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An Electrophoretic Method for the Quantitative Isolation of Human and Swine Plasma Lipoproteins†

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ABSTRACT: A procedure for the isolation and purification of human and swine plasma lipoproteins using the combination of ultracentrifugation and Geon–Pevikon block electrophoresis has been described. Normal human and swine lipoproteins isolated by this procedure were compared to lipoproteins isolated by the standard method of ultracentrifugation and were found to be essentially identical with respect to chemical composition, immunochemical reactivity, size by electron microscopy, and apoprotein content by polyacrylamide gel electrophoresis. This procedure allowed the isolation of plasma lipoproteins in a shorter period of time without subjecting the lipoproteins to repetitive ultracentrifugation and washing procedures. In addition, the plasma lipoproteins from cholesterol-fed swine, which could not be separated by ultracentrifugation alone, were purified by the Geon–Pevikon electrophoretic procedure. These swine developed a hyperlipoproteinemia characterized by the presence of two different lipoproteins which had overlapping densities in the low density fraction.

We have previously reported the use of Geon–Pevikon block electrophoresis for the isolation of canine plasma lipoproteins (Mahley *et al.*, 1973). The dog has two distinctly different lipoproteins with overlapping densities in the low density range which were easily purified by the block electrophoretic procedure. The relative ease, the rapidity of separation compared with repetitive ultracentrifugations, and the high degree of purity suggested that the Geon–Pevikon block electrophoretic procedure might be of value under certain circumstances for purification of the lipoproteins of other species. A method for the isolation of plasma lipoproteins from the human and swine is reported. In addition the method is applied to the separation and purification of lipoproteins of cholesterol-fed swine, where ultracentrifugation alone results in a mixture of lipoprotein types.

Materials and Methods

Human plasma was obtained from laboratory workers who had normal lipoprotein profiles as judged by lipid values and lipoprotein electrophoresis. Porcine plasma was obtained from miniature swine derived from the Hormel breed following an overnight fast. Control swine were on a low-fat commercial hog chow. A high-fat, high-cholesterol diet was prepared by blending 15% lard and 1% crystalline cholesterol by weight with commercial hog chow. The swine were fed this diet for 6 months to 1 year prior to lipoprotein analysis. Plasma lipid concentrations and type of atherosclerosis produced in similar animals have previously been reported from this laboratory (Flaherty *et al.*, 1972).

Ultracentrifugation. Plasma was subjected to ultracentrifugation using established procedures (Havel *et al.*, 1955). All centrifugations were performed in a Beckman 60 ti rotor at 59,000 rpm for variable periods of time. Fractions isolated at $d = 1.006$ were centrifuged for 12 hr; at $d = 1.02$, 1.04, 1.06, and 1.08 for 16–18 hr; and at $d = 1.21$ for 24 hr. The $d = 1.006$ fraction was washed in saline ($d = 1.006$) by ultracentrifugation. Following ultracentrifugation the fractions were dialyzed against 0.15 M NaCl–0.01% EDTA and concentrated using the Amicon Diaflo apparatus.

Immunochemical Analyses. Antisera were produced to purified fractions of lipoproteins as previously described (Bersot *et al.*, 1970). Immunoelectrophoresis was performed according to the method of Levy and Fredrickson (1965).

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Paper Electrophoresis. Paper electrophoresis was performed using the method of Hatch and Lees with Oil Red O staining (Hatch and Lees, 1968).

Negative Staining Electron Microscopy. Negative stains were prepared by placing a drop of the lipoprotein fraction on a grid followed by a drop of 1% potassium phosphotungstate. Excess solution was removed from the surface of the grid and the grid was dried (Hamilton *et al.*, 1967). Electron micrographs of random areas were taken with an AEI electron microscope.

Chemical Determinations. Protein was determined by the method of Lowry *et al.* (1951) using a bovine albumin standard. Lipid analyses included triglyceride (Fletcher, 1968), total cholesterol (Abell *et al.*, 1952), and phospholipid (Zilversmit and Davis, 1950).

