Thin-layer electrophoresis of serum lipoproteins

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ABSTRACT A method is described for semiquantitative study of serum lipoproteins, with particular reference to the very low density fractions involved in transport of triglycerides. Serum samples were subjected to electrophoresis in thin layers of starch granules; lipids were extracted from starch segments and subjected to thin-layer chromatography; lipid fractions were recovered from the adsorbent and quantified colorimetrically.

The procedure is applicable to the study of alimentary and endogenous hyperlipidemias in man, and to radioactive tracer experiments. Over-all recoveries of lipids were variable and averaged 81% for cholesterol and 76% for triglycerides.

RECENTLY THERE has been increased interest in the hyperlipidemias encountered in clinical practice. The types characterized by elevation of the serum triglycerides and triglyceride-bearing lipoproteins have attracted special attention because of improvements in classification of primary hyperlipidemias and because of recognition of hypertriglyceridemia as a secondary manifestation in a wide variety of diseases (1–6). The need for refinement of our knowledge of the foregoing conditions has stimulated efforts to achieve better methods for description and measurement of the very low density portion of the lipoprotein spectrum.

Techniques of zone electrophoresis have been applied to lipoprotein analysis with some success. Electrophoresis in blocks of starch granules permits fractionation of serum in amounts sufficient for recovery and analysis of the separated fractions (7–11). The absence of interference with migration of macromolecules in the starch, such as a sieving effect, is indicated by the similar results obtained by "free" electrophoresis in solution (Tiselius apparatus) (12, 13). The only major disadvantage of the starch block procedure is the prolonged period required for separation (the standard run takes about 24 hr). Recently Bierman and his collaborators have utilized this technique for extensive studies of lipoproteins in abnormal human sera and changes induced by dietary manipulations (14, 15).

We report here a procedure for serum electrophoresis in starch granules on thin-layer plates (16). The technique gives more rapid separations (2–4 hr) of sufficient serum to permit characterization of the lipoprotein fractions by extraction and quantitative TLC of the lipid constituents. Examples are given of applications to the study of triglyceride transport during alimentary fat absorption in man and to a radioactive tracer experiment in the rat.

MATERIALS

Serum Samples. Serum was obtained in the fasting state, or after meals to test fat tolerance, from healthy subjects and from one normolipemic patient with coronary heart disease. Electrophoresis was performed within a few hours after sampling; the serum was held at room temperature until that time and was mixed well before use.

Electrophoresis. The equipment was manufactured by the E-C Apparatus Corp., Philadelphia, Pa. The power supply (Cat. No. EC453) provided regulated voltage, and the horizontal tank (Cat. No. EC401) (12 inch size) was filled with barbital buffer, pH 8.6, ionic strength 0.1, containing 0.001 M EDTA.

Abbreviation: TLC, thin-layer chromatography.
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Thin-Layer Techniques. Apparatus of Brinkmann Instruments Inc., Westbury, N. Y., was used; it included the Desaga variable-thickness spreader.

For electrophoresis potato starch, purified grade, was obtained from Fisher Scientific Company, New York, N.Y., or J. T. Baker Chemical Co., Phillipsburg, N.J. For TLC Silica Gel HR was obtained from Brinkmann Instruments Inc., Westbury, N.Y.

All solvents and chemicals used for chromatography, extraction, and colorimetric methods were the best grades available commercially, e.g., "spectroquality" hexane.

Radioactive Tracer Experiment. The cisterna chyli of a 200 g rat was cannulated with polyethylene tubing. Tripalmitin-^{14}C was administered with corn oil by stomach tube. After 2 hr serum and lymph were obtained and subjected to electrophoresis.

EXPERIMENTAL PROCEDURES

Thin-Layer Electrophoresis

Glass plates, 20 × 20 cm, were cleaned in dichromate–sulfuric acid solution and dried. A slurry of potato starch in the barbital buffer described above (1 g/2 ml) was spread at a thickness of 0.37 mm. The plate was allowed to dry on the bench until the surface became dull and nonreflective in appearance. Portions of 20 μl of serum were applied immediately with the Spinco applicator (Beckman Instruments, Inc., Palo Alto, Calif.), either in four separate lanes for different samples or in a single band containing a total of 0.1 ml across the plate. The loading ratio, serum volume:starch bed volume, utilized during electrophoresis was estimated to be about one-third of that in the conventional starch block method. The serum was applied about 5 cm from the intended cathodal end of the plate.

