# Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions

M. BURSTEIN, H. R. SCHOLNICK, and R. MORFIN

Centre National de Transfusion Sanguine, Paris, France: and Department of Medicine, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461

ABSTRACT Procedures are described for the isolation of lipoproteins from human serum by precipitation with polyanions and divalent cations. A mixture of low and very low density lipoproteins can be prepared without ultracentrifugation by precipitation with heparin and either $MnCl_2$ alone or $MgCl_2$ plus sucrose. In both cases the precipitation is reversible, selective, and complete. The highly concentrated isolated lipoproteins are free of other plasma proteins as judged by immunological and electrophoretic methods. The low density and very low density lipoproteins can then be separated from each other by ultracentrifugation. The advantage of the method is that large amounts of lipoproteins can be prepared with only a single preparative ultracentrifugation. Polyanions other than heparin may also be used; when the precipitation of the low and very low density lipoproteins is achieved with dextran sulfate and $MnCl_2$, or sodium phosphotungstate and $MgCl_2$, the high density lipoproteins can subsequently be precipitated by increasing the concentrations of the reagents. These lipoproteins, containing small amounts of protein contaminants, are further purified by ultracentrifugation at d 1.22. With a single preparative ultracentrifugation, immunologically pure high density lipoproteins can be isolated from large volumes of serum.

SUPPLEMENTARY KEY WORDS heparin · dextran sulfate · sodium phosphotungstate · divalent cations · sucrose · immunology · ultracentrifugation · zonal electrophoresis

It is known that at neutral pH sulfated polysaccharides can form insoluble complexes with serum low density lipoproteins (1). With polysaccharides of high molecular weight, LDL can be precipitated without the addition of divalent cations; when lower molecular weight polysaccharides such as heparin are used, divalent cations must be present in order to precipitate LDL. The conditions of the lipoprotein precipitation by heparin depend upon the cation being used. With $Mn^{++}$ the precipitation can be achieved by direct addition of reagents to the serum (2), but with $Mg^{++}$ or $Ca^{++}$ it is necessary to lower the ionic strength (3) or to add sucrose (4). Otherwise, only triglyceride-rich lipoproteins (chylomicrons and VLDL) are precipitated (3).

By using certain polyanions other than heparin it is possible to precipitate not only LDL and VLDL ($\beta$- and pre $\beta$-lipoproteins), but also HDL ($\alpha$-lipoproteins) if higher concentrations of reagents are used. This difference in reagent concentration makes it feasible to prepare, as a first step, serum free of LDL and VLDL, and then by increasing the concentrations of the reagents, to bring about the reversible precipitation of HDL. The isolation of HDL can best be achieved by using either dextran sulfate and $MnCl_2$ (5), or sodium phosphotungstate and $MgCl_2$ (6).

In this report four procedures for the isolation of low and high density lipoproteins will be described. One of these procedures (method I, see below) has been published before (4) as a note, but it will be outlined again so that it can be readily compared with the other techniques. The precipitation procedures used in the remaining three methods have also been published before (precipitation of LDL plus VLDL by heparin and $Mn^{++}$ [2], precipitation of HDL by dextran sulfate and $MnCl_2$

Abbreviations: NaPhT, sodium phosphotungstate; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; PVP, polyvinylpyrrolidone.



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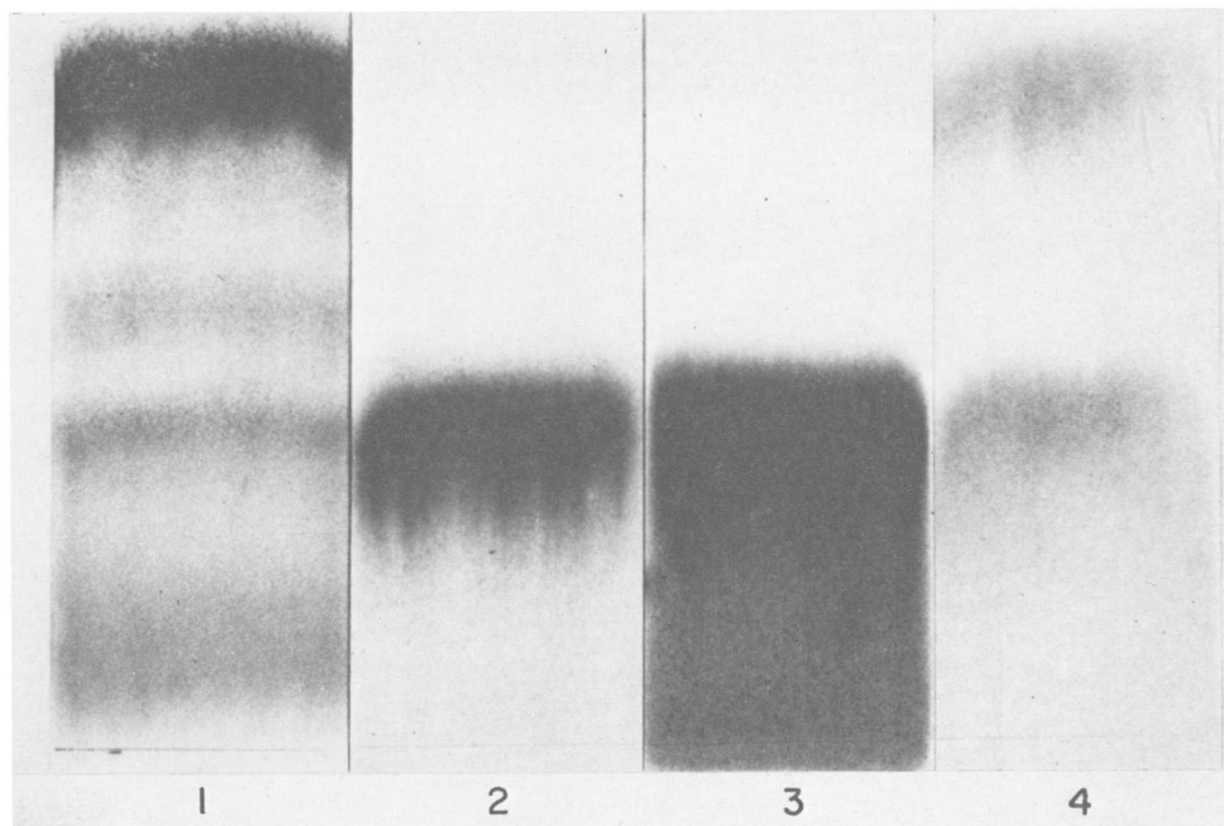


Fig 1. Paper electrophoresis. *1* and *4*, normal human serum; *2* and *3*, isolated LDL plus VLDL (cholesterol, 3.75 g/100 ml of solution); *1* and *2*, protein stain; *3* and *4*, lipid stain.

[5], and sodium phosphotungstate and $MgCl_2$ [6]), but the isolation of immunologically pure lipoproteins from the precipitates has not heretofore been described in the literature.

## EXPERIMENTAL PROCEDURE

### *Materials*

Pooled clear serum from fasting healthy donors was used.

The following solutions of the polyanions in the precipitations were used: (*a*) sodium salt of heparin (Produits Roche, S.A., Paris, France), 5% in 0.15 M NaCl; (*b*) dextran sulfate (mol wt approximately 15,000; Sochibo, Boulogne, France), 10% in 0.15 M Nacl; (*c*) sodium dextran sulfate 2000 (mol wt $2 \times 10^6$; Pharmacia, Uppsala, Sweden), 5% in 0.15 M NaCl; (*d*) Mepesulfate, sodium salt of sulfated polygalacturonic acid methyl ester–methyl glycoside (Produits Roche), 10% in 0.15 M NaCl; and (*e*) 4% sodium phosphotungstate, pH 7.6. To prepare this latter solution 40 g of phosphotungstic acid (Merck) were dissolved in 500 ml of distilled water, and 160 ml of 1 M NaOH were then added with stirring; the volume was made up to 1000 ml with distilled water. Two types of Tris buffers were used: Tris–HCl, 0.02 M, pH 7.7, and Tris–HCl–NaCl, the same buffer, but containing 1% NaCl.

Commercial horse and rabbit anti-whole human sera were used (Pasteur, Paris, France). For the preparation of anti-human HDL serum, rabbits were injected intramuscularly twice weekly for 6 wk with the isolated HDL (see below). For each injection 3 mg of protein mixed with Freund's complete adjuvant was used. Animals were bled 1 wk after the last injection.

Pure anti-$\beta$-lipoprotein antibodies were isolated from the specific precipitate, human $\beta$-lipoprotein—rabbit anti-human $\beta$-lipoprotein, by a method previously described (7). These antibodies were used to prepare a minimally diluted human serum specifically devoid of VLDL and LDL (8). They were also utilized to detect traces of $\beta$-lipoproteins since visible precipitation occurs in a serum diluted 200-fold. In addition, serum free of LDL and VLDL was prepared by ultracentrifugation at d 1.063.