Apoprotein Analysis. Analysis of the apoproteins of the purified lipoproteins by polyacrylamide gel electrophoresis was performed following delipidation and solubilization as described previously (Brown *et al.*, 1969). Approximately 50 μg of protein was applied to each gel. Gels were composed of 8% acrylamide in a Tris buffer at pH 8.9 in 8 M urea (Reisfeld and Small, 1966).

Geon–Pevikon Block Electrophoresis. Geon–Pevikon block electrophoresis was based on the method of Barth *et al.* (1964). The apparatus was composed of two buffer chambers with three baffles and a platinum electrode. The volume of each chamber was approximately 4 l. A plexiglass tray supporting the Geon–Pevikon media was placed between the buffer chambers and measured 29 cm wide × 38 cm long × 1.5 cm deep.[1] A power supply to deliver a constant current of 50 mA was required. Other apparatus included a coarse sintered-glass filter (125 mm) mounted on a 4-l. vacuum flask, several smaller coarse sintered-glass filters (45 mm), and sidearm test tubes (200 × 25 mm).

Reagents included approximately 8 l. of barbital buffer (pH 8.6, ionic strength 0.06). Lipoproteins used for markers were prestained with Sudan Black B. The stock solution of Sudan Black B, which could be stored for several months, was a 1% solution in diethylene glycol prepared by heating the stain to 110° and filtering. Prior to each experiment the stock solution was diluted 0.05 part to 1 part of distilled H_2O. Diluted stain (1 ml) mixed with 0.1 ml of Tween 20 (1:100) was used for prestaining lipoproteins.

Several different types of Geon[2] and Pevikon[3] were used and a specific type was found to vary from batch to batch. However, with only minor changes in the ratio of the Geon to Pevikon satisfactory results have been obtained with Geon Type 427 or Geon Type 101 and with Pevikon C870. In some cases Pevikon C870 alone gave suitable results, but in experiments reported in this paper equal parts of Geon and Pevikon were used.

The Geon and Pevikon were washed prior to use and 300 g of each were combined in a 2-l. beaker with 900 ml of distilled H_2O and stirred to a slurry. After allowing the slurry to stand 10 min, the fines were aspirated. The slurry was transferred to a large sintered-glass filter on a vacuum flask and washed two more times. After the last H_2O wash the mixture was almost dried by suction and then resuspended in the filter with 800 ml of barbital buffer. After this was sucked almost dry the stem of the filter was covered with Parafilm to stop draining and 500 ml of barbital buffer was added. The slurry was resuspended in the filter and poured into a 2-l. beaker. The plexiglass tray was lined with Parafilm and four wicks were placed across each end of the tray. The wicks (Telfa surgical pads, Kendall Surgical Supplies, 8 × 3 in.) soaked in barbital buffer were applied to the bottom of the tray and extended in about 1 in. from the end of the tray.

The Geon–Pevikon slurry was poured carefully into the tray. Care was taken to keep the wicks submerged in the medium. Excess buffer was allowed to drain from the medium through the wicks into towels. The time required for adequate drying of the medium depended upon the consistency of the Geon–Pevikon but was usually of the order of 20 min. Enough buffer was removed through the wicks so that when a slit was made in the medium with a spatula the walls of the slit did not collapse. Care was taken to avoid overdrying the block. Once the medium was of the correct consistency, the wicks were rolled up and placed on top of the Geon–Pevikon block to prevent further drying of the block while applying the sample.

A slit in the Geon–Pevikon, made with a flat spatula 7 cm from one end of the tray, extended to within about 2 mm of the bottom of the support medium. The sample was taken up in a syringe and applied slowly into the slit through a 19-gauge needle. When the entire width of the block was used, approximately 12 ml of total volume could be applied. The slit was then closed by packing its sides together with a small spatula. Good resolution could be obtained with a load of up to 200 mg of lipoprotein protein. Equal volumes of Sudan Black B and lipoprotein (~0.5 ml; 0.5–2 mg of lipoprotein protein) were mixed and applied to a small slit at one side of the block to serve as a marker for cutting and eluting the unstained bands of lipoproteins.