The plate was placed in the tank, and wicks of Whatman 3MM filter paper were applied between the buffer reservoirs and the ends of the starch layer. Spacer strips of paper were added over the wicks at the ends of the plate and a clean glass plate was placed above the starch layer. The top of the apparatus was put in place so that evaporation of buffer might be decreased. Cooling solution at 6°C was circulated through the lower coils only, which gave an operating temperature of approximately 20°C in the starch layer (thermistor measurement).

Electrophoresis was performed at 600 v and 18–20 ma for 2 hr, at which time serum albumin had migrated approximately 10 cm toward the anode.

The fractionation was qualitatively evaluated by examination of white bands that appeared transiently during drying of the plate. Lipoprotein bands appeared earliest, followed by fainter bands which seemed to represent the other major protein fractions. The latter

were obscured when markedly lactescent sera were run. The white bands were readily recorded by Polaroid photography. The plates could be wrapped in polyvinyl chloride film to delay the drying process if desired.

Direct lipoprotein staining has proved difficult because of the fragility of the starch layers, although with gentle handling the plates could be immersed in a warm, saturated solution of Oil Red O in 60% ethanol to give successful staining. In our experience the use of binding compounds to stabilize the layers disturbed lipoprotein mobility. The serum protein bands were readily demonstrated by spraying the dried plate with 0.4% ninhydrin in acetone solution and allowing color to develop at room temperature.

Starch segments were removed for chemical analysis by scraping with a razor blade. Demarcation of zones to be taken was based either upon the white bands described above or upon measurement of intervals (1 cm or $^{1}/_{2}$ inch). The scraping procedure was best done before the plates were completely dry.

A modification for fractionating larger amounts of serum is as follows. A thicker slurry of starch is prepared and the spreader is adjusted to give layers 1–2 mm thick. The plate is dried about 18 inches from a heat lamp until the surface appears slightly damp. A trough is made across the plate with a pointed spatula, and filled with a thin starch slurry containing 0.25–0.5 ml of serum. Electrophoresis at 500 v or lower, current 35–40 ma, is continued until the albumin band (usually visible because of its color) has migrated 12–14 cm (about 4 hr). Lipoproteins were less easily seen as thick plates dried. Accordingly, bands $^{1}/_{2}$ inch wide were scraped for analysis.

Extraction of Lipids

Lipids were extracted from the lipoproteins by placing each moist starch segment in a 30 ml round bottom, stoppered centrifuge tube. Chloroform–methanol 2:1 (20 ml) was added and the mixture was thoroughly and repeatedly mixed on a Vortex mixer for 1 hr, then allowed to stand for 1 hr more at 25°C. It was then filtered through "sharkskin" filter paper and the filter paper was rinsed with a small amount of solvent. The filtrate was collected in the same type of tube. Ten milliliters of 2% aqueous Na_2CO_3 was allowed to flow down the walls of the tube, which was then kept at 4°C overnight. Of the separated chloroform layer 10 ml was removed by means of a syringe and long needle and placed in a screw-capped test tube.

