Changes in Electrophoretic Mobilities of α- and β-Lipoproteins as a Result of Plasma Delipidation

Bill E. Cham and Brian R. Knowles

A two-phase system containing the ternary mixture butanol/diisopropyl ether/plasma in different proportions yields ordered delipidation of α-lipoproteins, pre-β-lipoproteins, and β-lipoproteins in plasma, as quantitated by densitometry after electrophoresis. As a consequence of delipidation the electrophoretic mobilities of pre-β-lipoprotein and β-lipoprotein increased, that of α-lipoprotein decreased.

In a previous communication (1) we reported that the ternary mixture butanol/diisopropyl ether/plasma provides a convenient two-phase system for extraction of unesterified fatty acids, triglycerides, cholesterols, and phospholipids from plasma. When the solvent components are mixed in suitable proportions, plasma or serum are completely delipidated in 30 min. After centrifugation the lipids distribute into the upper, non-polar phase, whereas the proteins and other chemical constituents remain completely soluble in the clear lower phase.

This paper reports that exposure of plasma for 30 min to a two-phase system consisting of these same solvents in different proportions yields ordered delipidation of α-lipoproteins, β-lipoproteins, and pre-β-lipoproteins as quantitated by densitometry after electrophoresis.

These observations may indicate varying affinities of the different apolipoprotein fractions for lipid in plasma. Changes in electrophoretic mobilities after delipidation of these lipoprotein classes are also reported.

Department of Medicine, University of Queensland, Royal Brisbane Hospital, Brisbane, 4029, Australia.

Materials and Methods

Plasma

We used outdated human acid citrate-dextrose plasma obtained from the district blood bank.

Reagents

Diisopropyl ether was laboratory-reagent grade and was made peroxide free by distilling in the presence of ferrous sulfate; all other reagents were analytical grade.

Procedures

Delipidation. Partially and totally delipidated plasma was prepared by the method of Cham and Knowles (1). For the present studies, 10-ml aliquots of plasma were added to 20 ml of organic phase consisting of 0, 2.5, 5, 10, 20, 30, or 40 ml of butanol per deciliter of diisopropyl ether.

The samples were mixed for exactly 30 min at room temperature in a blood-cell-suspension rotator. After removing the aqueous phase from the organic phase, we subjected the samples to quantitative lipid analysis and electrophoresis.

Chemical analysis. The lipids in the plasma, before and after exposure to the organic solvent, were measured by semiautomated continuous-flow methods. Cholesterol and triglyceride (triacylglycerol) were determined in isopropanol extracts. Cholesterol was estimated by method N-24a (2), and triglyceride by the method of Lofland (3), modified by using Fletcher's (4) zeolite mixture instead of the Doucil mixture to absorb phospholipid. Phospholipid was extracted by the method of Folch et al. (5) and, after sample digestion, phosphorus was measured by the method of Fiske and SubbaRow (6). The factor 25 was used in converting lipid phosphorus values to

Table 1. Delipidation of Human Plasma on 30-min Extraction

Extraction solvent, butanol/ diisopropyl ether	Triglycerides	Cholesterols	Phospholipids
	% of control[a]		
None	100	100	100
0/100	80	80	96
	(72–90)	(73–88)	(95–100)
2.5/97.5	57	64	82
	(45–70)	(54–75)	(56–95)
5/95	41	55	73
	(22–52)	(42–71)	(49–85)
10/90	10	27	63
	(4–25)	(17–42)	(40–79)
20/80	0	0	39
			(28–57)
30/70	0	0	18
			(13–21)
40/60	0	0	0

[a] Each value, as percent delipidation, is the mean of five experiments. Range is in parentheses. Range of initial concentrations (mg per deciliter of plasma) were: triglyceride, 100–276; cholesterol, 120–190; phospholipid, 131–276.
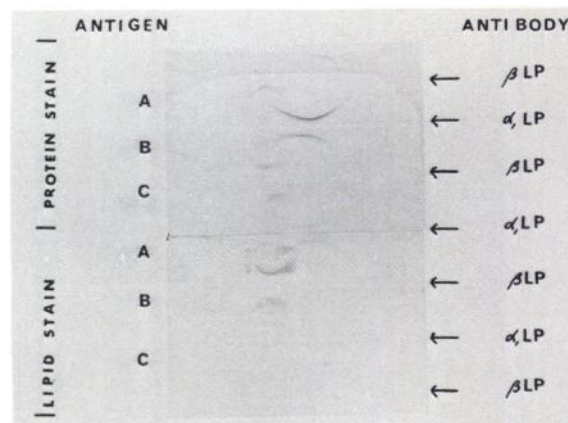