The tray was transferred to a cold room (5–10°), placed between the buffer chambers each containing approximately 3 l. of buffer, and the wicks were submerged in the buffer. The block was covered with a piece of plexiglass to prevent excessive evaporation from the surface during the electrophoresis. After the block was allowed to equilibrate approximately 5 min in the cold, a constant current of 50 mA was applied for 18 hr.

At the end of the run the tray was removed and excess buffer allowed to drain through the wicks into towels (~5 min). The tray was placed on an X-ray viewing box in a dark room and the lipoproteins were observed as sharp bands (Figure 1). The area of the block which contained the desired lipoprotein was cut out with a spatula, transferred to small sintered-glass filters draining into sidearm test tubes, and eluted with 30–40 ml of saline. The washing process could be hastened by applying a small amount of suction. The eluates were transferred to conical test tubes and centrifuged at 2000*g* for 20 min to remove small amounts of Geon–Pevikon particles from solution. Fractions were concentrated to desired volumes.

Results

Human Plasma Lipoproteins. The procedure used for the isolation of the human plasma lipoproteins included an initial centrifugation at plasma density (d = 1.006) to obtain VLDL.[4] The 1.006 infranatant was raised to d = 1.21 with solid KBr and centrifuged 36 hr at 59,000 rpm in a 60 ti rotor. Following an overnight dialysis of the 1.006–1.21 fraction against 8 l. of 0.15 M NaCl–0.01% EDTA, the fraction was subjected to Geon–Pevikon block electrophoresis as described. The sep-

[1] A diagram of the apparatus is available upon request.

[2] Geon was obtained from Goodrich Chemical Co., Cleveland, Ohio.

[3] Pevikon was obtained from Mercer Consolidated Corp., Yonkers, N. Y.

[4] Abbreviations used are: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

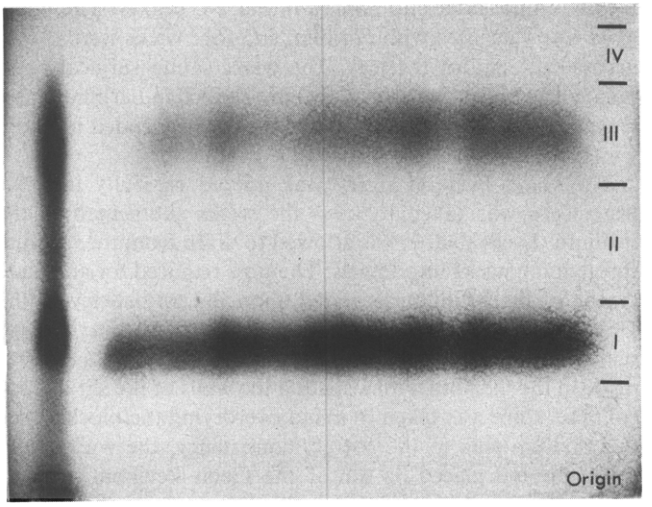

FIGURE 1: Photograph of the Geon–Pevikon block showing the separation of human LDL (zone I) and HDL (zone III) from the $d = 1.006$ to 1.21 density fraction. A small aliquot prestained with Sudan Black B is on the block to the left. The main bands represent unstained lipoproteins, with the centers of the bands being approximately 6 and 12 cm from the origin for LDL and HDL, respectively.

aration of the LDL and HDL in a representative experiment is shown in Figure 1. Based on total protein applied to the block ~70% was recovered in zones I (LDL) and III (HDL). Approximately 10% of the total protein applied to the block was isolated in zone IV which reacted strongly with anti-human albumin. No lipoproteins were visualized on paper electrophoresis of zone IV. Zone II accounted for about 1.5%. The material in this zone was a lipoprotein having α_2 mobility migrating slightly faster than VLDL (Figure 2) and is presently being further characterized.