TLC of Lipids

Lipid solutions were evaporated to dryness under nitrogen. To each tube 50 μl of chloroform was added and 5–30 μl (depending upon the expected lipid concentration) was applied to thin-layer plates of Silica Gel HR
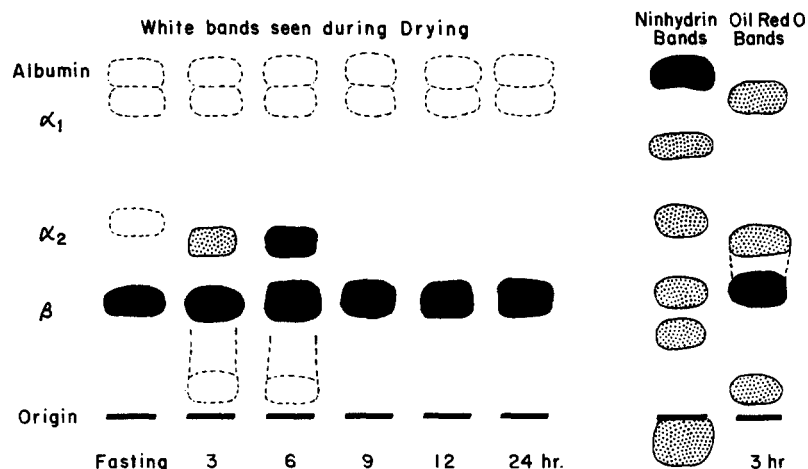
FIG. 1. Bands observed after thin-layer electrophoresis in starch granules. 100 g fat tolerance test, R. L. ♂ 30; barbital buffer: pH 8.6, Γ/2 0.1, EDTA 0.001 M, 600 v, 18–20 ma, 2 hr. The series at the left side of figure represent white bands on gray background. These were observed during drying of a plate to which serum samples obtained throughout a fat tolerance test had been applied. Note the transient increase of lipoproteins in the α_2-region and just anodal to the origin during alimentary lipemia. At the right are the serum protein bands revealed by ninhydrin spraying and lipoproteins in the 3 hr sample stained by Oil Red O dye. Dotted outlines, stippled zones, and filled zones signify increasing intensity of white or colored bands observed during drying or after staining of the plate.

(0.25 mm thick). Ten samples or standards could be applied across a 20 × 20 cm plate. The *standard solution* contained a mixture of 5 mg/ml each of cholesterol, cholesteryl oleate, and triolein; and 5, 10, 15, and 20 µl (25–100 µg) were applied with each set of samples (usually two silica gel plates were required for the segments from one electrophoretic plate). Phospholipid analyses were not attempted in this study.

Plates were developed to a height of 15 cm in hexane–diethyl ether–acetic acid 90:10:1. Solvents were completely removed in a stream of air and the plates briefly exposed to iodine vapor. The zones were demarcated by means of a pointed instrument. Particular care was taken to exclude phospholipid remaining at the origin from the cholesterol zone. After the iodine had sublimed away, the zones containing cholesterol esters, triglycerides, and free cholesterol were scraped from the plates into 15 × 100 mm test tubes.

Measurement of Triglycerides

To the silica gel samples from the triglyceride zones was added 5 ml of chloroform–methanol 2:1. The suspension was mixed thoroughly on a Vortex mixer and allowed to stand for 10 min. The tubes were centrifuged for 10 min at 3500 rpm and the supernatant solutions were poured into 18 × 150 mm test tubes. This extraction process was repeated once and the extracts combined.

The solvent was evaporated to dryness in a vacuum oven at 30°C. Triglycerides were measured by a slight modification of the method of Vioque and Holman (17). To the lipid residues were added 0.05 ml of 2.5% hydroxylamine in 95% ethanol, 0.05 ml of 2.5% NaOH in ethanol, and 5 ml of diethyl ether (peroxide-free). The contents of the tubes were mixed and immersed in a bath at 55°C, at which temperature the ether boiled away gently. The remnants of solvent were removed completely under a jet of nitrogen. The ferric perchlorate reagent was freshly diluted in an ice bath as directed, and 1.25 ml was added to the samples in the ice bath. The tubes were allowed to stand for 30 min at 25°C and read in a spectrophotometer at 520 mµ against a blank of absolute ethanol. The values obtained for the extracted lipoprotein fractions were read from the standard curve and corrected by calculation for the aliquots of serum and chloroform taken.

Measurement of Cholesterol and Cholesterol Esters (18, 19)

The lipids were extracted with chloroform from the scraped silica gel zones and evaporated as described for the triglycerides. Absolute ethanol, 1 ml, was added to each tube. Cholesterol and cholesterol esters were determined with a reagent which contained 1 mg of FeCl₃·6 H₂O per ml of concentrated H₂SO₄. The reagent was layered beneath the alcohol and the tubes were then mixed well and placed in a boiling water bath for 3 min, the tubes being covered with glass marbles. The tubes were subsequently cooled in ice for 30 min and read in a spectrophotometer at 560 mµ. A reagent blank was used.