### *Methods*

Zonal electrophoresis on paper (9), agarose (10), and cellulose acetate (11) was performed in barbital buffer at pH 8.6 (barbital 0.138 g/100 ml, sodium barbital



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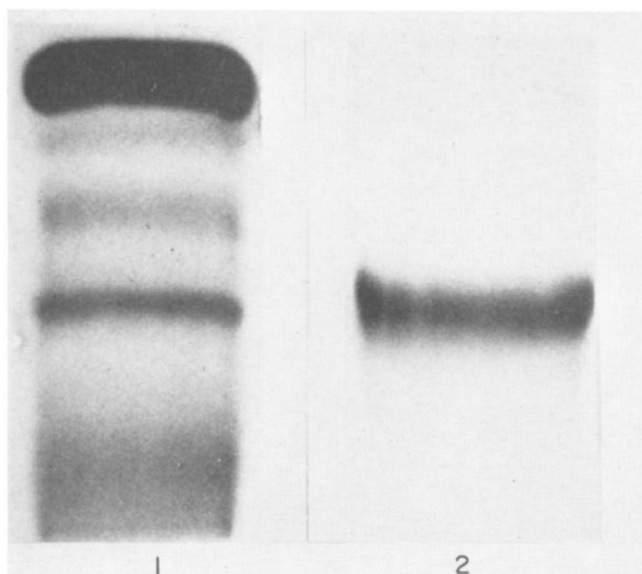


FIG. 2. Electrophoresis on cellulose acetate; protein stain. *1*, normal human serum; *2*, isolated LDL plus VLDL (cholesterol, 3.25 g/100 ml of solution).

0.876 g/100 ml). For the starch gel electrophoresis (12) the discontinuous system of buffers (13) was employed.

The paper and agarose strips were stained for lipid with Sudan Black (14). The cellulose acetate strips and starch gels were stained for protein with Amido Schwarz. The paper strips were stained for protein with Light Green, and the agarose strips with either Amido Schwarz or Light Green.

Immunochemical analyses were performed by the following gel diffusion methods: microimmunoelectrophoresis (15) in 1.5% agarose, single diffusion by the procedure of Oudin (16), and double diffusion in two dimensions by the method of Ouchterlony (17). The sedimentation constants were measured in a model E Spinco analytical centrifuge. Runs were made at 59,780 rpm at 20°C; the solvent used was Tris–HCl–NaCl buffer.

Total lipids were determined by the method of Delsal (18) after their extraction from serum with methylal–methanol. The protein content of the serum was estimated by the biuret method (19), and of the methylal–methanol-insoluble material by a modification of the biuret method (20). Lipid phosphorus was measured by the method of Zilversmit and Davis (21); a factor of 25 was used to convert phosphorus to phospholipid. Total cholesterol was determined by the method of Pearson, Stern, and McGarack (22).

## ISOLATION OF LIPOPROTEINS

All precipitations are carried out at room temperature. Centrifugation, if not otherwise indicated, is performed at 20°C. All dialyses are conducted at 4°C.

### *Method I: Isolation of LDL and VLDL by Precipitation with Heparin and $MgCl_2$ in the Presence of Sucrose*

500 g of sucrose is dissolved in 500 ml of human serum (1 g/ml of serum) expanding the volume to 800 ml. 8 ml of 5% heparin and 40 ml of 2 M $MgCl_2$ are then added (final concentrations: heparin 0.05%, $MgCl_2$ 0.1 M). Opacification occurs immediately. After 15 min the mixture is centrifuged in stoppered tubes for 30 min at 6000 *g*. Due to the high density (sucrose), the precipitated lipoproteins float to the top and form a pellicle on the surface of the clear subnatant. The subnatant solution is completely removed by aspiration with a syringe and needle. The tubes are then centrifuged again for 1 min in order to sediment the pellicles. The precipitates are combined and dissolved in 10 ml of 5% NaCl by incubation for 30 min at 37°C. To remove contaminating serum proteins, the lipoproteins are precipitated by adding 500 ml of the Tris–HCl buffer and 12.5 ml of 2 M $MgCl_2$. The final concentration of $MgCl_2$ is 0.05 M.

Since the ionic strength is low (0.1% NaCl), the complete precipitation of the lipoproteins occurs in the absence of sucrose. The precipitate sediments on centrifugation (10 min at 6000 *g*) and is dissolved again in 10 ml of 5% NaCl; the lipoproteins are reprecipitated as above to eliminate the last traces of impurities.

The washed precipitate is dissolved in 2.5 ml of 10% sodium citrate. The solution is dialyzed for 24 hr against 2000 ml of the Tris–HCl–NaCl buffer to remove the citrate and the $Mg^{++}$. In order to remove the heparin, the dialysis bag is transferred to another flask containing 5% $BaCl_2$. After 24 hr, the insoluble heparin–barium salt is removed by centrifugation at 4°C.

The supernatant is dialyzed against Tris–HCl–NaCl buffer in order to remove the $BaCl_2$. This results in a clear yellow solution of concentrated lipoproteins (15 g/100 ml or more) with a protein-free density of approximately 1.006. Such a highly concentrated solution of isolated lipoproteins remains clear even after prolonged dialysis.

The lipoproteins isolated in this way are a mixture of LDL and VLDL; these may be separated from each other by ultracentrifugation. After 24 hr at 100,000 *g*, the VLDL form an opalescent band at the top of the tube, and the clear yellow LDL sediment to the bottom.

The precipitation is selective and complete. No precipitation occurs when a serum devoid of LDL and VLDL (see Methods) is used. The subnatant is free of LDL and VLDL as judged by immunological techniques, and there is no further precipitate upon the addition of heparin, $MgCl_2$, or both.

The precipitation of lipoproteins is not affected by the pH in the range of 6–9. If the ionic strength of the noncentrifuged mixture is raised by addition of 1.0 g of NaCl per 100 ml, the precipitate dissolves almost com-



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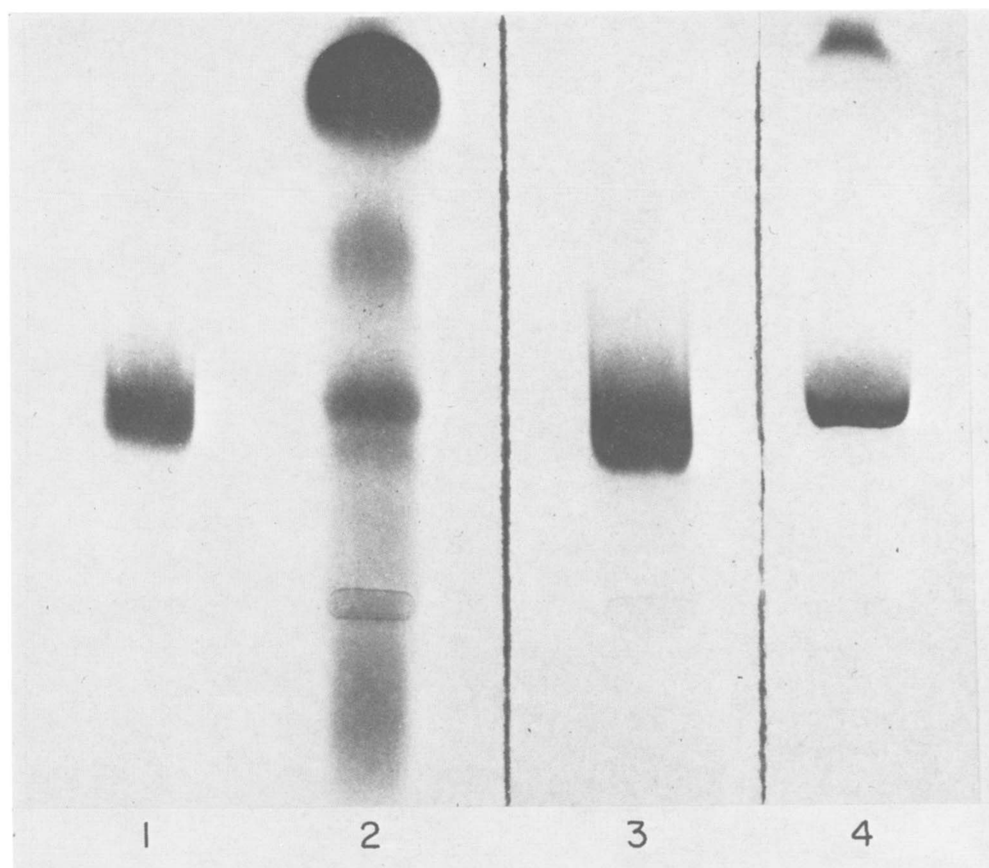


FIG. 3. Electrophoresis on agarose gel. *1* and *3*, isolated LDL plus VLDL (cholesterol, 4.0 g/100 ml of solution); *2* and *4*, normal human serum; *1* and *2*, protein stain (light green); *3* and *4*, lipid stain.

pletely. The minimum required final concentration of heparin is 0.025%; with less heparin only a fraction of the lipoproteins is precipitated. When less sucrose is used (for example, 0.5 g/ml), the minimum requirement of heparin is higher (0.1% final concentration). The minimum requirement of $MgCl_2$ is 0.02 M, but under these conditions higher centrifugal speeds are needed to separate the precipitated lipoproteins.

