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A simple and rapid method for the preparation of apolipoproteins for electrophoresis

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Summary A new method is described for the rapid preparation of apolipoproteins for polyacrylamide gel electrophoresis. It is suitable for all serum lipoproteins including chylomicrons. The procedure involves extraction with diethyl ether in the presence of trichloroacetic acid and sodium deoxycholate. The method gives an improved protein recovery, in particular with chylomicrons. In addition, samples do not require dialysis to remove salts (e.g., KBr) prior to processing; as a consequence, the procedure requires only 1 h. Due to this rapidity and the high yields, the procedure is superior to present methods utilizing ethanol–diethyl ether extraction.—**Mindham, M. A., and P. A. Mayes.** A simple and rapid method for the preparation of apolipoproteins for electrophoresis. *J. Lipid Res.* 1992. **33:** 1084–1088.

Supplementary key words lipoprotein delipidation • chylomicrons • polyacrylamide gel electrophoresis

One of the most commonly used methods for the analysis of apolipoprotein composition of lipoproteins is polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (1). Results are improved by delipidation of the lipoproteins, prior to application of the apolipoproteins to the gel (2). This delipidation can be achieved by extraction with a mixture of polar and nonpolar organic solvents of which the most commonly used is a mixture of ethanol and diethyl ether (3). We have compared this established method with a new method in which the lipoproteins are extracted in diethyl ether in the presence of trichloroacetic acid (TCA) and sodium deoxycholate (DOC) to precipitate the apolipoproteins. Our method does not require dialysis of the sample, even when high concentrations of salts are present (e.g., KBr used to prepare lipoproteins by ultracentrifugation). In addition, it can be used to concentrate diluted samples of lipoproteins without the need for large quantities of flammable solvents. The procedure is very simple and takes less than 1 h to complete compared with 40 h for the technique using ethanol–diethyl ether. It has been used successfully on rat and human serum lipoproteins and rat lymph chylomicrons.

METHODS

Preparation of lipoproteins

Lipoproteins were prepared from serum by ultracentrifugation. One ml of serum was adjusted to the required density by addition of solid KBr, layered under a solution of similar density in a 6.5-ml cellulose acetate tube (Kontron, Watford, UK), and centrifuged for 24 h at 40,000 rpm in a fixed-angle rotor (MSE-MFT 50.6) at 4°C. The top 1 ml was harvested by tube-slicer.

Chylomicrons were prepared from rats fed 0.5 ml of corn oil by intubation. After 1–2 h, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt.) and the thoracic duct was cannulated with polyethylene tubing (external diam. 1.52 mm) according to Bollman, Cain, and Grindlay (4). Chyle was collected for 15 h, and layered under NaCl (1.006 g/ml) in 6.5-ml tubes and centrifuged for 6×10^5 *g·min (rav.* = 64.2 mm) in a fixed-angle rotor (MFT 50.6) at 12°C. The top fraction was harvested and used at once.

Preparation of apolipoproteins using TCA/DOC/diethyl ether

One ml of lipoprotein sample, obtained by ultracentrifugation and containing up to 1 mg of protein, was

Abbreviations: TCA, trichloroacetic acid; DOC, sodium deoxycholate; SDS, sodium dodecyl sulfate.

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shaken with 10 ml of diethyl ether for 2 min. DOC (0.2 ml of 3.6 mM) and TCA (0.2 ml of 4.9 M) were added and mixed briefly. The contents were allowed to settle and the top layer of diethyl ether was removed by aspiration and a stream of nitrogen. The precipitated apolipoproteins, in the lower layer, were pelleted by centrifugation at 3000 *g* for 20 min and the supernatant was decanted. The apolipoproteins were then dissolved in 0.25 ml of sample buffer, neutralized by dropwise addition of 0.5 M NaOH until the indicator bromophenol blue turned to blue, incubated for 5 min at 80°C, and finally made up to 0.5 ml with distilled water. The sample buffer consisted of 0.125 M Tris buffer (pH 6.8) containing 41 mg of SDS/ml, 0.02 mg of bromophenol blue/ml, 20% (v/v) glycerol, and 10% (v/v) 2-mercaptoethanol.