The LDL and HDL isolated from the electrophoretic block had β and α mobility, respectively, on paper electrophoresis (Figure 2). By immunoelectrophoresis the LDL and HDL exhibited the characteristic immunoprecipitin arcs when allowed to react to anti-LDL or anti-HDL antisera. By negative staining electron microscopy the LDL ranged in size from 170 to 240 Å and the HDL from 60 to 130 Å in diameter.

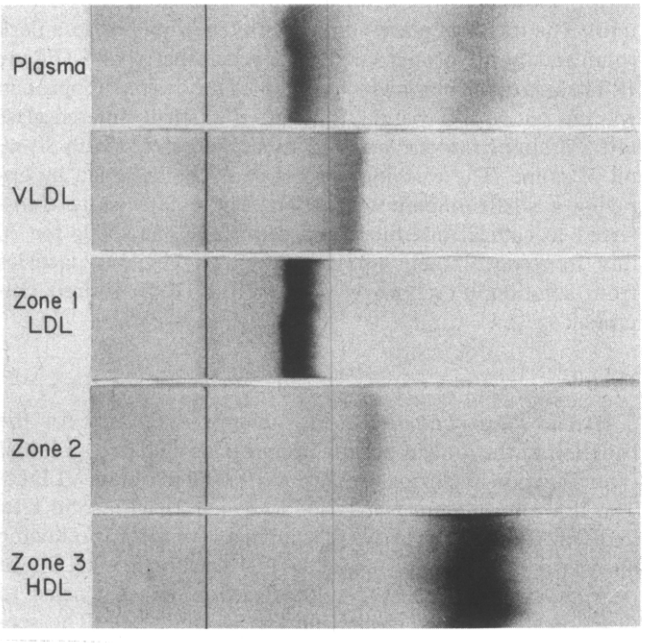

FIGURE 2: Paper electrophoretic patterns of human plasma, VLDL, and the lipoproteins isolated in the Geon–Pevikon electrophoretic procedure.

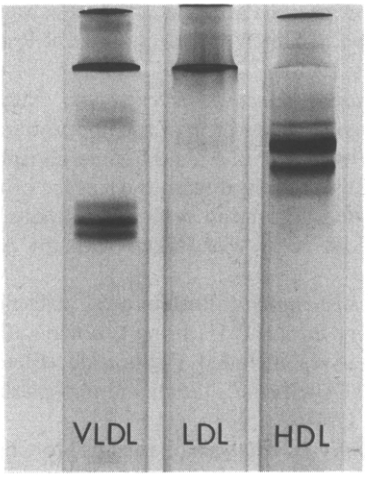

FIGURE 3: Polyacrylamide gel electrophoresis patterns of human lipoproteins. The LDL and HDL were isolated by the Geon–Pevikon electrophoretic procedure.

The chemical composition of human VLDL, LDL, and HDL isolated as described is shown in Table I. The apoprotein pattern by polyacrylamide gel electrophoresis is shown in Figure 3.

Swine Plasma Lipoproteins. The distribution of lipoproteins in the plasma of miniature swine was determined by sequentially centrifuging the plasma from $d = 1.006$ to $d = 1.21$. The paper electrophoretic patterns for the multiple density cuts are shown in Figure 4. Swine LDL were detected from $d = 1.02$ to approximately 1.080. In the several normal swine studied plasma LDL were not detected above $d = 1.09$. The extension of swine LDL to higher densities than normally ascribed to human LDL has been previously reported (Janado *et al.*, 1966). Swine HDL were detected as low as $d = 1.070$.