### *Method II: Isolation of LDL and VLDL by Precipitation with Heparin and $MnCl_2$*

To 500 ml of serum are added 20 ml of 5% heparin solution and 25 ml of 1 M $MnCl_2$ (final concentrations: heparin 0.2%, $MnCl_2$ 0.05 M). A precipitate appears immediately. The mixture is centrifuged for 10 min at 6000 *g* (the centrifugation can be performed immediately or after several hours). The precipitated lipoproteins sediment; the supernatant is clear, but there is a white pellicle of manganese oxide which floats on the surface. The supernatant is decanted, and the precipitates are combined and dissolved in 5 ml of 10% sodium bicarbonate. The manganese associated with the lipoprotein precipitates as the bicarbonate salt and is removed by centrifugation. 500 ml of Tris–HCl buffer is added to the clear yellow supernatant, and the lipoproteins are completely precipitated by addition of 12.5 ml of 2 M $MgCl_2$ (final concentration 0.05 M). The precipitate is separated by centrifugation (10 min at 6000 *g*), redissolved in 10 ml of 5% NaCl, and handled in the same way as the lipoproteins isolated in the presence of sucrose, that is, 500 ml of Tris–HCl buffer, 12.5 ml of 2 M $MgCl_2$, etc., centrifugation at 6000 *g* for 10 min, etc.

As in method I (heparin–Mg–sucrose), this precipitation is complete and selective, and is independent of the source of the heparin. After adding $MnCl_2$ to the serum, the pH drops from 7.8 to 7.2. Adjusting the pH of the mixture to either pH 4.0 with HCl or pH 8.6 with NaOH causes the redissolution of the precipitate. The precipitate can also be dissolved by increasing the ionic strength by the addition of 1.0 g of NaCl per 100 ml of the mixture. The minimum final concentration of heparin is 0.1%, and in a serum diluted 10-fold with a serum devoid of VLDL and LDL, it drops to 0.02%; the minimum concentration is 0.005% in a serum diluted 10-fold with buffered saline (pH 7.7). Thus, there is a correlation between the minimum heparin requirement and both the concentration of lipoproteins and the concentration of other serum proteins.



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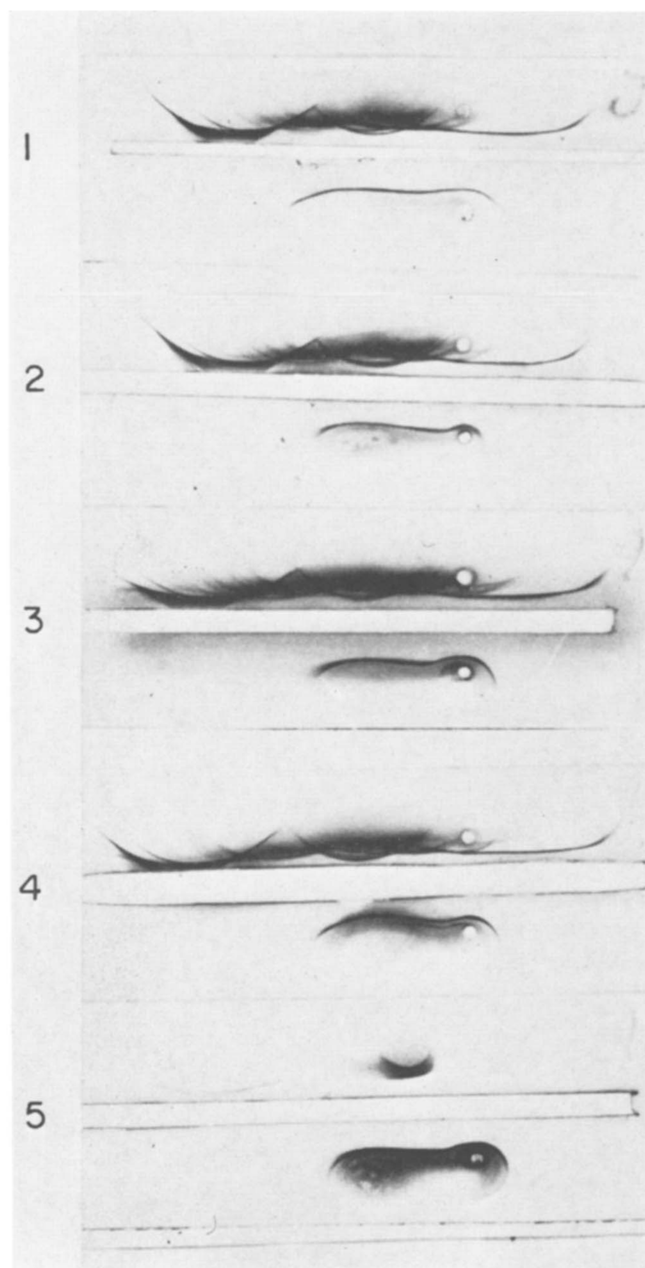


FIG. 4. Immunoelectrophoresis. Upper wells, normal human serum; lower wells *1–4*, four different samples of isolated LDL plus VLDL; lower well 5, same preparation as 4. Troughs: horse anti-whole human serum. Slides *1–4*, protein stain; slide *5*, lipid stain.

Further precipitation does not occur with addition of more heparin, $MnCl_2$, or of both to the supernatant. The clear supernatant becomes turbid with standing because of the precipitation of manganese oxide. However, $MnCl_2$ can be removed from the supernatant by dialysis against Tris–HCl–NaCl buffer, and heparin is precipitated by dialysis against 5% $BaCl_2$ (see above). This serum, free of both heparin and $Mn^{++}$, is devoid of VLDL and LDL as judged by agarose electrophoresis and subsequent lipid staining. There is no immunological reaction with anti–β-lipoprotein (LDL plus VLDL) antibodies, and the immunoelectrophoretic pattern against anti-whole human serum is normal except for the absence of the β-lipoprotein precipitin line. After adsorption of a rabbit anti-whole human serum with this preparation, a specific anti–β-lipoprotein serum is obtained (8). The cholesterol and phospholipid content is the same as in that of serum from which LDL and VLDL have been eliminated by addition of anti–β-lipoprotein antibodies.

In this method, one starts with $MnCl_2$ because the ionic strength of the serum is too high to use $MgCl_2$. For the reprecipitations, $MgCl_2$ can be used since there is a low ionic strength. The substitution of $MgCl_2$ for $MnCl_2$ is necessary because there is some coprecipitation of proteins when $MnCl_2$ is used.

### *Method III: Isolation of HDL by Precipitation with Dextran Sulfate and $MnCl_2$*

To 500 ml of serum are added 2.5 ml of 10% dextran sulfate and 25 ml of 1 M $MnCl_2$ (final concentrations: dextran sulfate 0.05%, $MnCl_2$ 0.05 M). With these concentrations, the LDL and VLDL are completely and selectively precipitated. The precipitate is then removed by centrifugation for 10 min at 6000 *g*. The lipoproteins may be isolated from the precipitate by a procedure similar to that used in the heparin–$Mn^{++}$ method. The precipitate is redissolved in 10 ml of 10% sodium bicarbonate; after removing the $MnCO_3$ by centrifugation, the lipoproteins are precipitated by adding 500 ml of Tris–HCl buffer and 12.5 ml of 2 M $MgCl_2$. The precipitate is redissolved in 10 ml of 5% NaCl, and the lipoproteins are reprecipitated by addition of 500 ml of 0.5% NaCl buffered with Tris–HCl (0.02 M, pH 7.7) and 25 ml of 2 M $MgCl_2$; this procedure is repeated. The washed precipitate is treated in the same way as in the heparin–Mg–sucrose method except that the dextran sulfate is removed by dialysis against 1% $BaCl_2$ in 1% NaCl (instead of 5% $BaCl_2$ in distilled water for removal of heparin).