For some lipoprotein samples, with low triacylglycerol content, e.g., HDL, the addition of diethyl ether was omitted. To these samples 0.2 ml of DOC and 0.1 ml of TCA were added. The resulting precipitate was recovered after centrifugation and treated with sample buffer as described above.

Preparation of apolipoproteins using ethanol/diethyl ether

Lipoprotein fractions, obtained by ultracentrifugation of serum, were dialyzed for 15 h against 0.9% (w/v) NaCl. Rat chylomicrons were not dialyzed but were extracted with 10 vol of diethyl ether which was removed by aspiration and discarded. This procedure was necessary to reduce triacylglycerol content before ethanol–diethyl ether extraction. A sample of 1 ml of lipoproteins, containing up to 1 mg of protein, was added to 50 ml of ice-cold ethanol–diethyl ether 3:2 (v/v). After shaking, the precipitate was allowed to settle by standing for 24 h at −20°C. Most of the solvent was removed by aspiration, and the precipitate was washed twice with 20 ml of diethyl ether. The ethanol–diethyl ether removed was added to a further 25 ml of diethyl ether and any precipitated protein was added to the main batch. The diethyl ether–apolipoprotein suspension remaining after aspiration of the bulk of the final wash was transferred to a test tube and dried down by a stream of nitrogen. The apolipoproteins were immediately dissolved in 0.25 ml of electrophoresis sample buffer, incubated for 5 min at 80°C, and finally made up to 0.5 ml with distilled water.

Analytical methods

Total protein was measured by the method of Lowry et al. (5) using bovine serum albumin as standard and modified Lowry reagent (L1013, Sigma Diagnostics, Poole, Dorset, UK). The apolipoprotein samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS as described by Laemmli (1) and modified for use in vertical slab gel electrophoresis using 160 × 200 × 1.5 mm gels (system LKB 2001, Pharmacia-LKB, Milton Keynes, UK). A portion of the sample, containing up to 500 μg of protein, was layered in wells in the stacking gel and run at a constant current of 20 mA/gel at 10°C. Gels were stained with Coomassie Blue (R250, Sigma Chemical Co., Poole, Dorset, UK) and finally scanned at 550 nm by spectrophotometer.

RESULTS

Lipoproteins treated by either procedure for delipidation gave similar resolution of apolipoprotein bands when examined by polyacrylamide gel electrophoresis (**Fig. 1**). The protein recovery was greater when using the TCA–DOC–diethyl ether procedure (**Table 1**). A portion of the protein lost during preparation with ethanol–diethyl ether was due to the fact that it was necessary to dialyze the sample to remove KBr prior to extraction. The preparation of apolipoproteins from chylomicrons using ethanol–