Fig. 1. Immunoelectrophoresis on agarose in control, partially delipidated, and totally delipidated plasma

Antibody troughs contain antisera to human α-lipoprotein or β-lipoprotein. Antigen wells contain control plasma, A; plasma, partially delipidated with butanol/diisopropyl ether (10/90 by vol) for 30 min, B; plasma, totally delipidated with butanol/diisopropyl ether (40/60 by vol) for 30 min, C. Proteins and lipids are stained with Coomassie Blue and Fat Red 7B, respectively. C contains no detectable lipid

phospholipid values. Percentage delipidation was calculated by difference.

Zonal electrophoresis. Zonal electrophoresis on agarose (7) was performed in barbital buffer (50 mmol/liter, pH 8.60). We used Fat Red 7B (Analytical Chemists, Palo Alto, Calif. 94303) and Coomassie Blue (ICI, Australia) to stain lipid and protein, respectively.

Densitometry. After destaining, the gels were scanned with a Quick Scan Jr. Densitometer incorporating a built-in integrator unit (Helena Laboratories, Beaumont, Tex. 77704). We integrated the areas stained with Fat Red 7B in the α-, pre-β-, and β-lipoprotein fractions of plasma, before and after exposure to the organic solvent, by the method of McTaggart and Dwyer (8). The percentage of lipid removed by the organic solvent from each fraction was calculated by difference.

Immunoelectrophoresis. For this we used pre-cast agarose (10 g/liter) slides (Corning-Eel, Halstead, Essex, England). The conditions for electrophoresis and immunodiffusion were described by Keyser (9). Rabbit antisera to human α-lipoproteins and β-lipoproteins were obtained from Behringwerke AG (Farbwerke Hoechst AG, Frankfurt/Main, Germany).

Results

Increasing proportions of butanol in the butanol/ diisopropyl ether extraction mixture produce increasing degrees of delipidation of human plasma in 30 min (Table 1). Both triglyceride and cholesterol are completely extracted by butanol diisopropyl ether

(20/80 by vol). A 40/60 mixture is required to extract all phospholipids from their plasma lipoproteins into the organic phase.

On immunoelectrophoresis, control plasma showed single precipitin arcs against antisera to α_1- and β-lipoprotein (Figure 1, A) and defined the electrophoretic mobility of these fractions under the experimental conditions. The partially and totally delipidated samples undergoing immunoelectrophoresis simultaneously with the control plasma produced precipitin arcs of differing mobility and intensity to the control plasma (Figure 1, B and C). Specifically, fractions reacting with α_1-lipoprotein antibody moved more slowly in both the protein-stained partially and totally delipidated samples. Additionally, two precipitin arcs were apparent in the partially delipidated plasma. Fractions reacting with β-lipoprotein antibody migrated more rapidly in the partially and totally delipidated plasma than did the corresponding fraction in the control plasma. Lipid-stainable material was demonstrated at sites corresponding to the precipitin arcs in the control and partially delipidated specimens, but no lipid-stainable material was demonstrable at these sites in the totally delipidated plasma.