To the LDL- and VLDL-free supernatant A are added 30 ml of 10% dextran sulfate and 75 ml of 1 M $MnCl_2$ (final concentrations: dextran sulfate 0.65%, $MnCl_2$ 0.2 M). The increase of the concentration of $MnCl_2$ causes the pH to drop from 7.2 to 6.9. Precipitation begins immediately and is complete after 2 hr. The mixture is centrifuged at 20,000 *g* for 30 min. The supernatant B is decanted, and the precipitate is washed in 250 ml of the Tris–HCl–NaCl buffer containing 0.1% dextran sulfate and 0.1 M $MnCl_2$. The washed precipitate which contains the HDL, some other proteins, and manganese oxide, is recovered by centrifugation (10



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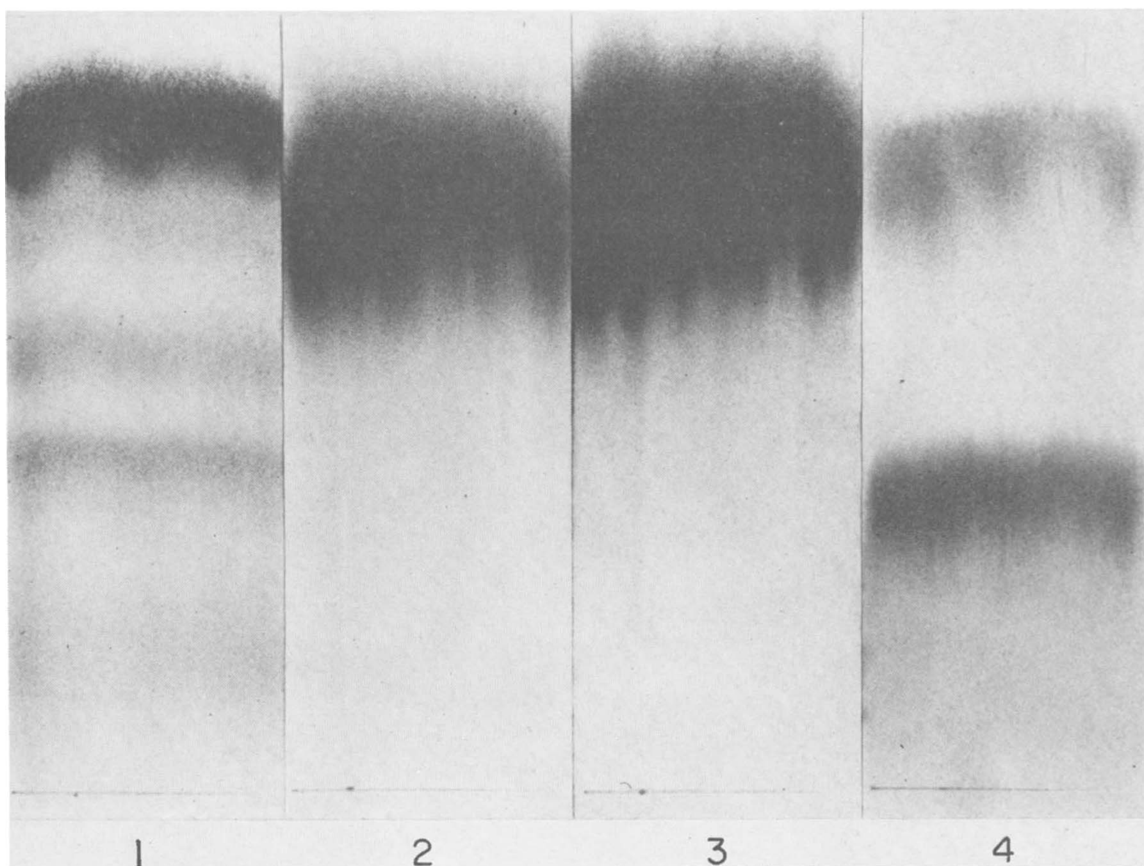


FIG. 5. Paper electrophoresis. *1* and *4*, normal human serum; *2* and *3*, isolated HDL (protein, 2 g/100 ml of solution); *1* and *2*, protein stain; *3* and *4*, lipid stain.

min, 6000 *g*) and is suspended in 50 ml of 2% sodium citrate in 1% NaCl.

The proteins are dissolved by stirring the suspension (magnetic stirrer) for 30 min and by adjusting the pH to 8.0 with 1 N NaOH. The turbid solution is centrifuged at 6000 *g* for 10 min to remove the white precipitate of manganese oxide. The supernatant is a clear yellow solution of HDL contaminated by small amounts of serum proteins. To prepare pure HDL it is necessary to perform one ultracentrifugation at the appropriate density (approximately 1.22). This density is achieved by adding 10 g of NaCl and 23 g of KBr to the supernatant and by bringing the volume up to 100 ml with distilled water. In this hypertonic medium, dextran sulfate precipitates and is removed by low-speed centrifugation. After ultracentrifugation of the supernatant (Spinco model L-2, rotor 50-Ti, 105,000 *g*, 18°C, 24 hr), the HDL float to the top and are removed. The yellow-green color of the subnatant is due to the $Mn^{++}$. The clear solution of concentrated HDL (8 g/100 ml or more) is dialyzed against the Tris–HCl–NaCl buffer. Larger amounts of HDL (from 1 liter of serum) may also be isolated with one preparative ultracentrifugation by performing the flotation at d 1.22 in a final volume of 150 ml.

Analysis of the supernatant B reveals that the HDL are almost completely precipitated by dextran sulfate and $MnCl_2$. This supernatant is dialyzed[1] against 1% saline to remove $Mn^{++}$, and against 1% $BaCl_2$ in 1% NaCl to precipitate dextran sulfate. After centrifugation at 4°C, the solution is dialyzed against Tris–HCl–NaCl buffer to remove $BaCl_2$; it is then concentrated to its original protein content by dialysis against PVP. This serum contains approximately 30 mg of phospholipid per 100 ml and only traces of cholesterol. With a specific anti-HDL serum it is possible to demonstrate by immunodiffusion and immunoelectrophoresis (protein staining) that small amounts of HDL are still present; the residual HDL cannot be eliminated by increasing the dextran sulfate concentration to 1.0%. Immunoelectrophoresis of supernatant B against anti-whole human serum reveals all the precipitin bands of normal human serum except the lipoproteins.

[1] In the undialyzed supernatant there is a spontaneous precipitation of manganese oxide.



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TABLE 1 COMPOSITION OF LIPOPROTEINS

| | Protein | Lipid | Cholesterol/Lipid | Phospholipid/Lipid | Cholesterol/Phospholipid |
|---|---|---|---|---|---|
| | | | % | | |
| VLDL plus LDL | 17.5–20 | 80–82.5 | 0.35–0.38 | 0.24–0.26 | 1.4 –1.5 |
| LDL | 19–21.5 | 78.5–81 | 0.37–0.39 | 0.24–0.26 | 1.45–1.55 |
| HDL | 44–47 | 53–56 | — | — | 0.57–0.61 |

TABLE 2 LIPOPROTEINS PRECIPITATED BY VARIOUS SULFATED POLYSACCHARIDES AND DIVALENT CATIONS

| | Sulfated Polysaccharide | | |
|---|---|---|---|
| Cation | Heparin | Dextran Sulfate | Dextran Sulfate 2000 |
| $Mg^{++}$, $Ca^{++}$ | Chylomicrons<br>VLDL | Chylomicrons<br>VLDL<br>LDL | Chylomicrons<br>VLDL<br>LDL<br>HDL |
| $Mn^{++}$ | Chylomicrons<br>VLDL<br>LDL | Chylomicrons<br>VLDL<br>LDL<br>HDL | |

TABLE 3 FINAL CONCENTRATIONS OF REAGENTS REQUIRED FOR THE PRECIPITATION OF LIPOPROTEINS

| Reagents | Chylomicrons plus VLDL | LDL | HDL |
|---|---|---|---|
| Heparin<br>$Mn^{++}$ | 0.01%<br>0.05 M | 0.1%<br>0.05 M | no ppt |
| Dextran sulfate<br>$Mg^{++}$ ($Ca^{++}$) | 0.01%<br>0.1 M | 0.1%<br>0.1 M | no ppt |
| Dextran sulfate<br>$Mn^{++}$ | | 0.05%<br>0.05 M | 0.65%<br>0.2 M |
| Dextran sulfate 2000<br>$Ca^{++}$ | | 0.05%<br>0.1 M | 0.55%<br>0.2 M |
| NaPhT<br>$Mg^{++}$ | 0.05%<br>0.1 M | 0.2%<br>0.1 M | 2.0%<br>0.2 M |
| NaPhT<br>$Mn^{++}$ | | 0.08%<br>0.05 M | 0.6%<br>0.2 M |

To precipitate the HDL from the serum devoid of both LDL and VLDL (supernatant A), it is necessary to increase the concentrations of both reagents; increasing either dextran sulfate or $MnCl_2$ yields no precipitation. Intermediate concentrations produce only partial precipitation (approximately 20% with dextran sulfate 0.1%, and $MnCl_2$ 0.1 M). Increasing the ionic strength by addition of NaCl (1.0 g/100 ml) to the serum free of LDL and VLDL inhibits the precipitation of HDL.

