Electroimmunoassay of rat apolipoproteins A-I, A-IV, and E. A procedure for sample treatment to increase the sensitivity in diluted fractions

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Summary Methods for the quantitative determination of rat apolipoproteins A-I, A-IV, and E by electroimmunoassay are described. Apolipoproteins present in diluted samples of biological fluids (approx. 2 ml) were concentrated by precipitation with deoxycholate and trichloroacetic acid. The protein pellets were solubilized in 0.1 ml of 0.5 M NaOH and these samples were delipidated with tetramethylurea and assayed. This protocol enables the measurement of apolipoprotein concentrations that are at least 10 times lower than normally detectable; 0.2 μg of apolipoprotein A-IV, 0.2 μg of apolipoprotein A-I, and 0.8 μg of apolipoprotein E can be easily detected in samples of 2 ml. — **Dallinga-Thie, G. M., P. H. E. Groot, and A. van Tol.** Electroimmunoassay of rat apolipoproteins A-I, A-IV, and E. A procedure for sample treatment to increase the sensitivity in diluted fractions. *J. Lipid Res.* 1985. **26:** 889–892.

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Quantitative measurements of specific apolipoproteins in biological fluids are possible with a variety of immunochemical procedures, e.g., radioimmunoassay, enzyme-linked immunosorbent assay, radial immunodiffusion, immunoelectrophoresis, and nephelometry. If the number of samples to be analyzed is limited, radial immunodiffusion and immunoelectrophoresis are the methods of choice. Their use is, however, limited to samples with substantial concentrations of apolipoproteins due to the relatively low sensitivity of these methods. To make them applicable to diluted samples, we developed a procedure of sample treatment that enables measurement of apolipoprotein concentrations by electroimmunoassay, which are at least ten times lower than normally detectable. Apolipoproteins present in diluted samples were concentrated by precipitation with deoxycholate-trichloroacetic acid (DOC-TCA); the precipitates were then delipidated with TMU and assayed. This method has been successfully used to analyze the distribution of apolipoproteins A-I, A-IV, and E in rat serum and mesenteric lymph, fractionated by agarose gel column chromatography.

MATERIALS AND METHODS

Isolation of apolipoproteins

HDL was isolated from serum of chow-fed male Wistar rats (body weight 350–400 g) by sequential density ultracentrifugation at 4°C in the density interval of 1.063–1.21 g/ml (1), washed once by reflotation at a density of 1.21 g/ml, and dialyzed extensively against 0.15 M NaCl, containing 2 mM Na-phosphate buffer (pH 7.4).

The HDL was delipidated by extraction with 20 vol of ethanol–diethyl ether 3:1 (v/v) for 20 hr at 4°C (2) and the apolipoproteins were dissolved in 0.05 M NaCl containing 5 M urea and 2 mM Na-phosphate buffer (pH 7.4).

ApoE was isolated from the apoHDL fraction by affinity chromatography on Sepharose CL-4B, containing covalently bound heparin, as described by Shelburne and Quarfordt (3) and further purified by chromatography at 4°C on Sephadex G 200 (column size 0.9 × 70 cm, flow 7 ml/hr), equilibrated with 150 mM Tris-HCl (pH 8.2) containing 1 mM EDTA, 0.02% NaN_3, and 6 M urea. The fraction of HDL not bound to the heparin-Sepharose column was used for the isolation of apoA-I and apoA-IV. ApoA-I and apoA-IV were separated by gel filtration at 4°C on Sephadex G-200 (column size 2.5 × 90 cm, flow 15 ml/hr), equilibrated with the same buffer as described above. The isolated apolipoprotein fractions were dialyzed against 0.15 M NaCl containing 1 mM EDTA (pH 7.4) and stored at −70°C. The purity was checked by urea polyacrylamide gel electrophoresis (apoA-I and apoA-IV) (4) and by Na-dodecylsulfate polyacrylamide gel electrophoresis (apoA-I, apoA-IV, apoE) (5). Each apolipoprotein showed a single band after staining with Coomassie Brilliant Blue R-250.