Figure 2 shows the electrophoretic patterns of partially and totally delipidated samples, obtained after 30-min extraction of plasma aliquots with increasing concentrations of butanol in diisopropyl ether, as well as an unextracted control. When the electrophoretograms were simultaneously stained for protein, it was seen that increasing quantities of lipid removed from the plasma correspond to a broadening in the α-globulin band and a narrowing of the β-globulin band. A second series of electrophoretograms, prepared concurrently from the same samples stained for protein, were simultaneously stained with Fat
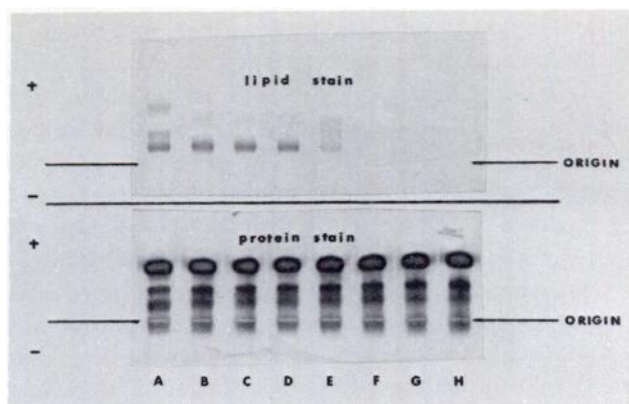
Fig. 2. Agarose electropherograms comparing control unextracted human plasma with human plasma extracted for 30 min with increasing concentrations of butanol in diisopropyl ether

Lipid and protein were stained with Fat Red 7B and Coomassie Blue, respectively. *A*, unextracted control; *B–H*, respective extraction mixtures (butanol/diisopropyl ether) were 0/100, 2.5/97.5, 5/95, 10/90, 20/80, 30/70, and 40/60 by vol. *F* and *G* contained some phospholipid, undetectable with the lipid stain. No lipid was present in sample *H* on quantitative estimation
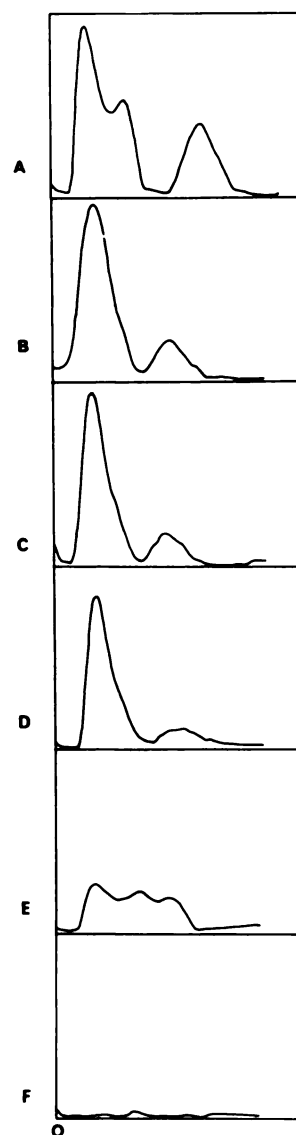

Fig. 3. Densitometric scans of agarose electropherograms, stained for lipid, illustrating the effect on electrophoretic mobility and the degree of delipidation of human plasma on extraction for 30 min with increasing concentrations of butanol in diisopropyl ether

A, unextracted control; *B–F*, respective proportions of solvents in extraction system are 0/100, 2.5/97.5, 5/95, 10/90, and 20/80. *O*, origin. The peaks in *A* are (*left to right*) beta-, pre-beta, and alpha-lipoproteins

Red 7B to demonstrate the lipid bound in the lipoprotein fractions (Figure 2) and scanned with the densitometer, at 520 nm (Figure 3).

As more lipid was removed from plasma, electrophoretic mobilities of the β- and pre-β-lipoprotein fractions increased; that of the α-lipoprotein fraction decreased. Integration of the densitometric scans demonstrates that α-lipoproteins are more readily delipidated by our solvent system than are the β- and pre-β-lipoprotein fractions. In some cases, because of the change in electrophoretic mobilities, it was not possible precisely to distinguish the pre-β-lipoprotein peak, which was masked by incomplete resolution from β-lipoprotein band (Table 2).

Because the lipid components—cholesterols, triglycerides, and phospholipids—add up to give a value almost the same as that for the total lipid present in the α-, β- and pre-β-lipoprotein fractions in plasma, lipid remaining in the various lipoprotein classes after partial delipidation correlated well, whether determined by chemical analysis or by densitometry (Table 3). This correlation did not apply over the whole range of delipidation, because some phospholipid still present in the plasma after 30-min delipidation with butanol/diisopropyl ether (20/80 by vol) was not detectable by Fat Red 7B stain.