Unlike VLDL and LDL (3), HDL are not precipitated when $Ca^{++}$ or $Mg^{++}$ is used instead of $Mn^{++}$. However, with dextran sulfate of a higher molecular weight (dextran sulfate 2000), almost complete precipitation of HDL is achieved in the presence of $Ca^{++}$; the precipitation is only partial with $Mg^{++}$. Higher concentrations of reagents are required for the precipitation of HDL. Thus VLDL plus LDL and HDL can be precipitated separately be stepwise addition of reagents. After precipitation of VLDL plus LDL by dextran sulfate 2000 (0.05%) and $CaCl_2$ (0.1 M), and removal of the precipitate by centrifugation, HDL are precipitated by adding to the supernatant more dextran sulfate 2000 and $CaCl_2$ (final concentrations: dextran sulfate 2000 0.55%, $CaCl_2$ 0.2 M). The precipitated HDL are soluble in 0.5 M potassium oxalate (20 ml is used for the HDL obtained from 500 ml of serum). The HDL can then be purified by ultracentrifugation as described above.

### *Method IV: Isolation of HDL by Precipitation with Sodium Phosphotungstate and $MgCl_2$*

To 500 ml of serum are added 50 ml of 4% NaPhT and 12.5 ml of 2 M $MgCl_2$ (final concentrations: NaPhT

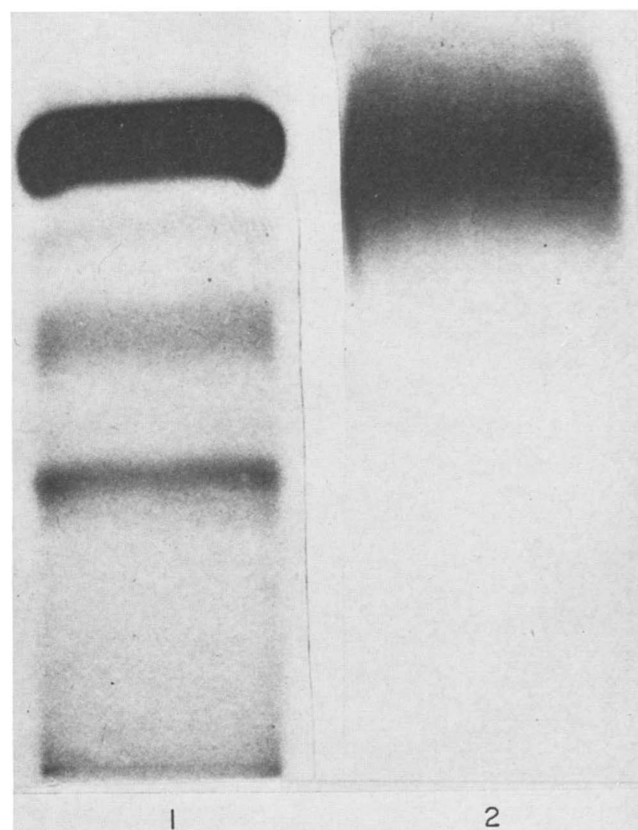


FIG. 6. Electrophoresis on cellulose acetate; protein stain. *1*, normal human serum; *2*, isolated HDL (protein, 2 g/100 ml of solution).



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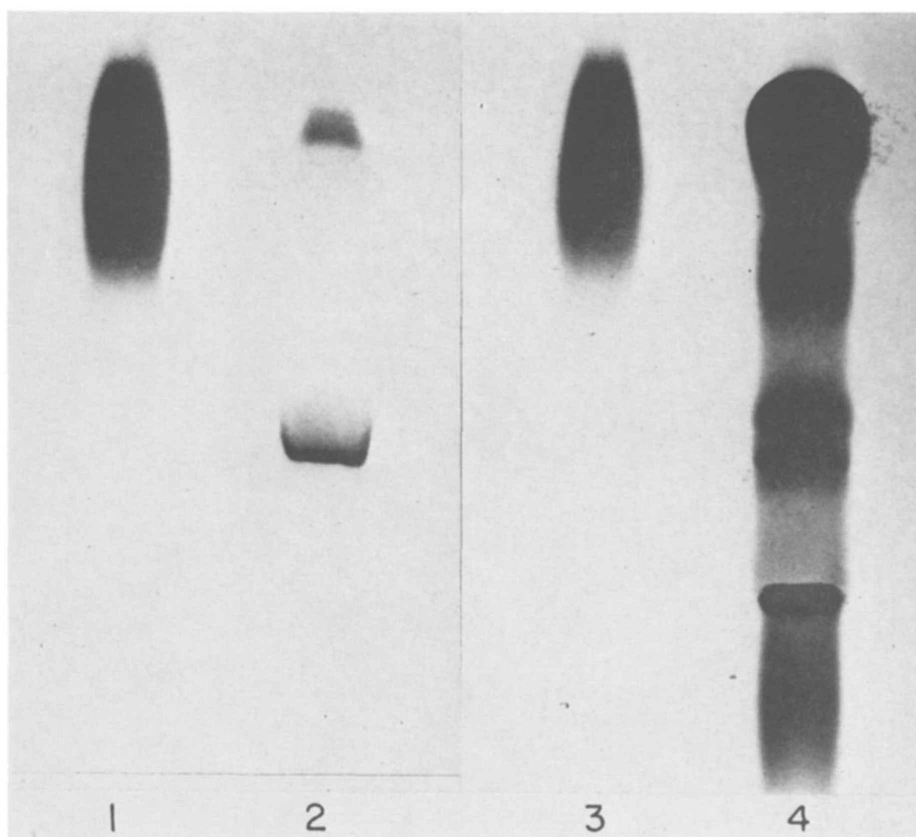


FIG. 7. Electrophoresis on agarose gel. *1* and *3*, isolated HDL (protein, 2 g/100 ml of solution); *2* and *4*, normal human serum; *1* and *2*, lipid stain; *3* and *4*, protein stain (Amido Schwartz).

0.4%, $MgCl_2$ 0.05 M). There is an immediate, complete, and selective precipitation of both LDL and VLDL. This precipitate is removed by centrifugation (10 min, 6000 *g*).[2] 450 ml of 4% NaPhT is added to the clear supernatant I (final concentrations: NaPhT 2%, $MgCl_2$ 0.025 M). The precipitate, which appears immediately, is free of lipid and contains mostly γ-globulin; it is removed by centrifugation (10 min, 6000 *g*). To supernatant II is added 87.5 ml of 2 M $MgCl_2$ (final concentrations: NaPhT, 2%, $MgCl_2$ 0.2 M). The precipitation is complete after 2 hr, and the mixture is centrifuged for 30 min at 20,000 *g*. The clear supernatant III (pH 7.1) is decanted, and the precipitate is washed in 250 ml of a solution of the following composition: 1% NaCl, 0.4% NaPhT, and 0.1 M $MgCl_2$. The washed precipitate is recovered by centrifugation (10 min, 6000 *g*) and is suspended in 40 ml of 1% NaCl. 10% sodium carbonate is added dropwise with stirring on a magnetic stirrer until redissolution is achieved; approximately 10 ml is required. This concentrated neutral solution of HDL contaminated by small amounts of serum protein is further purified by ultracentrifugation in the same manner as in the method with dextran sulfate and $MgCl_2$.

Prior to further analysis, supernatant III is dialyzed, first against 10% NaCl which facilitates the removal of NaPhT and then against Tris–HCl–NaCl buffer. The serum is concentrated to its original protein content by dialysis against PVP. As in the case of supernatant B of method III (dextran sulfate–$MnCl_2$), supernatant III contains about 30 mg of phospholipid per 100 ml and only traces of cholesterol. The results of immunochemical studies are similar.

The precipitation of HDL can be achieved even with half the concentration of $MgCl_2$ used above (that is 0.1 M), but only after the solution stands for 24 hr; with 0.05 M $MgCl_2$ partial (10–15%) precipitation is obtained. Unlike the precipitation with dextran sulfate, the precipitation of HDL and VLDL plus LDL with NaPhT is not affected by raising the ionic strength to 5% NaCl with NaCl (5 g/100 ml).

By using $MnCl_2$ instead of $MgCl_2$ (0.05 M for LDL, 0.2 M for HDL) the precipitation of the lipoproteins can be obtained with a lower final concentration of NaPhT (0.08% for LDL plus VLDL, 0.6% for HDL). However, these precipitates are poorly soluble, and for this reason $MgCl_2$ is preferable.

[2] Pure lipoproteins can be prepared from this precipitate without ultracentrifugation by a method previously described (23).



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## RESULTS

### *LDL and VLDL*

Zonal electrophoresis of a concentrated solution of the isolated LDL plus VLDL on paper (Fig. 1), cellulose acetate (Fig. 2), and agarose gel (Fig. 3) revealed a single band in the $\beta$ region by both protein and lipid staining. After immunoelectrophoresis of this preparation against anti-whole human serum, only one precipitation arc was demonstrated (Fig. 4). Likewise, no protein impurities could be shown by using the Oudin or Ouchterlony methods. Anti-whole human serum adsorbed by LDL plus VLDL showed on immunoelectrophoresis all the precipitation arcs except that of $\beta$-lipoprotein (LDL plus VLDL).

