

# Lipoprotein fractionation

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Most of the methods used in the separation and fractionation of serum proteins have also been applied to the lipoproteins. Salt precipitation was used as early as the last century to fractionate serum proteins, and with this technique a lipoprotein was isolated in fairly pure form for the first time by Macheboeuf (1929). The precipitation techniques most commonly used today are based on the precipitation of very-low-density and low-density lipoproteins by sulphated polysaccharides (Burstein and Samaille, 1955) (see below). Much of our present knowledge of the serum lipoproteins is based on the studies of Gofman and coworkers, who used a combination of analytical and preparative ultracentrifugation (DeLalla and Gofman, 1954). The analytical ultracentrifuge is not widely available but preparative ultracentrifugation which, in combination with lipid analysis of the separated fractions, remains one of the best tools for the study of serum lipoproteins, is quite widely used (Havel, Eder, and Bragdon, 1955; Furman, Howard, and Norcia, 1959; Hatch, 1968). Electrophoresis is one of the most important modern techniques for studying lipoproteins; an important milestone in its development, which led to the classification of hyperlipoproteinaemias of Fredrickson, Levy, and Lees (1967), was the inclusion of albumin in the buffer for paper electrophoresis (Lees and Hatch, 1963). More recent techniques with agarose or cellulose acetate gel as supporting medium give better resolution of the lipoprotein pattern. Of other techniques, both ultrafiltration in combination with nephelometry and adsorption chromatography on glass powder or calcium phosphates have been used to a limited extent. Gel filtration techniques are fairly new and have mainly been used in the study of the apoproteins of the lipoproteins (Brown, Levy, and Fredrickson, 1970). Immunological methods such as immunoelectrophoresis and immunodiffusion have been invaluable in the study of the protein moiety of the lipoproteins. Such studies have been mainly qualitative (Hatch, 1968; Brown *et al.*, 1970), but there are some quantitative studies (Lees, 1970).

An excellent review of this field has been published by Hatch (1968).

## Practical Methods for Lipoprotein Fractionation

To be acceptable any technique must separate the four major lipoprotein families in serum, namely, the chylomicrons, pre- $\beta$  or very-low-density lipoproteins (VLDL),  $\beta$ - or low-density lipoproteins (LDL), and  $\alpha$  or high-density lipoproteins (HDL), and must in addition permit identification of floating or broad  $\beta$ -lipoprotein and sinking pre- $\beta$  or Lp(a) lipoprotein.

The methods currently used in our laboratory for the analysis of human serum lipoproteins will be described. These are preparative ultracentrifugation in combination with agarose gel electrophoresis, and inspection of serum after standing overnight at 4°C as a qualitative test for chylomicrons.

The sample is obtained from fasting subjects and after separation of the serum by low speed centrifugation EDTA is added to a final concentration of 0.05%. If analysis is not started on the same day, the samples are stored at 4°C and never deep frozen.

### ULTRACENTRIFUGATION

The procedure for fractionating the lipoproteins by ultracentrifugation, based on the above mentioned review of Hatch and summarized in fig. 1, is as follows: 4 ml of serum is transferred to a 6.5 ml centrifuge tube (0.5 × 2.5 in) which is then filled by layering saline of density 1.006<sup>1</sup> on top of the serum. After centrifugation for 16 hours at 105 000 *g*,<sup>2</sup> three zones can be seen in the tube, a narrow top layer which is opalescent, a clear middle layer, and a yellow bottom layer. The top layer (fig 1, A) contains the VLDL, which is separated from the rest (fig 1, B) by slicing the tube. Both fractions, A and B, are transferred to separate volumetric flasks, carefully rinsing the two parts of the centrifuge tube with the saline of density 1.006. Fraction B contains the LDL and HDL; 4 ml of this is transferred to a clean centrifuge tube and its density raised to 1.063 by the addition of 2 ml of a NaCl-NaBr solution of density 1.182.<sup>3</sup> After careful mixing the tube is centrifuged

<sup>1</sup>To make this solution 11.4 g of NaCl, 0.1 g of EDTA, and 1 ml of 1M-NaOH are dissolved and made up to 1 litre with water, and an additional 3 ml of water is added.

<sup>2</sup>40 000 rpm in a type 40.3 rotor in the Beckman model L2-65B at 15°C.

<sup>3</sup>Made by adding 24.98 g of NaBr to 100 ml of the density 1.006 solution.