The procedure used for the isolation of lipoproteins from swine fed a commercial hog chow included an initial centrif-

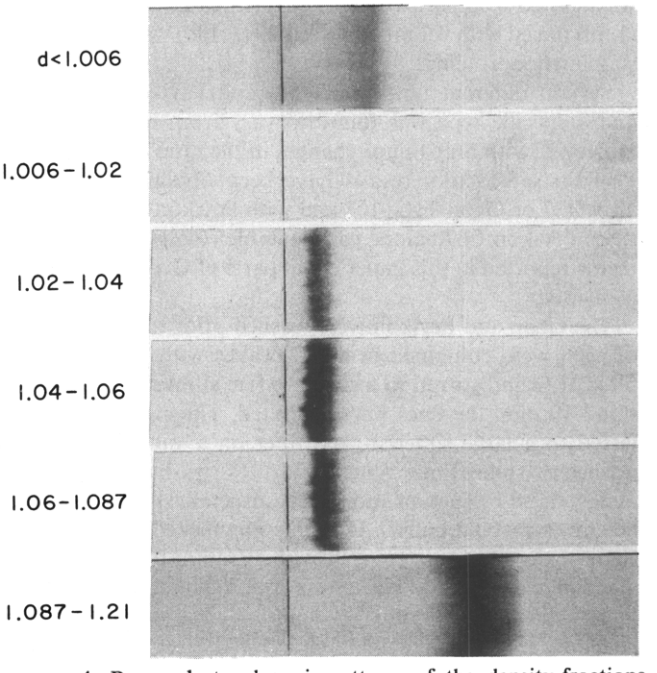

FIGURE 4: Paper electrophoresis patterns of the density fractions obtained from the plasma of control miniature swine.

TABLE I: Per Cent Composition of Human Lipoproteins.

		Tri-glyceride	Cholesterol	Phospho-lipid	Protein
VLDL	I	60.0	10.4	19.9	9.7
	II	56.6	8.6	20.7	14.1
LDL	I	6.4	48.0	23.4	25.7
	II	4.2	46.1	22.3	23.9
HDL	I	5.7	21.6	23.4	49.2
	II	3.1	21.1	29.9	45.9

ugation of the plasma at $d = 1.006$ to obtain VLDL. The 1.006 infranatant was raised to 1.21 and the fractions were isolated as described for human lipoproteins. Geon–Pevikon block electrophoresis of the 1.006–1.21 fraction resulted in excellent resolution between the LDL and HDL. LDL and HDL isolated on Geon–Pevikon block electrophoresis were compared with corresponding lipoproteins isolated by ultracentrifugation. Swine LDL were isolated from $d = 1.019$–1.060 and washed by ultracentrifugation. Swine HDL were isolated from $d = 1.09$–1.21 and washed by ultracentrifugation. The chemical compositions of LDL and HDL isolated by electrophoresis and ultracentrifugation were essentially identical, as shown in Table II. The immunochemical reactivities were also identical. Negative staining electron microscopy demonstrated that swine LDL and HDL isolated by the electrophoretic procedure or by ultracentrifugation ranged in size from 180 to 220 and 70 to 130 Å in diameter, respectively. Swine VLDL ranged in size from 270 to 750 Å in diameter. The apoprotein patterns on polyacrylamide gel electrophoresis of swine LDL and HDL isolated by the electrophoretic procedure or by ultracentrifugation were essentially identical (Figure 5).

Plasma Lipoproteins from Cholesterol-Fed Swine. Feeding the miniature swine a diet high in fat and cholesterol resulted in a marked increase in plasma cholesterol from a normal of ~90 mg/100 ml to levels ranging from 350 to 600 mg/100 ml as previously reported (Flaherty *et al.*, 1972). Analyses of the multiple density cuts obtained by ultracentrifugation revealed a marked change in the distribution of the plasma

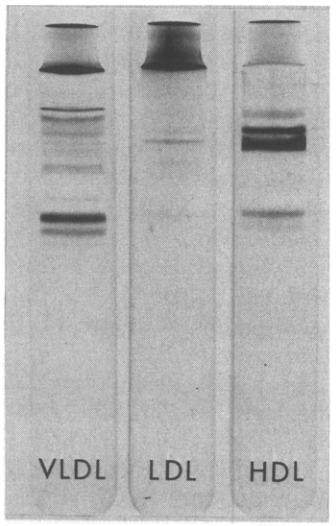

FIGURE 5: Polyacrylamide gel electrophoresis patterns of swine lipoproteins. The LDL and HDL were isolated by the Geon–Pevikon electrophoretic procedure.