The chemical analysis of LDL plus VLDL is shown in Table 1. The preparation is practically free of heparin, and there is no precipitation after adding $MgCl_2$ when the ionic strength is very low. There is no metachromatic staining with toluidine blue. The chemical analysis of the LDL separated by ultracentrifugation at d 1.006 from the LDL plus VLDL preparation, is also shown in Table 1. The concentrated solution of LDL (15% and greater) in Tris–HCl–NaCl buffer can be kept at −10°C for several months without denaturation.[3] The amount of LDL isolated by this method is about 0.40 g/100 ml of serum.

### *HDL*

Electrophoresis of the concentrated HDL on paper (Fig. 5), cellulose acetate (Fig. 6), agarose gel (Fig. 7), and starch gel (Fig. 8) revealed only one component with the mobility corresponding to $\alpha$-lipoprotein. One precipitin line in the $\alpha$ region was found after immunoelectrophoresis of the HDL preparation against anti-whole human serum (Fig. 9). The absence of protein impurities was confirmed by Oudin and Ouchterlony methods. Adsorption of anti-whole human serum by the isolated HDL caused the disappearance only of the antibodies against $\alpha$-lipoprotein as demonstrated by immunoelectrophoresis.

On immunoelectrophoresis against human serum, the antiserum prepared in rabbits against this isolated lipoprotein, showed only one precipitation line; there was also only one precipitin line against the isolated HDL (Fig. 10). Ultracentrifugal analysis (Fig. 11) demonstrated the presence of one sharp peak. The sedimentation constant of this single component was 4.9–5.0 Svedberg units ($s_{20,w}$). The chemical analysis of the HDL is in Table 1. The ratio of protein:cholesterol was 3.1–3.4, and the ratio of protein:phospholipid was 1.8–2.0.

[3] In the absence of NaCl, LDL become insoluble after freezing at −10°C.

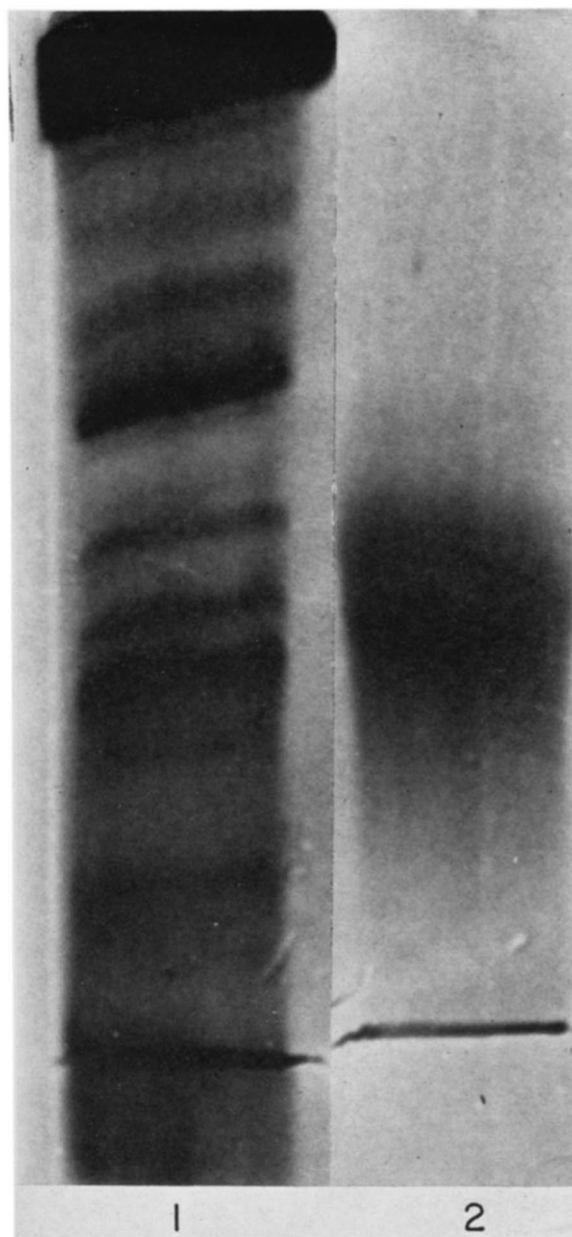


FIG. 8. Starch gel electrophoresis; protein stain. *1*, normal human serum; *2*, isolated HDL (protein, 2 g/100 ml of solution).

The HDL are practically free of polyanions and are not precipitated by addition of $MnCl_2$ even at low ionic strength. There is no metachromatic staining with toluidine blue. The HDL can be kept for up to 1 yr at −10°C. The amount of HDL isolated by this method is about 0.25 g/100 ml of serum.

## DISCUSSION

The four major classes of serum lipoproteins (chylomicrons, VLDL, LDL, and HDL) are generally separated by ultracentrifugation at appropriate densities (20,



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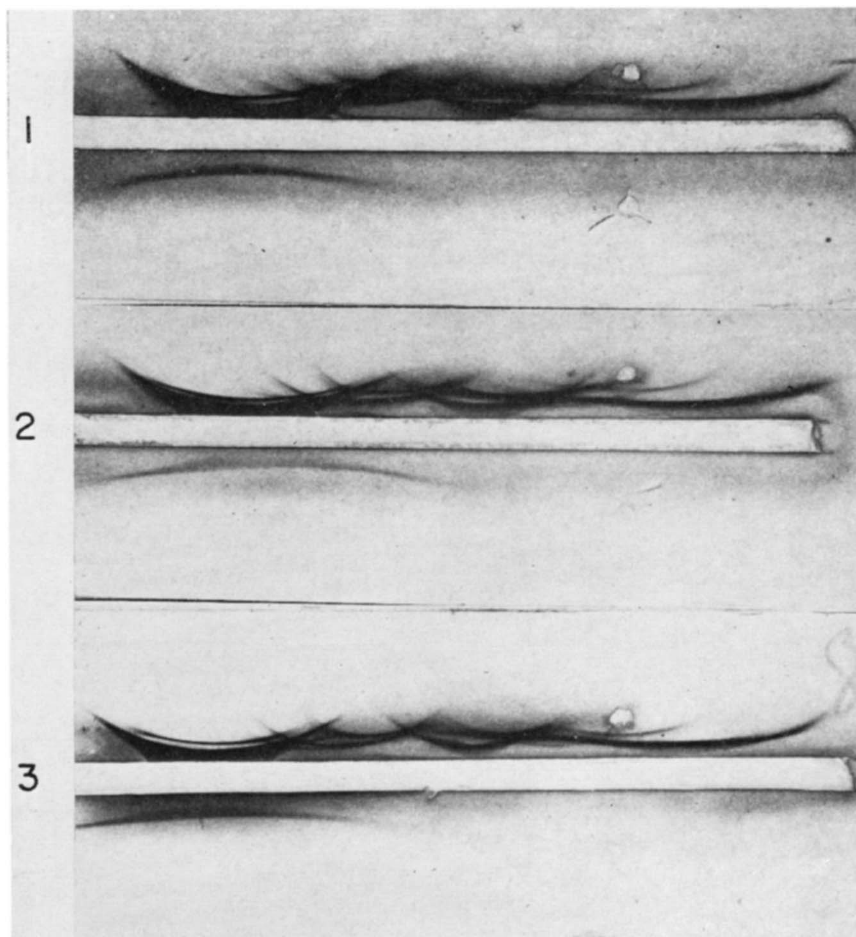


FIG. 9. Immunoelectrophoresis; protein stain. Upper wells, normal human serum; lower wells, three different preparations of isolated HDL (protein, 2 g/100 ml of solution). Troughs: rabbit anti-whole human serum.

24–29). The methods discussed in this report for the isolation of LDL plus VLDL and HDL depend not upon density but rather upon the precipitability of lipoproteins by polyanions in the presence of divalent cations. It is shown not only that low density but also that high density lipoproteins can be isolated by precipitation with certain polyanions and divalent cations. The polyanions are either sulfated polysaccharides ($SO_3^-$) or NaPhT ($WO_3^-$).[4]

The precipitation of lipoproteins at neutral pH depends upon several factors: (*a*) the nature of the polyanion and cation used, (*b*) the concentration of reagents, (*c*) ionic strength, and (*d*) the presence of other serum proteins. Varying these factors enables one to precipitate the classes of lipoproteins separately. Table 2 illustrates the effect of different combinations of sulfated polysaccharides and cations on the precipitation of the lipoproteins.[5] The final concentrations of reagents necessary for the precipitation of the lipoproteins with different combinations of polyanion and cation are shown in Table 3. Noteworthy is the fact that with the same polyanion and cation, the reagent requirements for the precipitation of the major classes of lipoproteins differ widely. It has been shown above that it is possible to isolate LDL plus VLDL and HDL by the stepwise addition of the same reagents. In a similar way, chylomicrons plus VLDL can be separated from LDL in a

[4] In the presence of $Ca^{++}$, the precipitation of low density lipoproteins can also be achieved by polysulfates other than sulfated polysaccharides, such as neoarsphenamine or sodium polyanetholsulfonate (3), and by a long-chain inorganic polyphosphate ($PO_3^-$) in neutral solution (30).