## SERUM LIPOPROTEIN ANALYSIS ULTRACENTRIFUGATION

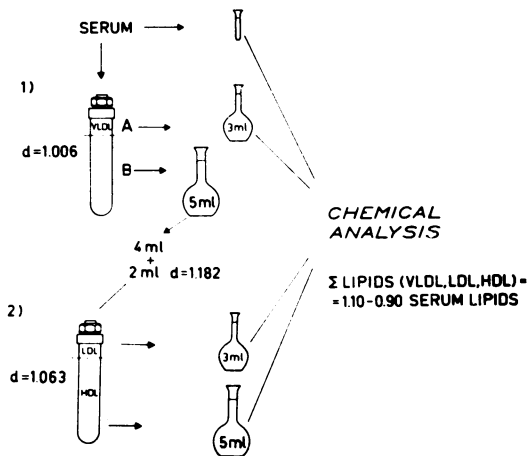


Fig 1 The ultracentrifugation procedure: at (1) centrifugation for 16 hours at 105 000 g; at (2) centrifugation for 20 hours at 105 000 g.

for 20 hours at 105 000 g. After centrifugation there is again a narrow top layer, which is now yellow and contains the LDL. The middle zone is completely clear and the bottom yellow fraction consists mainly of serum proteins but contains also the HDL. The top fraction and the remainder are transferred to separate volumetric flasks as after the first centrifugation (fig 1). The three separate VLDL, LDL, and HDL fractions are then extracted for determination of triglyceride and cholesterol. A blank is run through the whole procedure and the original serum is analysed at the same time to calculate recovery. A recovery of 90-110% is considered acceptable, the average in the results presented below being 95%. To obtain good reproducibility it is necessary to check the densities of the different salt solutions carefully at defined temperature, eg, by pycnometry. It should be noted that the density 1.063 is defined at 26°C, whereas other densities are measured at 20°C.

The error of the entire ultracentrifugation procedure, obtained by running 60 different sera in duplicate, is shown in table I. The within-batch coefficient of variation varies from about 4 to 8%, except for HDL triglyceride where it is 15.4%. The error of the chemical estimations alone is about 3%.

The second centrifugation to separate the LDL from the HDL can be replaced by precipitation of the LDL by heparin (Burstein and Samaille, 1960), a beautifully simple and quick method, which is performed as follows:

To 1 ml of the bottom fraction from the first ultracentrifugation add 0.04 ml of heparin (5000 IU/ml). Add 0.05 ml of 1M-MnCl<sub>2</sub> and mix by vortexing. Stopper the tube and leave overnight at 4°C. Centrifuge for 10 min at 3000 rpm at 4°C. The supernatant contains HDL and may be extracted for lipid analysis.

Results obtained by this method agree well with those obtained by ultracentrifugation (fig 2). If this technique is used instead of the second ultracentrifugation step, LDL must be determined by an

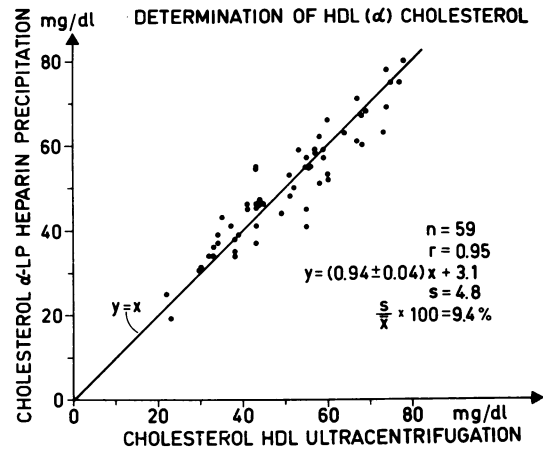


Fig 2 Relationship between HDL (α-lipoprotein) cholesterol determined by ultracentrifugation and by heparin precipitation.

	VLDL		LDL		HDL	
	Triglyceride (mmol/l)	Cholesterol (mg/100 ml)	Triglyceride (mmol/l)	Cholesterol (mg/100 ml)	Triglyceride (mmol/l)	Cholesterol (mg/100 ml)
Mean	1.62	34	0.63	176	0.26	47
Standard deviation	0.11	2.4	0.05	8.0	0.04	1.8
Coefficient of variation (%)	6.8	7.1	7.9	4.6	15.4	3.9

Table I The within-batch precision of triglyceride and cholesterol estimation in the lipoprotein fractions obtained by ultracentrifugation<sup>1</sup>

<sup>1</sup>The values were obtained by analysing 60 different sera in duplicate.

indirect method, namely, by estimating triglyceride and cholesterol in the bottom fraction of the first centrifugation and subtracting the corresponding values for HDL obtained by the precipitation technique.