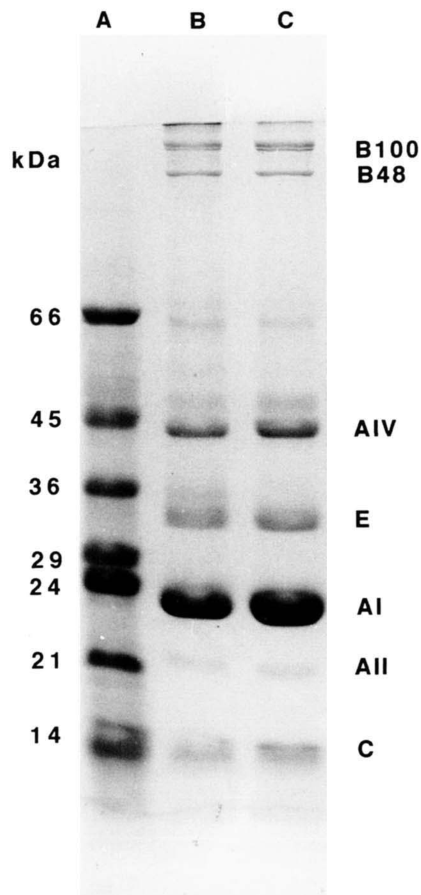

Fig. 1. Polyacrylamide gel electrophoresis of rat apolipoproteins prepared by two different methods. The apolipoprotein samples were prepared from serum lipoproteins (d < 1.21 g/ml) by ethanol–diethyl ether extraction (lane B) or by DOC–TCA method (lane C) as described in the text. Three hundred twenty μg of protein was added to each lane, as determined before sample preparation. Lane A, standard proteins.

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TABLE 1. Recovery of protein after delipidation of rat lipoproteins by two different procedures

Procedure	Recovery of Apolipoproteins			
	Chylomicrons	VLDL/IDL	HDL	Total Serum Lipoproteins
			%	
TCA-DOC-diethyl ether extraction	93.2 ± 5.6***	96.6 ± 7.4	100.0 ± 11.6	98.4 ± 7.2*
Ethanol-diethyl ether extraction	64.8 ± 4.4	88.5 ± 6.8 (91)	90.3 ± 8.2 (93)	87.1 ± 5.8 (94)

Apolipoproteins were prepared from lipoproteins obtained from 1 ml of rat serum or chyle by ultracentrifugation. The mean protein content of each sample, determined by modified Lowry assay, was as follows: 0.032 mg/ml chylomicrons, 0.33 mg/ml VLDL/IDL, 1.15 mg/ml HDL, 1.48 mg/ml total serum lipoproteins. Values in parentheses represent protein recovery after dialysis but before extraction (no dialysis was performed during the TCA/DOC/diethyl ether method). Values are means ± SD of four determinations. Significance of difference between means: *, $P < 0.05$; ***, $P < 0.001$

diethyl ether resulted in a large loss of protein of approximately 60%. This recovery was improved by first extracting the chylomicrons in 10 volumes of diethyl ether to reduce their lipid content, a technique employed by other workers (6). Using this additional extraction, protein recovery was still only 65% compared to more than 90% recovery with the TCA-DOC-diethyl ether procedure (Table 1). Comparison of the proportions of chylomicron apolipoproteins recovered by each procedure indicated that ethanol-diethyl ether extraction resulted in some loss of all apolipoproteins but in particular those of low molecular mass, such as apolipoproteins C and A-I (**Table 2**).

Many lipoprotein samples, particularly those with low lipid content, do not require delipidation prior to application to the gel, since there is sufficient detergent (SDS) in the electrophoresis reagents to achieve this. Thus, in many instances the addition of diethyl ether in the TCA-DOC-diethyl ether method can be omitted with no deterioration in results (**Fig. 2**). Whether the new procedure was used with or without diethyl ether extraction, it was found to be advantageous over existing methods since it did not require dialysis of the lipoprotein sample even when high concentrations of salts were present.

Analysis of chloroform-methanol extracts of serum apolipoproteins prepared by the TCA-DOC-diethyl ether method indicated complete delipidation with respect to triacylglycerol. However, up to 12% of the phospholipid and cholesterol remained associated with the protein, although this would be expected to be removed during incubation with the sample buffer containing SDS.

DISCUSSION

Protein precipitation by TCA in the presence of DOC has been shown by present results to be an effective method for the preparation of apolipoproteins for electrophoresis. DOC increases the precipitation of proteins by TCA, particularly at low protein concentrations and has been used prior to protein estimation in the Lowry method (7) and to remove substances that would otherwise interfere with the assay (8). A comparison of protein recovery following TCA-DOC-diethyl ether or ethanol-diethyl ether preparation of apolipoproteins indicated that the former gave a superior protein recovery. In the analysis of serum lipoproteins, the majority of protein

TABLE 2. Apolipoprotein composition of rat chylomicrons after delipidation by two different procedures