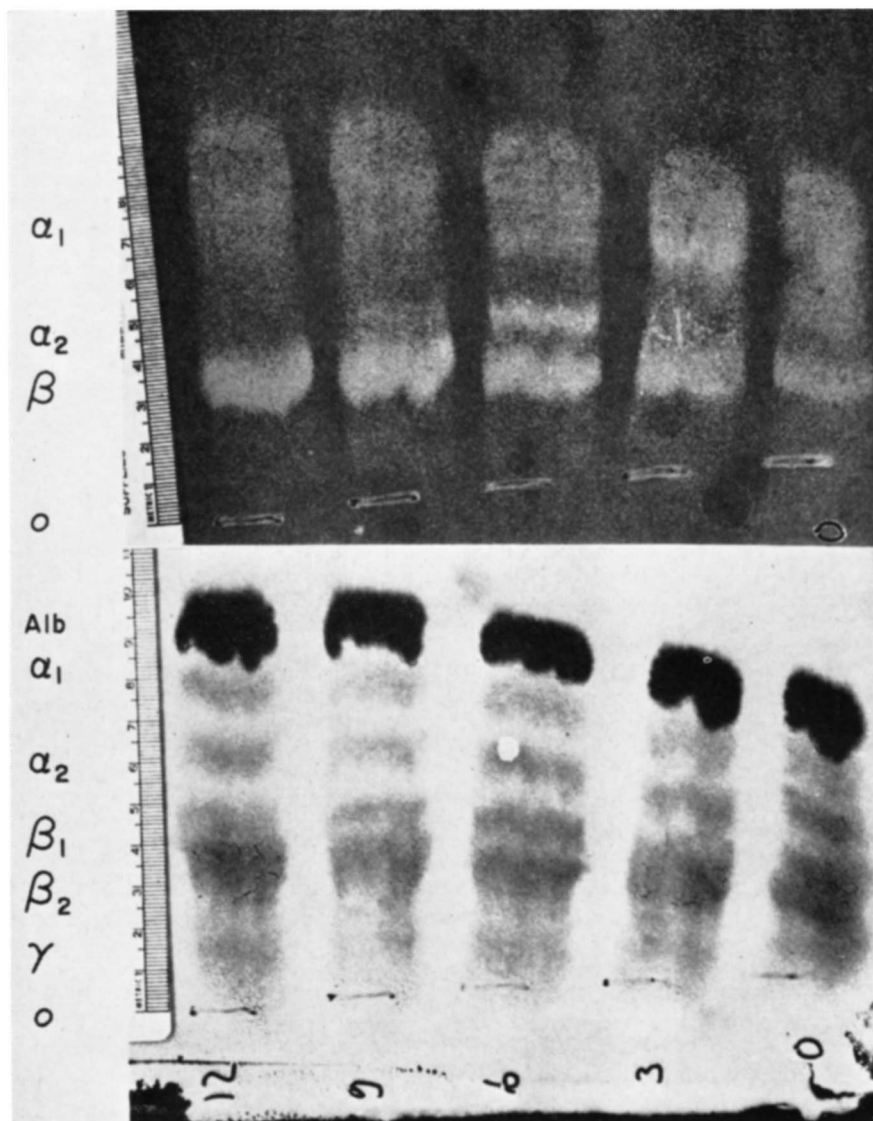FIG. 2. Thin-ayer electrophoresis in starch granules. Serum samples obtained before and 3, 6, 9, and 12 hr (reading from right to left) after ingestion of a meal containing 250 g of fat by a healthy 26-year-old male. Top: pattern observed during drying of plate after electrophoresis (originally recorded by Polaroid photography). Note the heavy white band near α_1-region in 3-hr sample, which had diminished at 6 hr; and the second white band in α_2-region, which was maximal in 6-hr sample. Chemical analysis revealed that each of these zones transported triglycerides. Bottom: protein bands developed by spraying plate with 0.4% ninhydrin in acetone.

Standard curves were constructed from the standard cholesterol and cholesteryl oleate zones on the plates.

Other Methods

The polyvinylpyrrolidone gradient tube method of Gordis (20) was applied to lactescent sera.