In 60 control males the mean cholesterol was 245 mg/100 ml, the range of  $\pm 2$  standard deviations being 169 to 321 mg/100 ml, whereas the mean triglyceride was 1.64 m mol/l ranging from 0.74 to 2.54.<sup>1</sup> However, the distribution of the triglyceride values is skewed in a log-normal fashion. Calculation of the mean and standard deviation using logarithmic values gives a normal range for triglyceride of 0.93 to 2.69 mmol/l. The values for cholesterol and triglyceride in the separated lipoprotein fractions are given in tables II and III respectively. When age and sex are taken into account (fig 3), females are seen to have lower VLDL and LDL but considerably higher HDL levels; there is also some increase in concentration with age, except for men over 60.

The results from a population study of 131 males aged 50 years attending a health control clinic in Uppsala show a wider range of total triglyceride and cholesterol values (fig 4), the former again showing a log-normal distribution. Of the individual lipo-

<sup>1</sup>1mmol/ = 88.5 mg/100 ml

		Lipoprotein Fraction		
		VLDL	LDL	HDL
Cholesterol content (mg/100 ml)	Upper	35	227	78
	Mean	17	162	54
	Lower	0.6	98	30

Table II Cholesterol content of the lipoprotein fractions in 60 male controls<sup>1</sup>

<sup>1</sup>The upper and lower values indicate  $\pm 2$  standard deviations.

protein fractions VLDL shows a log-normal distribution of both triglyceride and cholesterol, and all fractions show a wide range of concentration of these substances, including the HDL cholesterol (Carlson, L. A., and Hedstrand, unpublished).

		Lipoprotein Fraction		
		VLDL	LDL	HDL
Triglyceride content (m mol/l)	Upper	1.56 (1.77)	0.77 (0.90)	0.35
	Mean	0.84 (0.78)	0.51 (0.49)	0.23
	Lower	0.12 (0.34)	0.25 (0.26)	0.11

Table III Triglyceride content of the lipoprotein fractions in 60 male controls<sup>1</sup>

<sup>1</sup>The upper and lower values indicate  $\pm 2$  standard deviations. The values in parentheses are those derived from logarithms of the original values (see text).

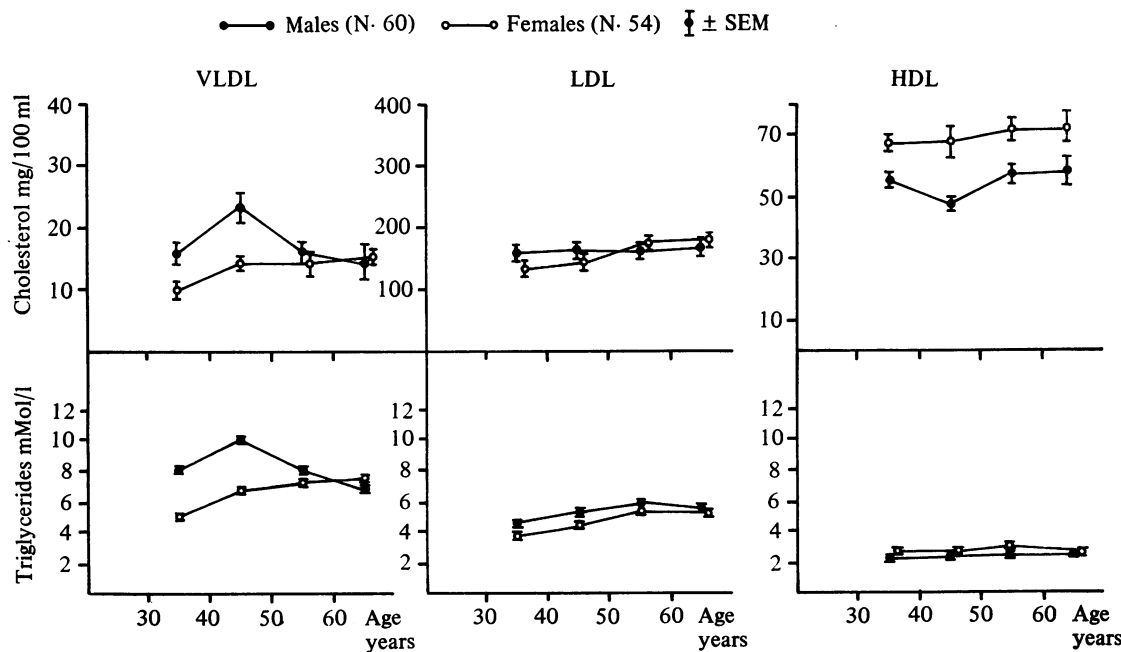


Fig 3 The variation of the concentration of triglyceride and cholesterol with age and sex in a control group.