Apolipoprotein	TCA-DOC-Diethyl Ether Procedure		Ethanol-Diethyl Ether Procedure	
	Protein	% of Total Protein	Protein	% of Total Protein
	μg		μg	
B-100	0.15	0.75	0.15	0.76
B-48	9.02	45.1	10.64	53.2
A-IV	1.20	6.0	1.52	7.6
E	0.90	4.5	1.08	5.4
A-I	7.22	36.1	5.90	29.5
C	1.50	7.5	0.72	3.6
Total	20.0	100.0	20.0	100.0

Apolipoproteins were prepared from 2 ml of chylomicrons by TCA-DOC-diethyl ether or ethanol-diethyl ether extraction. The gels were run, stained, and scanned as described in the text. The mean amount of protein applied to the electrophoresis gels was 22.4 and 15.6 μg, respectively. Results have been normalized to 20 μg of protein and the relative chromogenicity of the proteins is assumed to be equal. Over the range of quantities of protein applied to the gels, the areas under the peaks scanned were proportional to the amounts applied. Protein samples to be compared were always run on the same gels.

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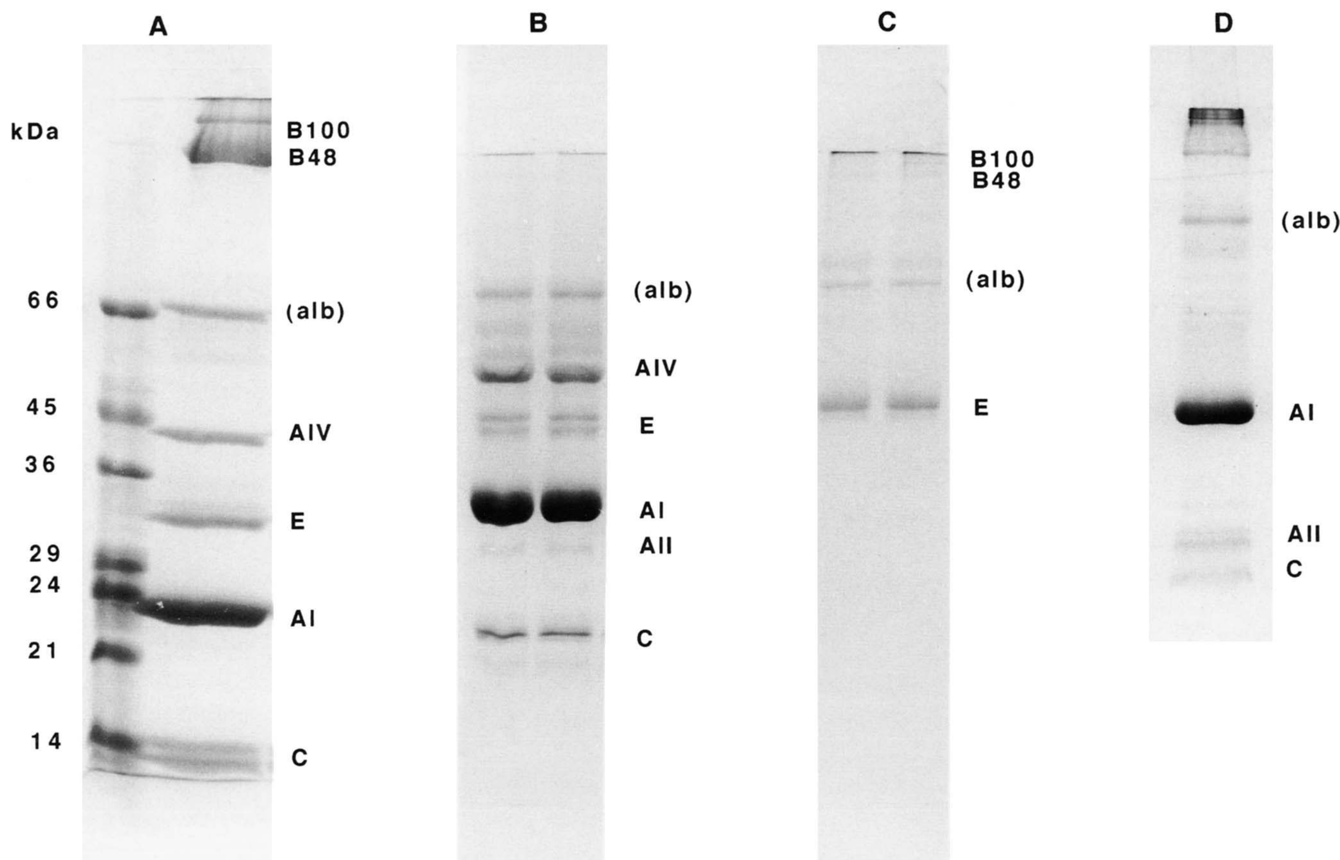