For assay of samples containing radioactive tracer compounds, each starch zone, after it was completely dry, was transferred into a counting vial to which 10 ml of a toluene scintillation mixture was then added. This simple technique permits determination of the distribution of the labeled compound in the starch zones, but since the actual counting efficiency might be dependent upon the degree of extraction of the material from the lipoproteins into toluene, quantitative determination of radioactivity might require more elaborate procedures.

If confirmation of the chemical form of the tracer was desired, the lipids were extracted with chloroform–methanol, subjected to TLC on silica gel, and counted by lipid class.
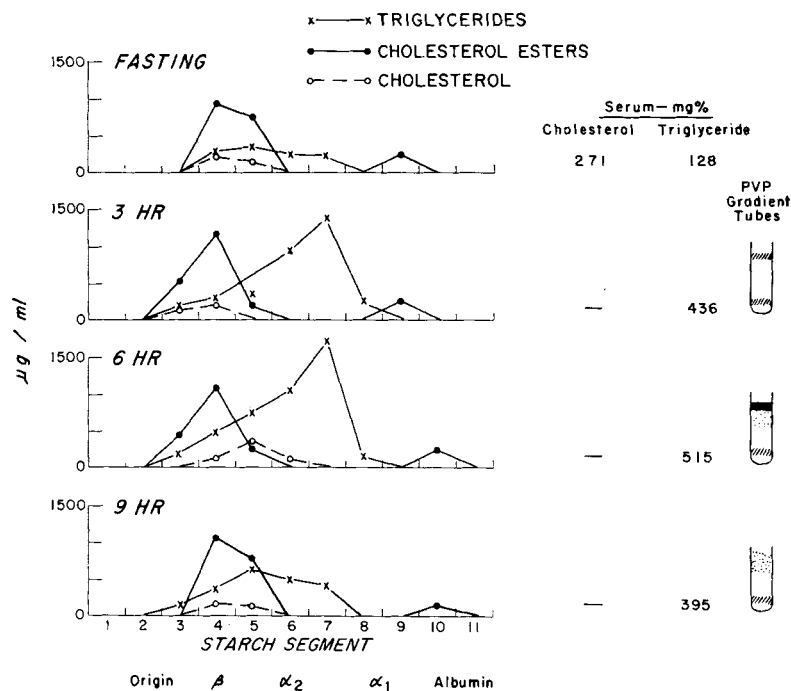
FIG. 3. Lipoprotein electrophoresis during alimentary lipemia. 150 g fat tolerance test, H. F. ♂ 39; 2 mm starch layers, 0.25 ml serum per lane, 200 v, 13 ma, 4 hr, two lanes per plate. In the fasting sample, small amounts of triglycerides were found within and just anodal to the leading edge of the β-lipoprotein. During alimentary lipemia a large peak of triglyceride-rich, cholesterol-poor lipoprotein was present in the α_2-region. As lipemia cleared, the triglyceride-bearing fraction returned toward the fasting distribution. Also shown are the polyvinylpyrrolidone gradient tubes (20) for the same serum samples.
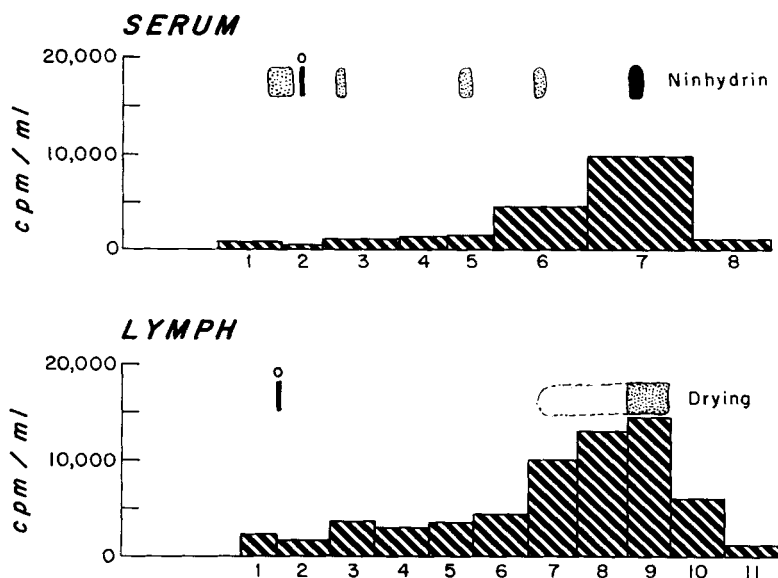