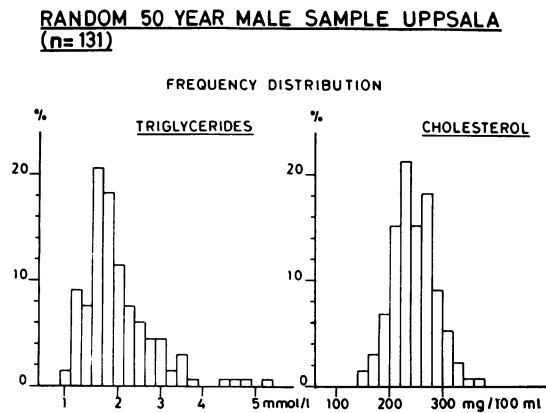


Fig 4 Frequency distribution of serum triglyceride and cholesterol concentration in a random sample of 131 50-year-old males.

The relationship between the concentration of triglyceride and cholesterol in the lipoproteins has been investigated in this material. There is a good correlation between triglyceride and cholesterol in the VLDL fraction (fig 5). There is an inverse correlation between VLDL triglyceride and HDL cholesterol (fig 6) (the major lipids in these particular lipoproteins), which may be explained by the fact that they have an apoprotein in common (LaRosa, Levy, Brown, and Fredrickson, 1971).

**ELECTROPHORESIS**

The results presented below are obtained in agarose

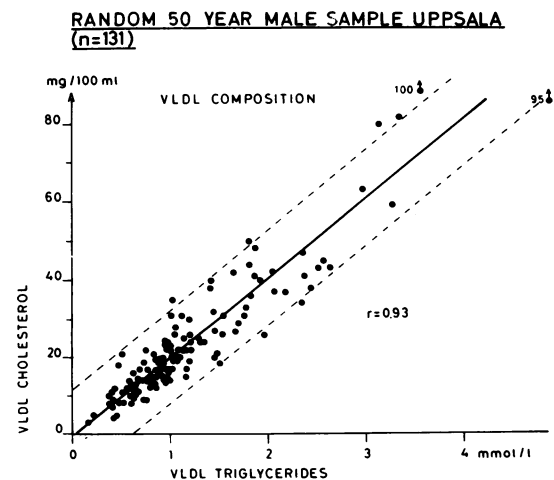


Fig 5 The relationship of cholesterol to triglyceride concentration in the VLDL.

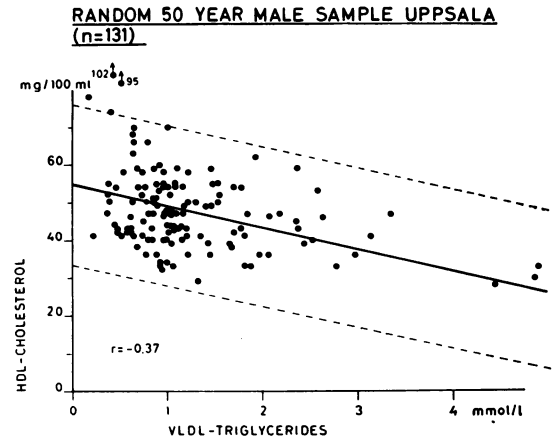


Fig 6 The relationship of HDL cholesterol to VLDL triglycerides.

gel (10 g/l) after electrophoresis for one hour using a voltage of 16 V/cm and Sudan Black as stain.

Whole serum usually shows three bands, the  $\beta$ , pre- $\beta$ , and  $\alpha$ -lipoproteins, the top fraction from the first ultracentrifugation shows one pre- $\beta$  band, and the bottom fraction shows two bands, the  $\beta$ - and  $\alpha$ -lipoproteins (fig 7). Chylomicrons, if present, are found as a band at the origin on electrophoresis of whole serum or of the top fraction. The most common variations of this pattern are shown in fig 8 where the first pattern (P1) shows a normal serum. In type III hyperlipoproteinaemia a band with  $\beta$  mobility is found in the top fraction and is called the 'floating  $\beta$ ' (fig 8, P2). The type III disorder has been called 'broad- $\beta$ -disease', referring to the pattern sometimes, but not always, seen on paper electrophoresis of whole serum and infrequently on agarose gel electro-

**SERUM LIPOPROTEIN ANALYSIS**

ULTRACENTRIFUGATION AGAROSE GEL ELECTROPHORESIS

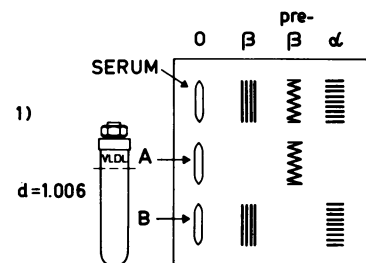


Fig 7 Agarose gel electrophoresis of serum and its ultracentrifugal fractions.