Preparation of antisera

Apolipoprotein solutions (50 μg in 1.5 ml of 0.15 M NaCl) were mixed with an equal volume of Freund's complete adjuvant and injected intradermally at multiple spots in New Zealand white rabbits weighing about 2 kg. After 14 days this procedure was repeated once. Then booster injections without the adjuvant were given every 14 days, until 5 μl of a 1:30 dilution of the rabbit serum caused the formation of a visible precipitation arc against 5 μl of undiluted rat serum in a Ouchterlony double immunodiffusion test. Animals were bled and serum was prepared. The IgG fraction of these antisera was isolated by affinity chromatography on Sepharose-4B, con-

Abbreviations: apo, apolipoprotein; EDTA, ethylene diamine tetraacetic acid; DOC, deoxycholate; TCA, trichloroacetic acid; TMU, tetramethylurea; IgG, γ-immunoglobulins; HDL, high density lipoprotein.

taining covalently bound protein A (Pharmacia, Uppsala, Sweden) according to standard procedures. The titers (μg of IgG protein needed to precipitate 1 μg of apolipoprotein) of the resulting IgG preparations were 78 (apoA-I), 140 (apoA-IV), and 598 (apoE). The specificity of the antibody preparations was checked by double immunodiffusion against pure rat apoA-I, apoE, and apoA-IV and against rat serum albumin. All antibody preparations reacted only with their specific antigen.

Electroimmunoassays

Immunoelectrophoresis according to Laurell (6) was performed for each of the rat apolipoproteins. The antibody-supporting gel in the apoA-I assay contained 1% agarose dissolved in 80 mM Tris-HCl, 24 mM Tricine-HCl, 0.5 mM Ca-lactate (pH 8.6), 5% (v/v) polyethylene glycol (mol wt 300), and anti-rat apoA-I rabbit IgG. Sixty μl of anti-apoA-I, containing 1.2 mg of IgG, was mixed with 30 ml of agarose solution. For the assay of apoA-IV, 2 mM Ca-lactate was used, while for the apoE assay neither Ca-lactate nor polyethylene glycol was added. One hundred eighty μl of anti-apoA-IV (5.1 mg of IgG) or 420 μl of anti-apoE (15.9 mg of IgG) was mixed with 30 ml of agarose solution. Samples of 13 μl were applied to the wells in the agarose gels. The electrophoresis of apoA-I and apoA-IV was carried out at 15°C and 7 V/cm for 17 hr. The apoE assay was carried out at 2 V/cm for 12 hr. The gels were washed in saline, dried on overhead projector sheets (3M, St. Paul, MN), stained with Coomassie Brilliant Blue R-250 (0.034%) in ethanol–acetic acid–water 1:2:4 (v/v) and destained in ethanol–acetic acid–water 3:1:5 (v/v). The within-day coefficients of variation for rat serum were 3%, 6%, and 6% for apoA-I, apoA-IV, and apoE, respectively; the between-day coefficients of variation were 4%, 7%, and 7% for apoA-I, apoA-IV, and apoE, respectively.

Sample treatment

Proteins, including (apo) lipoproteins, in the samples to be analyzed were routinely precipitated with TCA in the presence of DOC as a carrier (7). This precipitation step increased the sensitivity of the assay by a factor of 10, because the precipitate could be solubilized in a small volume. Samples were diluted, if necessary, to a final volume of 1.975 ml, and 0.025 ml of 2% DOC solution was added. After standing for 30 min at room temperature, 1 ml of 18% TCA solution was added, and after 15 min the resulting precipitate was collected by centrifugation at 3000 g for 30 min. The supernatant was discarded and the precipitate was dissolved in 0.1 ml of 0.5 M NaOH. Freshly distilled TMU (0.1 ml) was added and the samples were incubated for 30 min at 30°C (4). Next, 0.1 ml of 20 mM Tris-HCl (pH 8.3), containing 8 M urea, was added and the samples were heated at 100°C for 2 min. The resulting volume was measured and accounted for in the final calculations. A sample of pooled rat serum and pure apolipoprotein solutions were treated identically and used as standards in each plate. Protein was determined according to Lowry et al. (8), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