Discussion

Application of a biphasic extraction method for plasma lipids, without first precipitating proteins, has permitted a comparative study of the extraction of lipids from the individual lipoprotein fractions in plasma.

Stained protein-bound lipid was directly measured densitometrically after agarose electrophoresis of plasma samples with various degrees of delipidation. An objection to direct densitometry is the nonlinear relationship between concentration of unextracted lipoprotein and density of bound dye (*10*). Nevertheless, Adlersberg et al. (*11*) successfully quantitated lipoproteins by densitometry. Individual pure lipids stain with various intensities, increasing the difficulty of quantitating lipid bound to the lipoprotein fractions. Notwithstanding these inadequacies, densitometry is the method of choice for quantitating serum lipoproteins (*8, 10–13*), in contrast to quantitative estimation of lipid fractions.

Lipids stainable with Fat Red 7B were more readily extracted with butanol/diisopropyl ether from α-lipoprotein than from the pre-β- and β-lipoprotein fractions in plasma. We saw little difference in lipid extractability of β- and pre-β-lipoprotein fractions in

Table 2. Effect on Electrophoretic Mobility of 30-min Extraction of Human Plasma by Various Solvent Mixtures[a]

Extraction solvent, butanol/ diisopropyl ether	Lipo-protein fraction	Distance migrated, mm	Integration units	Lipid extract-ed, %
No extraction	α	61.5	130	0
	pre-β	32	150	0
	β	16	210	0
0/100	α	48	85	35
	pre-β	—)	360)	0
	β	18.7)		
2.5/97.5	α	46	50	62
	pre-β	—)	280)	23
	β	19)		
5/95	α	46	40	69
	pre-β	—)	230)	36
	β	19)		
10/90	α	46	30	77
	pre-β	35	60	60
	β	19	90	58

[a] Electrophoresis in agarose gels, pH 8.6, of α-, β-, and pre-β-lipoprotein fractions. The concentrations of these fractions relative to the corresponding unextracted control plasma lipoprotein fractions are expressed as percent lipid extracted. Percent lipid extracted was derived from densitometric integration units.

plasma. These results compare favorably with those of Scanu and Schiano (13), who delipidated serum by a continuous cold-extraction method and obtained precipitated proteins, which were partly soluble in physiological saline. They found that the α-lipoprotein fraction was more easily delipidated than the β-lipoprotein fraction in serum. However, it is possible that all the apolipoprotein fractions obtained in the precipitated form by this continuous cold-extraction method were not soluble in physiological saline, because Tris-HCl buffer at pH 8.2 is required to re-dissolve similarly treated individual lipoprotein fractions that have been separated ultracentrifugally

(14). Our butanol/diisopropyl ether extraction of lipid from plasma leaves apolipoproteins in solution (1). We surmise from our experience with the butanol/diisopropyl ether extraction system that less energy is required to break the bond between the Fat Red 7B stainable lipid and apolipoproteins of the α-lipoprotein fraction than is required for β- and pre-β-lipoprotein fractions, when these are in plasma.

Our extraction of lipid from plasma results in greater electrophoretic mobilities of the pre-β- and β-lipoprotein fractions, as compared with the same fractions in unextracted control plasma. The reverse is true for α-lipoprotein: removal of plasma lipids results in an electrophoretic mobility that is less than that of α-lipoprotein in unextracted plasma. Magnitude of net charge determines the velocity of migration; the sign of the charge dictates the direction of movement; the algebraic sum of the charges on the particle and those surrounding it determines net charge; shape, size, and mass of the molecule influence the absolute rate of migration. At least some of these factors are altered when lipid is extracted from the apolipoprotein moiety.

The demonstration (Figure 1B) that partly delipidated α-lipoprotein yields two immunoelectrophoretic boundaries with different mobilities suggests heterogeneity of α-lipoprotein. When delipidation is complete, an apparently uniform electrophoretic mobility occurs, but the precipitin arc is less intense, a difference unlikely to be the result of loss of protein, because protein loss by this delipidation technique is insignificant (1). Previous observations on the immunoreactivity of delipidated α-lipoprotein (1, 15) are in accord with the current findings.