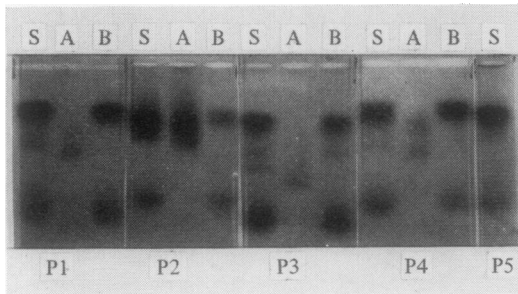


Fig 8 Agarose gel electrophoresis. *S* = whole serum, *A* and *B* = top and bottom fractions respectively after the first ultracentrifugation (at density 1.006). Note that the constituents of fraction *A* migrated rather faster than in whole serum.

*P1*—Normal serum; *P2*—floating  $\beta$ ; *P3*—sinking pre- $\beta$ ; *P4*—slow pre- $\beta$ ; *P5*—normal serum showing artefact at the origin due to freezing.

For full explanation see text.

phoresis. This means that electrophoresis of the top fraction is necessary to identify type III. Another variant is a lipoprotein with pre- $\beta$  mobility which occurs in the bottom fraction and which is therefore called the 'sinking pre- $\beta$ ' (fig 8, P3). This is sometimes seen as an extra pre- $\beta$  band in electrophoretograms of whole serum and is now well defined. It has been well studied by Berg and coworkers using immunological techniques (Berg, 1963) and was named by them Lp(a) lipoprotein. In contrast to the VLDL pre- $\beta$ -lipoprotein the sinking pre- $\beta$  contains much protein and very little triglyceride, and failure to separate the sinking pre- $\beta$  from VLDL pre- $\beta$  by electrophoresis of whole serum will result in a dense pre- $\beta$  band likely to be misinterpreted as a type IV pattern. A true type IV has a high triglyceride level, which is not present when an increased pre- $\beta$  band is due to sinking pre- $\beta$ . In the electrophoretograms of the top fraction, two bands are sometimes seen both at normal and elevated levels of VLDL (fig 8, P4). The first has normal pre- $\beta$  mobility, the second a mobility between pre- $\beta$  and  $\beta$ . The nature of this slow pre- $\beta$  is uncertain. It may be due to a small amount of the floating  $\beta$  lipoprotein, so small that there is no change in the ratio of cholesterol to

triglyceride in the VLDL fraction. Another possibility is that slow pre- $\beta$  consists of particles which are so large that they are retarded by a gel filtration effect. The remaining pattern in fig 8 (P5) shows a normal serum which has been kept deep frozen; the material at the origin is an artefact due to denaturated protein and does not represent chylomicrons, thus demonstrating why frozen serum should not be used for electrophoresis.

When ultracentrifugation is combined with electrophoresis of the separated fractions, the sinking pre- $\beta$  is found in about 20% of the random sample of 50-year-old men, which is a slightly lower incidence than the 35% reported from immunological studies in other Scandinavian populations (Monn, Berg, Reinskou, and Teisberg, 1971). The slow pre- $\beta$  occurs in 26% of cases, whereas type III is found in 1%.

The amount of  $\beta$ -lipoprotein apoprotein in VLDL and LDL can be determined by the quantitative immunological rocket technique of Laurell (1966). Electrophoresis is performed in agarose gel which contains a monospecific antiserum and the samples are compared with standards of purified  $\beta$ -lipoprotein. Some data obtained with this technique are given in table IV.

To summarize, a complete lipoprotein analysis requires a combination of ultracentrifugation and electrophoresis. In many cases, however, electrophoresis combined with analysis of triglyceride and cholesterol in the serum will be adequate: in others the need for the added information from ultracentrifugation can be left for the physician to decide.

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Fredrickson Type	VLDL				LDL			
	Triglyceride (mmol/l)	Cholesterol (C) (mg/100 ml)	Protein (P) (mg/100 ml)	C/P	Triglyceride (mmol/l)	Cholesterol (C) (mg/100 ml)	Protein (P) (mg/100 ml)	C/P
Normal	0.36	8	7	1.1	0.45	170	80	2.1
IIb	3.96	42	32	1.3	1.58	251	130	1.9
IV	2.40	34	15	2.3	0.46	99	69	1.4
V	21.30	332	48	6.9	0.57	41	35	1.2

Table IV Protein, cholesterol, and triglyceride content of isolated lipoproteins in normal and abnormal serum

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