FIG. 4. Application of thin-layer liproprotein electrophoresis to a radioactive tracer experiment in which tripalmitin-^{14}C was fed with corn oil to a rat. After 2 hr, serum and lymph were obtained and subjected to electrophoresis. Zones from the dried starch plate were scraped into liquid scintillation vials and covered with 10 ml of toluene counting solution. In the rat, triglyceride-bearing lipoproteins of alimentary origin in both serum and lymph migrated approximately at the rate of albumin. Most of the ^{14}C was shown by TLC to be in triglycerides rather than in free fatty acids.

RESULTS

Thin-layer electrophoresis in starch granules was applied to the study of lipoproteins either qualitatively or quantitatively. The former technique is illustrated in Figs. 1 and 2. The bands that developed transiently on the plates during drying were correlated with the positions of protein bands demonstrated with ninhydrin.

Application of the semiquantitative method to the study of human alimentary lipemia is illustrated in Fig. 3. After the lipids had been extracted from the lipoproteins in the starch segments and separated by chromatography on silica gel, colorimetric methods were applied to the separated lipids. In our study cholesterol, cholesterol esters, and triglycerides were determined. The amounts of free cholesterol were usually so small as to be near the limits of sensitivity of the method. However, the cholesterol esters served to delineate the positions of β- and α_1-lipoproteins, while triglycerides revealed the location of chylomicrons and very low density lipoproteins.

When the amounts of lipid obtained from the starch segments were summed and compared with lipid determinations made directly on the original sera, the recoveries were incomplete and variable. In a series of eight separate plates (fat tolerance test samples at 0, 3, 6, and 9 hr in two subjects), the recovery of total cholesterol averaged 81.1 ± 13.3% (SD) and of triglycerides, 76.3 ± 18.5%.

During alimentary lipemia in man, the triglycerides were observed predominantly in the α_2-region in starch electrophoresis (Figs. 1–3). In three subjects we have noted an additional lipoprotein band just anodal to the origin (Fig. 1). In one healthy subject after a very large fat load (250 g), independent triglyceride-bearing bands were present in the α_1- and α_2-regions (Fig. 2). The significance of these variations is not yet clear.

In the rat administered radioactive tripalmitin, the chylomicrons of both serum and lymph migrated in electrophoresis predominantly to the region of albumin (Fig. 4). Although there was some trailing of radioactivity in both cases, there was no significant zone of label remaining at the origin.

DISCUSSION

In order to supplement the available techniques for study of the very low density lipoproteins of serum, we have presented an adaptation of electrophoresis in starch granules to the thin-layer mode. When coupled with quantitative TLC, the method describes the location of different lipid constituents in the electrophoretic pattern. Illustrative examples are given for alimentary lipemia in man and a radioactive tracer experiment in the rat. The technique should also be applicable to the study of hyperlipidemia, but we have so far undertaken only limited trials of it for this purpose.

The method has advantages over paper electrophoresis: sufficient material can be processed to permit approximate chemical analysis of the lipid composition of separated fractions or the counting of radioactivity at low levels; all types of "fat particles" (i.e., chylomicrons and very low density lipoproteins) migrated away from the origin and were found in fairly discrete zones (21–23). The latter feature may be particularly important in isotopic tracer studies in which confusion between metabolically significant material and dissociated artifact must be avoided. The advantages of this thin-layer procedure over conventional starch block electrophoresis are the shorter period of electrophoresis and the smaller amounts of supporting material from which the lipoprotein constituents must be recovered.

Four factors are believed to contribute to the incomplete and variable recoveries of the lipids from the lipoprotein fractions: (a) incomplete extraction of the lipids from the starch segments, (b) incomplete extraction of the lipids from the silica gel plates, (c) losses in handling small amounts of material, and (d) neglect of small amounts of lipids present in the starch segments outside the main fractions. Insofar as we could determine, the losses were randomly distributed and did not result in obscuring any particular component.

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