In order to make the immunoassays suitable for assaying apolipoproteins in a variety of biological fluids, we developed a procedure for sample treatment that makes it possible to measure apolipoprotein concentrations in very diluted samples. Apolipoproteins in diluted samples are quantitatively precipitated with TCA in the presence of DOC. The small pellet, collected by centrifugation, is routinely solubilized in 0.1 ml of 0.5 M NaOH.

Proteins are quantitatively recovered in the pellet. The use of a rather concentrated NaOH solution is necessary to neutralize the TCA-containing pellet and to dissolve the protein pellet in a small volume. If the NaOH concentration is lowered, the rocket immunoprecipitate becomes less clear and, because larger volumes (0.2 ml of 0.2 M NaOH and 0.2 ml of TMU) are needed to dissolve the protein pellet, the sample concentration is decreased. The solubilized pellet is subsequently delipidated with TMU and apolipoproteins are fully unfolded by the addition of 8 M urea followed by boiling for 2 min. The described sample treatment, using 0.1 ml of 0.5 M NaOH, results in a 10-fold concentration of apolipoproteins. This factor can be increased much further if larger samples are used, assuming that a reproducible method for the collection of the precipitate is available.

We used the buffer Tris-Tricine instead of the frequently used Veronal buffer because the use of the former buffer results in sharper rockets and essentially prevents current-induced changes in the pH of the medium during electrophoresis.

The described procedure for sample treatment could affect the immunoreactivity of the different apolipoproteins. Therefore, standards treated identically were always included in the assay. Rockets obtained with pure rat apolipoprotein standards treated according to the above-mentioned procedure are shown in **Fig. 1.** Immunoprecipitates are shown in assay plates containing anti-rat apoA-IV (top), anti-rat apoA-I (middle), or anti-rat apoE (bottom) rabbit IgG. The surface areas of the precipitates (height × width at half-maximal height) were always strictly linearly related to the antigen concentration as illustrated in the figures. The morphological appearance of the rockets obtained with rat serum was identical to that obtained with pure apolipoprotein standards (not shown).