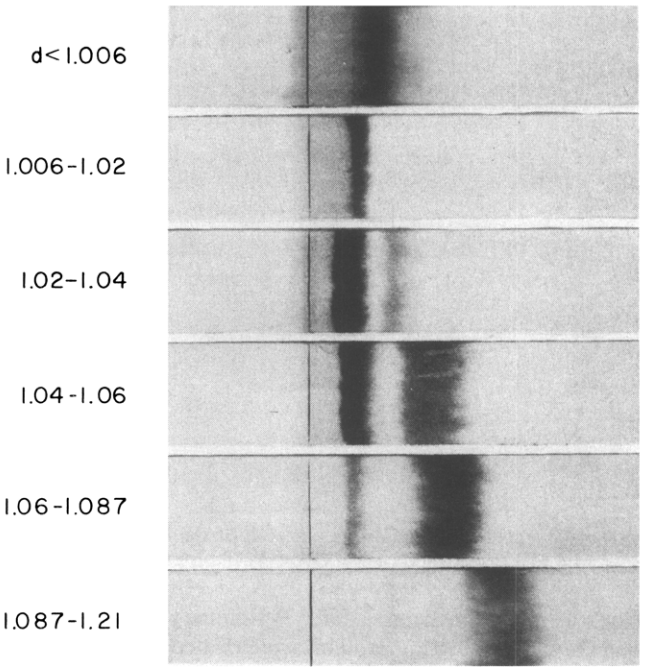

FIGURE 6: Paper electrophoresis patterns of the density fractions obtained from the plasma of cholesterol-fed miniature swine.

lipid concentrations and the types of plasma lipoproteins. As shown in Figure 6 the density range from 1.006 to 1.087 contained two differently migrating lipoproteins which could not be isolated in pure form by ultracentrifugation alone. The occurrence of an α-migrating lipoprotein in the low density fraction from the fat-fed swine is similar to the pattern obtained for the fat-fed dog (Mahley *et al.*, 1973). In the dog this α-migrating lipoprotein in the low density range has been shown to be similar to HDL and has been referred to as HDL_1. For this reason we will refer to this lipoprotein in the swine as HDL_1.

With the Geon–Pevikon block electrophoretic procedure it was possible to separate the lipoproteins in the low density range. For example, from the density fraction 1.04–1.06, the β-migrating and the α-migrating lipoproteins were isolated without cross contamination as judged by immunoelectrophoresis and paper electrophoresis (Figure 7). Complete resolution of the two lipoprotein classes in the $d = 1.02$–1.04 and $d = 1.06$–1.087 fractions was also accomplished by the

TABLE II: Per Cent Composition of Swine Lipoproteins.

		Triglyc-eride	Choles-terol	Phos-pholipid	Protein
Isolated by Ultracentrifugation					
VLDL	I	54.7	9.1	23.2	12.9
($d < 1.006$)	II	59.0	9.6	23.8	7.7
LDL	I	5.2	41.8	27.9	25.1
(1.019–1.060)	II	4.8	43.6	26.3	25.3
HDL	I	1.0	18.9	37.4	42.7
(1.090–1.21)	II	0.9	20.1	34.2	44.8
Isolated by Geon–Pevikon Procedure					
LDL	I	5.5	40.2	25.4	28.9
	II	3.5	46.4	29.3	20.7
HDL	I	0.8	22.1	33.1	43.9
	II	0.8	18.5	35.6	45.1