[5] Chylomicrons plus VLDL may be isolated from lipemic serum by precipitation with heparin and $Mg^{++}$ (final concentrations: heparin, 0.25%; $MgCl_2$, 0.1 M). The chylomicrons are subsequently separated from VLDL by centrifugation at 40,000 *g* for 2 hr (31). The chylomicrons isolated in this way have a protein: lipid ratio of 0.018–0.03 and remain at the origin on cellulose acetate electrophoresis. VLDL have a protein:lipid ratio of 0.05–0.06 and on cellulose acetate migrate in the $\alpha$-2 globulin region (31). In addition, chylomicrons can be selectively precipitated with heparin–$Mg^{++}$, either by increasing the ionic strength or by lowering the heparin concentration to 0.05%.



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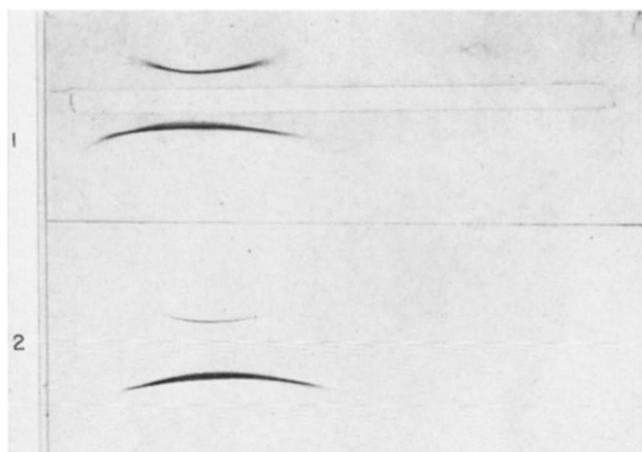

FIG. 10. Immunoelectrophoresis. Upper wells, normal human serum; lower wells, isolated HDL (protein, 2 g/100 ml of solution). Troughs: rabbit antiserum prepared against isolated HDL. Slide *1*, protein stain; slide *2*, lipid stain.

lipemic serum (alimentary or carbohydrate-induced lipemia). In fact, the precipitation of chylomicrons plus VLDL requires a lower concentration of polyanions than does the precipitation of LDL. This is demonstrated for heparin–$Mn^{++}$, dextran sulfate–$Mg^{++}$, and NaPhT–$Mg^{++}$ (Table 3). It can be seen that there is a parallel between the polyanion precipitation method and the ultracentrifugal method of isolation of the serum lipoproteins; in the former the concentration of reagents is increased stepwise, but in the latter it is the solvent density which is increased.

The increase of ionic strength by the addition of a sufficient amount of NaCl prevents the precipitation of lipoproteins by sulfated polysaccharides but not by NaPhT. On the other hand, decreasing the ionic strength by dialysis of the serum against Tris–HCl buffer pH 7.7, facilitates the precipitation by sulfated polysaccharides. Table 4 shows that only after dialysis can the precipitation of LDL be achieved by heparin–$Mg^{++}$, and the precipitation of HDL by heparin–$Mn^{++}$ (partial) or Mepesulfate–$Mn^{++}$. In addition, in a dialyzed serum the precipitation of LDL is obtained with a lower concentration of polyanions (dextran sulfate–$Mg^{++}$, Mepesulfate–$Mg^{++}$) than in native serum.

In the absence of other serum proteins, the precipitation of the lipoproteins by polyanions is facilitated. The minimal reagent requirement sufficient to preicipitate pure LDL increases when serum free of LDL is added. The reagent requirement is very low in the case of pure LDL in distilled water due to the low ionic strength and the absence of other serum proteins.

In a serum dialyzed against distilled water, only traces of HDL are precipitated by dextran sulfate–$Ca^{++}$ (Table 4); by simultaneously lowering the ionic strength and the protein content (serum diluted 10-fold with distilled water), more HDL are precipitated. Likewise, pure HDL in Tris–HCl buffer, pH 7.7, are precipitated by dextran sulfate–$Mg^{++}$ (but not by heparin–$Mg^{++}$); the addition of lipoprotein-free serum dialyzed against distilled water prevents this precipitation.

TABLE 4 FINAL CONCENTRATIONS OF REAGENTS REQUIRED FOR THE PRECIPITATION OF LDL AND HDL BEFORE AND AFTER DIALYSIS AGAINST TRIS–HCl 0.02 M, pH 7.7

| | LDL | | HDL | |
|---|---|---|---|---|
| Reagents | Normal Serum | Dialyzed Serum | Normal Serum | Dialyzed Serum |
| Heparin<br>$Mg^{++}$ ($Ca^{++}$) | no ppt | 0.2%<br>0.05 M | no ppt | no ppt |
| Heparin<br>$Mn^{++}$ | 0.1%<br>0.05 M | | no ppt | 0.5%*<br>0.1 M |
| Dextran sulfate<br>$Mg^{++}$ ($Ca^{++}$) | 0.1%<br>0.1 M | 0.05%<br>0.05 M | no ppt | 0.5%†<br>0.1 M |
| Mepesulfate<br>$Mg^{++}$ ($Ca^{++}$) | 0.4%<br>0.1 M | 0.1%<br>0.05 M | no ppt | no ppt |
| Mepesulfate<br>$Mn^{++}$ | 0.1%<br>0.05 M | | 0.5%*<br>0.2 M | 0.5%<br>0.1 M |

* Partial precipitation.
† Trades of HDL precipitated.

TABLE 5 FINAL CONCENTRATIONS OF POLYANIONS REQUIRED FOR THE PRECIPITATION OF LDL IN THE PRESENCE OF 0.1 M $MgCl_2$

| Polyanion | Without Sucrose | With Sucrose* |
|---|---|---|
| Heparin | no ppt† | 0.025% |
| Dextran sulfate | 0.1% | 0.007% |
| Mepesulfate | 0.4% | 0.015% |
| NaPhT | 0.2% | 0.05% |

* 1 g/ml of serum.
† Only chylomicrons (lipemic serum) and VLDL are precipitated.

After addition of sucrose (1 g/ml of serum) not only chylomicrons plus VLDL, but also LDL are precipitated by heparin–$Mg^{++}$. At the same time sucrose considerably reduces the minimal final concentrations of reagents required for the precipitation of LDL, especially in the case of sulfated polysaccharides (Table 5). Sucrose also facilitates the precipitation of HDL; after adding sucrose to the serum there is a partial precipitation of HDL by dextran sulfate–$Mg^{++}$ (32). Sucrose has the same effect on the precipitation of LDL and HDL as the simultaneous lowering of the ionic strength and the protein content.

The conditions of precipitation of HDL from the serum can be summarized as follows: HDL are precipitated by dextran sulfate–$Mn^{++}$, dextran sulfate 2000–$Ca^{++}$, and NaPhT–$Mg^{++}$; a partial precipitation can be obtained by dextran sulfate–$Mg^{++}$ after addition



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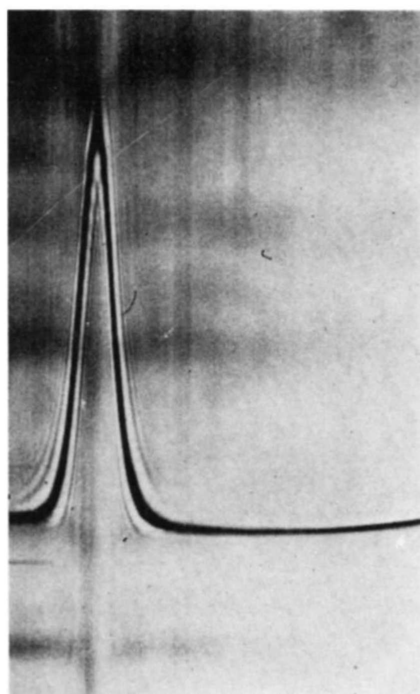

FIG. 11. Analytical ultracentrifugation. Isolated HDL (protein, 0.5 g/100 ml of solution). Solvent: 1% NaCl in 0.02 M Tris–HCl buffer pH 7.70. Photo taken after 34 min at 59,780 rpm.

of sucrose to the serum or by Mepesulfate–$Mn^{++}$. In a serum dialyzed against distilled water, partial precipitation is achieved by heparin–$Mn^{++}$, and total precipitation by Mepesulfate–$Mn^{++}$.

