract or by the procedures used to concentrate the urine. Such a particle may be similar to that described by Levy and Fredrickson (22). They found that ultracentrifugation of HDL produced a particle in the d > 1.21 g/ml fraction which they designated α LP_B. It had a slower electrophoretic mobility on agarose gel than HDL (as does the particle we have described). Nichols et al. (23) were able to produce a particle with similar electrophoretic mobility by dehydration and subsequent solubilization of HDL. This lipoprotein consisted chiefly of A-I and contained no apoC in contrast to the particle we have studied, which contains immunochemically detectable C apoproteins as well as A-I. Levy and Frederickson (22) did not determine the apoprotein composition of α LP_B; thus the presence of apoC in our material does not preclude its similarity to the α LP_B described by these investigators.

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