Electrophoretic mobility of the delipidated forms of the pre-β- and β-lipoproteins in plasma may be increased because of removal of lipid. If the lipids removed are neutral, then one would expect the apolipoproteins to migrate faster than the lipoproteins because they have lost about 80% of their mass. Alternatively, pre-β- and β-lipoprotein in plasma may be

Table 3. Comparison of Chemical Analysis and Densitometry for Lipid Components of Plasma Lipoproteins after Partial Delipidation by 30-min Extraction

Analysis of plasma lipoprotein-bound lipids

Extraction solvent, butanol/ diisopropyl ether	Triglyceride	Cholesterol	Phospholipid	Total lipids determined by chemical analysis[a]	Residual total lipid, %	Total lipids determined by densitometry,[a] integration units	Residual total lipids, %
	mg/dl						
No extraction	164	163	189	516	100	490	100
0/100	130	132	182	444	86	445	90
2.5/97.5	90	105	160	354	69	330	67
5/95	60	90	142	292	57	270	55
10/90	14	42	123	179	35	180	36

[a] Total lipids determined by chemical analysis correspond to the sum of cholesterols, triglycerides, and phospholipids in mg per 100 ml plasma. Total lipids determined by densitometry correspond to the sum of integration units of alpha, beta, and pre-beta-lipoproteins strained by Fat Red 7B after electrophoresis.

dissociated into smaller apolipoprotein units as a result of delipidation with our extraction mixture; β- and pre-β-apolipoproteins evidently contain heterogeneous units (14, 16). If the net charge of the parent lipoproteins is not evenly distributed between derived heterogeneous units, then electrostatic forces will also produce differences in migration rate, with the possibility of producing less distinct, or even merging, electrophoretic zones.

Either of these explanations would be compatible with our observations, as also would be a net change in mass, charge, and (or) molecular configuration. A clearer difference in electrophoretic mobility would be anticipated if one were to study separated apolipoproteins containing no other proteins.

References

1. Cham, B. E., and Knowles, B. R., A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.*, in press.

2. Technicon Instrument Corp., Tarrytown, N. Y. 10591; method N-24a.

3. Lofland, H. B., A semiautomated procedure for the determination of triglycerides in serum. *Anal. Biochem.* 9, 393 (1964).

4. Fletcher, M. J., A colorimetric method for estimating serum triglyceride. *Clin. Chim. Acta* 22, 393 (1968).

5. Folch, J., Lees, M., and Sloane Stanely, G. H., A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497 (1957).

6. Fiske, C. H., and SubbaRow, Y., The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375 (1925).

7. Noble, R. P., Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* 9, 693 (1968).

8. McTaggart, W. G., and Dwyer, J. L., Improved method for densitometry of electrophoretic lipoprotein fractions. *Clin. Chem.* 21, 183 (1975).

9. Keyser, J., Immunoelectrophoresis—applications and recent developments. *Chem. Br.* 8, 480 (1972).

10. Jencks, W. P., and Durrum, E. L., Paper electrophoresis as a quantitative method; the staining of serum lipoproteins. *J. Clin. Invest.* 34, 1437 (1955).

11. Adlersberg, D., Bossak, E. T., Sher, I. H., and Sobotka, H., Electrophoresis and monomolecular layer studies with serum lipoproteins. *Clin. Chem.* 1, 18 (1955).

12. Straus, R., and Wurm, M., A new staining procedure and a method for quantitation of serum lipoproteins separated by paper electrophoresis. *Am. J. Clin. Pathol.* 29, 581 (1958).

13. Scanu, A., and Schiano, N., Su di una nuova metodica di estrazione continua a freddo con etere dei lipidi del siero. Applicazione allo studio dei complessi lipoproteici. *Ist. Sierot, Ital.* 29, 457 (1954).

14. Scanu, A. M., Edelstein, C., and Aggerbech, L., Application of the technique of isoelectric focussing to the study of human-serum lipoproteins and their apoproteins. *Ann. N. Y. Acad. Sci.* 209, 311 (1973).

15. Levy, R. I., and Fredrickson, D. S., Heterogeneity of plasma high density lipoproteins. *J. Clin. Invest.* 44, 426 (1965).

16. Shore, V. G., Shore, B., and Hart, R. G., Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesterolemia. *Biochemistry* 13, 1579 (1974).