The precipitation of the lipoproteins by heparin depends upon the divalent cation used, the ionic strength, and the presence of sucrose. With heparin, chylomicrons plus VLDL are selectively precipitated by $Mg^{++}$; LDL are precipitated by $Mn^{++}$ and also by $Mg^{++}$, but only after the addition of sucrose or lowering the ionic strength of the serum. HDL are partially precipitated by heparin–$Mn^{++}$ at low ionic strength. Dextran sulfate is more active than heparin (that is, precipitation of LDL by $Mg^{++}$ and HDL by $Mn^{++}$ without lowering the ionic strength), and dextran sulfate 2000 is more active than dextran sulfate (that is, precipitation of HDL not only with $Mn^{++}$ but also with $Ca^{++}$). $Mn^{++}$ is more active than $Ca^{++}$ or $Mg^{++}$ (Tables 2–4).

In the presence of divalent cations and at neutral pH, chylomicrons, VLDL, LDL, and HDL form an insoluble complex: lipoprotein–polyanion–cation;[6] the formation of this complex is more easily achieved with chylomicrons than with LDL and in turn more easily with LDL than with HDL (that is, precipitation with a lower concentration of reagents, and in a medium with a higher ionic strength). The protein:lipid ratio decreases from HDL to chylomicrons; the insoluble complex forms more readily when this ratio is low.

The nature of the lipid moiety is unimportant. In fact, abnormal serum lipoproteins of jaundiced patients (38) are precipitated by heparin and divalent ca ions under the same conditions as VLDL (39). The lipid moieties in these lipoproteins are quite different: the abnormal lipoprotein is high in phospholipid and very low in triglycerides (40), and the VLDL are very rich in triglycerides. However, in both cases the protein:lipid ratio is low. Thus, the ease of precipitation depends not upon the lipid moiety per se but rather upon the protein:lipid ratio.

After delipidation of HDL by alcohol–ether, the apoprotein is not precipitated by either dextran sulfate–$Mn^{++}$ or NaPhT–$Mg^{++}$; there is no precipitation when this protein moiety is added to supernatants B or III.[7] One possible explanation is that the polyanion combines not with the protein moiety but rather with the phospholipids (41); another is that the polyanions do combine with the protein moiety to form a soluble complex, but in the absence of lipids the latter is not precipitated by divalent cations.

It is well known that the proteins in the d > 1.21 fraction obtained after removing the lipoproteins by ultracentrifugation, contain a significant amount of phospholipids (20, 25, 42–46) composed mostly of lysolecithin (42) bound to albumin (43). The presence of a small amount of HDL in this fraction has been shown by immunological methods (44–46). As in the case of the d > 1.21 fraction, supernatant B (dextran sulfate–$Mn^{++}$) and supernatant III (NaPhT–$Mg^{++}$) contain phospholipids (0.03%) and react on immunodiffusion with a specific anti-HDL serum.[8]

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[6] At neutral pH and in the absence of divalent cations, sulfated polysaccharides of high mol wt (1,000,000 or more), like dextran sulfate (33, 34), or sulfated amylopectin (35, 36), precipitate LDL plus VLDL. In the absence of divalent cations, low molecular weight polyanions (heparin, dextran sulfate, NaPhT) can also precipitate LDL plus VLDL but only when the pH is lowered (37).

[7] Burstein, M., H. R. Scholnick, and R. Morfin. Unpublished results.

[8] After a one-step precipitation of VLDL plus LDL and HDL by chlortetracycline in the presence of $Ca^{++}$ (47), or by an anionic or cationic detergent and a polyanion of opposite charge (48), the supernatant contains less phospholipids (0.015–0.02%) and no immunologically detectable HDL.



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### References

1. Cornwell, D. G., and F. A. Kruger. 1961. *J. Lipid Res.* **2:** 110.
2. Burstein, M., and J. Samaille. 1960. *Clin. Chim. Acta* **5:** 609.
3. Burstein, M., and J. Samaille. 1957. *J. Physiol.* (*Paris*). **49:** 83.
4. Burstein, M. 1962. *C. R. Acad. Sci.* **255:** 605.
5. Burstein, M., and R. Morfin. 1969. *Nouv. Rev. Fr. Hematol.* **9:** 231.
6. Burstein, M., and R. Morfin. 1969. *Life Sci.* **8:** 345.
7. Burstein, M. 1964. *Vox Sang.* **9:** 22.
8. Burstein, M., and H. R. Scholnick. 1970. *Nouv. Rev. Fr. Hematol.* **10:** 181.
9. Grassmann, W., and K. Hannig. 1959. *Klin. Wochenschr.* **32:** 838.
10. Burstein, M., P. Amouch, and J. M. Fine. 1968. *Nouv. Rev. Fr. Hematol.* **8:** 809.
11. Kohn, J. 1957. *Clin. Chim. Acta.* **2:** 297.
12. Smithies, O. 1955. *Biochem. J.* **61:** 629.
13. Poulik, M. D. 1957. *Nature* (*London*). **180:** 1477.
14. Swahn, B. 1953. *Scand. J. Clin. Lab. Invest. Suppl.* **5:** 9.
15. Scheidegger, J. J. 1955. *Int. Arch. Allergy Appl. Immunol.* **7:** 103.
16. Oudin, J. 1955. *Ann. Inst. Pasteur Paris.* **89:** 531.
17. Ouchterlony, O. 1962. *Progr. Allergy.* **6:** 30.
18. Delsal, J.-L. 1944. *Bull. Soc. Chim. Biol.* **26:** 99.
19. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. *J. Biol. Chem.* **177:** 751.
20. Havel, R. J., H. A. Eder, and J. M. Bragdon. 1955. *J. Clin. Invest.* **34:** 1345.
21. Zilversmit, D. B., and A. K. Davis. 1950. *J. Lab. Clin. Med.* **35:** 155.
22. Pearson, S., S. Stern, and T. McGarack. 1953. *Ann. Chem.* **25:** 813.
23. Burstein, M. 1963. *Nouv. Rev. Fr. Hematol.* **3:** 139.
24. DeLalla, O. F., and J. W. Gofman. 1954. *Methods Biochem. Anal.* **1:** 459.
25. Hillyard, L. A., C. Entenman, M. Feinberg, and J. L. Chaikoff. 1955. *J. Biol. Chem.* **214:** 79.
26. Bragdon, J. H., R. J. Havel, and E. Boyle. 1956. *J. Lab. Clin. Med.* **48:** 36.
27. Oncley, J. L., and S. E. Allerton. 1961. *Vox Sang.* **6:** 201.
28. Ayrault-Jarrier, M., G. Levy, R. Wald, and J. Polonovski. 1963. *Bull. Soc. Chim. Biol.* **45:** 349.
29. Ewing, A. M., N. K. Freeman, and F. T. Lindgren. 1965. *Advan. Lipid Res.* **3:** 25.
30. Burstein, M., and R. Morfin. 1968. *Nouv. Rev. Fr. Hematol.* **8:** 65.
31. Burstein, M., and M. Gouzi. 1969. *Rev. Fr. Transfus.* **12:** 271.
32. Burstein, M. 1962. *Life Sci.* **1:** 739.
33. Oncley, J. L., K. W. Walton, and D. G. Cornwell. 1957. *J. Amer. Chem. Soc.* **79:** 4666.
34. Antoniades, H. N., J. L. Tullis, L. H. Sargeant, R. B. Pennell, and J. L. Oncley. 1958. *J. Lab. Clin. Med.* **51:** 630.
35. Bernfeld, P., V. M. Donahue, and M. E. Berkowitz. 1957. *J. Biol. Chem.* **226:** 51.
36. Bernfeld, P., C. D. Bonner, and B. J. Berkeley. 1960. *J. Clin. Invest.* **39:** 1864.
37. Burstein, M., and R. Morfin. 1969. *Nouv. Rev. Fr. Hematol.* **9:** 645.
38. Eder, H. A., E. M. Russ, R. A. Pritchett, M. M. Wilbert, and D. P. Barr. 1955. *J. Clin. Invest.* **34:** 1147.
39. Burstein, M., and J. Caroli. 1969. *Rev. Med. Chir. Mal. Foie. Rate Pancreas.* **44:** 125.
40. Switzer, S. 1967. *J. Clin. Invest.* **46:** 1855.
41. Canal, J., and M. L. Girard. 1968. *Bull. Soc. Chim. Biol.* **50:** 1523.
42. Phillips, G. B. 1959. *Proc. Soc. Exp. Biol. Med.* **100:** 19.
43. Switzer, S., and H. A. Eder. 1965. *J. Lipid Res.* **6:** 506.
44. Levy, R., and D. S. Fredrickson. 1965. *J. Clin. Invest.* **44:** 426.
45. Scanu, A., and J. L. Granda. 1966. *Biochemistry.* **5:** 446.
46. Alaupovic, P., S. S. Sanbar, R. H. Furman, M. L. Sullivan, and S. L. Walraven. 1966. *Biochemistry.* **5:** 4044.
47. Burstein, M., and R. Morfin. 1968. *Nouv. Rev. Fr. Hematol.* **8:** 45.
48. Burstein, M., and R. Morfin. 1968. *Rev. Fr. Etud. Clin. Biol.* **13:** 274.


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