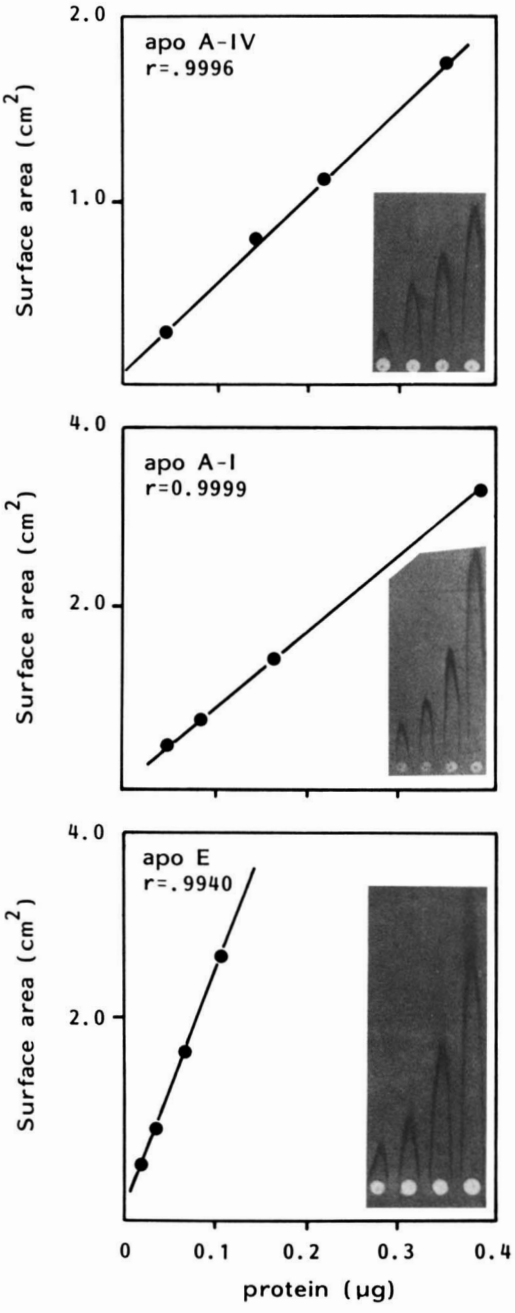

Fig. 1. Relationship of rocket surface area (height × width at half-height (cm^2)) and protein concentration (μg) of pure apoA-IV (top), apoA-I (middle), and apoE (bottom) standards, applied to the wells in a sample volume of 13 μl.

For further evaluation of the method, we studied the effect of sample concentration on the recovery of immunoreactive apolipoproteins, using prediluted pure apolipoprotein solutions and prediluted rat serum. The recovery of pure apolipoproteins after predilution followed by precipitation and subsequent resolubilization appears to be slightly less than 100%, if compared with untreated apolipoprotein solutions. However, we observed a comparable loss (4–14%) in the serum samples (see **Table 1**). Apolipoprotein concentrations measured in biological fluids with or without the precipitation with DOC-TCA are therefore identical. Two NaOH concentrations were used to resolubilize the precipitates. Using the highest concentration (0.5 M NaOH), the volume could be limited to 0.1 ml and the resulting rockets had sharper edges and were easier to quantitate. Using the protocol as described above (using a 2-ml sample volume), apolipoprotein masses in biological fluids as low as 0.2 μg of apoA-IV, 0.2 μg of apoA-I, and 0.8 μg of apoE can be measured.

The apolipoprotein concentrations in sera of male Wistar rats fasted overnight (body weight 250–300 g) were found to be 31.8 ± 3.8, 9.0 ± 2.2, and 20.4 ± 3.5 mg/100 ml for apoA-I, apoA-IV, and apoE, respectively (n = 12). Previous literature values for rat serum apoA-I range from 34 mg/100 ml, measured by radioimmunoassay (9), to 59 mg/100 ml measured by electroimmunoassay (10, 11). The apoA-IV concentration in rat serum was reported to be 24.4 mg/100 ml (12), but this level was increased due to the addition of 40% sucrose to the drinking water. Serum apoE levels in male rats of about 17 mg/100 ml were measured using either electroimmunoassay (13) or radioimmunoassay (14). It appears that the serum apolipoprotein levels measured by our assay are in reasonable agreement with the reported literature values, keeping in mind that fasting causes a decrease in apoA-I as well as apoA-IV concentration (unpublished observations).

In conclusion, a precipitation step including addition of DOC-TCA, preceeding TMU delipidation, makes it possible to measure apolipoprotein concentrations in very dilute samples. This method was specially designed to

TABLE 1. Effects of DOC-TCA precipitation and subsequent resolubilization in NaOH on the recovery of immunoreactive apolipoproteins

	0.2 M NaOH	0.5 M NaOH
Pure apoA-I standards	93 ± 4[a]	86 ± 2
ApoA-I in rat serum lipoproteins	93 ± 3	88 ± 3
Pure apoA-IV standards	90 ± 7	88 ± 8
ApoA-IV in rat serum lipoproteins	93 ± 4	92 ± 4
Pure apoE standards	96 ± 12	86 ± 9
ApoE in rat serum lipoproteins	96 ± 4	86 ± 5

[a]Values are % ± SD (three experiments) of concentrations of standards or rat serum assayed without precipitation and resolubilization. All samples were delipidated. Two concentrations of NaOH were tested. Precipitates were dissolved in 0.2 ml of 0.2 M NaOH or 0.1 ml of 0.5 M NaOH.

measure apolipoprotein concentrations in samples obtained after fractionation of serum and mesenteric lymph on 6% agarose columns. The results of these experiments will be published elsewhere.

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