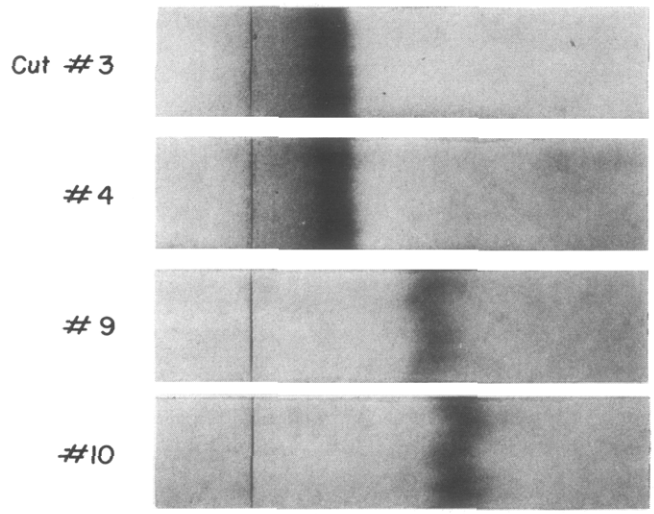

FIGURE 7: Paper electrophoresis patterns of the lipoproteins from $d = 1.04$ to 1.06 purified by the Geon–Pevikon procedure.

block electrophoretic procedure. Preliminary data suggested that the α-migrating lipoproteins were related to but not identical with the HDL of the swine.

Discussion

The Geon–Pevikon block electrophoretic method has been used by several investigators to isolate plasma proteins and is recommended because of the mildness of the procedure particularly when the proteins are to be used for metabolic studies (Barth *et al.*, 1964; Blaese *et al.*, 1971). It has not been used to our knowledge for the purification of plasma lipoproteins. This procedure based on the method of Barth *et al.* (1964) is relatively simple and can be used to prepare large quantities of pure plasma lipoproteins. In addition, it can be used when ultracentrifugation cannot separate lipoproteins because of overlapping densities, as occurs with cholesterol feeding of dogs (Mahley *et al.*, 1973) and, as reported in this paper, with cholesterol feeding of swine.

Isolation of homogeneous classes of plasma lipoproteins in most species has required the sequential raising of plasma density and long periods of ultracentrifugation including resuspension and washing steps to eliminate plasma proteins. The method reported in this paper allows the isolation of human LDL and HDL after removal of VLDL by ultracentrifugation. The LDL and HDL appear to be identical with the corresponding lipoproteins isolated by ultracentrifugation with respect to their physical and chemical properties. The compositions of the lipoproteins prepared by the Geon–Pevikon electrophoretic procedure (Table I) are very similar to values reported for human lipoproteins isolated by ultracentrifugation (Skipski, 1972; Hatch and Lees, 1968).

The human LDL and HDL isolated as described give characteristic immunoprecipitin reactions. Antisera prepared to human albumin do not react with these lipoproteins. Negative staining reveals that the LDL are about 200 Å in diameter and the HDL about 100 Å in diameter as has been previously reported (Forte *et al.*, 1968). Apoproteins on polyacrylamide gel electrophoresis demonstrate patterns for VLDL, LDL, and HDL similar to those previously described (for review, see Fredrickson *et al.*, 1972).

Studies by several investigators have suggested that swine plasma lipoproteins resemble those of man in several respects (Janado *et al.*, 1966; Jackson *et al.*, 1973; Fidge, 1973). However, it has been pointed out that the density ranges established for human lipoproteins do not apply to the swine (Janado *et al.*, 1966). We have investigated the distribution of plasma lipoproteins in the miniature swine and confirmed these results. The classes of swine lipoproteins isolated by ultracentrifugation have been compared with the lipoproteins isolated by Geon–Pevikon block electrophoresis and appear essentially identical. In addition, swine VLDL, LDL, and HDL were found to be comparable to the corresponding human lipoproteins with respect to their physical and chemical properties.

The distribution and type of plasma lipoproteins in the swine are markedly altered by feeding a diet high in fat. In the density range from 1.006 to 1.087 there are at least two major different types of lipoproteins, one having β mobility (LDL) and a faster α-migrating lipoprotein (HDL_1). The overlapping densities made it impossible to isolate either in pure form by ultracentrifugation. They have been purified by Geon–Pevikon block electrophoresis. Characterization of these lipoproteins and their metabolic significance awaits further analysis.

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