Fig. 2. Polyacrylamide gel electrophoresis of apolipoproteins prepared by the TCA-DOC-diethyl ether method. Apolipoproteins were prepared from lipoproteins obtained from serum or rat chyle by ultracentrifugation as described in the text. The addition of diethyl ether during the preparation was omitted where indicated. The identity of the apolipoprotein bands is indicated on right of gels. Alb denotes band corresponding to albumin. Gel A: left-hand lane, standard proteins; right-hand lane, rat chylomicrons; Gel B: rat HDL (d 1.050-1.21 g/ml), diethyl ether omitted in preparation of right-hand lane; Gel C: rat VLDL/IDL (d < 1.050 g/ml), diethyl ether omitted in preparation of right-hand lane; Gel D: human HDL (d 1.063-1.21 g/ml). The amount of protein applied to each well was: Gel A, 230 μg; Gel B, 290 μg; Gel C, 24 μg; Gel D, 160 μg.

losses occurred when it was necessary to dialyze the sample before ethanol-diethyl ether extraction due to their preparation in high-density solutions for ultracentrifugation. However, the ethanol-diethyl ether extraction process itself also resulted in some protein loss. It has been suggested that losses of protein during the ethanol-diethyl ether preparation are mainly those of smaller molecular mass (e.g., apolipoprotein C), due to solubility in the organic phase (3). However, in the present studies this loss was minimized by recovering the protein dissolved in the extraction solvent by addition of excess diethyl ether. Although we have used mainly rat lipoproteins for these studies, the method also works well with human lipoproteins (Fig. 2). In addition, apolipoproteins of normal rat serum lipoproteins, analyzed at the concentrations used here, can be prepared omitting the initial extraction step with diethyl ether, with no deterioration in results.

When rat chylomicrons were delipidated using the TCA-DOC-diethyl ether extraction, there was a large increase in recovered protein, demonstrating this method to be of particular value for triacylglycerol-rich lipoproteins. Apolipoprotein loss during ethanol-diethyl ether extraction was greatest for those apolipoproteins of low molecular mass, e.g., apolipoproteins C and A-I, implicating loss in the organic phase (3). In addition, delipidated apolipoproteins absorb strongly to glass and plastic surfaces (9). Therefore, the large surface area of vessels used for the ethanol-diethyl ether extraction may have contributed to protein loss, especially with lipoproteins containing a low protein content such as chylomicrons. The new method has also been used successfully in this laboratory for the preparation of apolipoproteins from chylomicron remnants (10).

The new procedure described here also has other practical advantages over ethanol-diethyl ether extraction. The whole procedure can be conducted in centrifuge tubes, and does not require facilities for storage of flammable solvents at −20°C. This, in conjunction with it not being necessary to dialyze the samples, reduces analysis time from approximately 40 h to 1 h.

In summary, the new procedure has several advantages.

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It is rapid with no loss of resolution compared with existing procedures. The use of highly flammable solvents is minimal, and for many samples can be omitted. In addition, protein recovery is improved, particularly for chylomicrons where this procedure is clearly the method of choice.

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