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Improved Techniques for Assessment of Plasma Lipoprotein Patterns. I. Precipitation in Gels after Electrophoresis with Polyanionic Compounds

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A method is described for making lipoprotein fractions visible by polyanion precipitation in situ in the gels after electrophoresis. With the new technique the pattern and its interpretation in the differential diagnosis of all primary forms of hyperlipoproteinemia is the same as for the other lipid-staining procedures. The new technique is simpler to perform, reliable, and provides the required information 60 min after electrophoresis. It also allows a very fast determination of the abnormal lipoprotein (LP-X) that characterizes cholestasis, without use of a specific antiserum.

Additional Keyphrases: *electrophoresis in agarose, in agar gel, in polyacrylamide • serum lipoprotein pattern • hyperlipoproteinemia • Ca-dextran sulfate as lipoprotein precipitant • immunodiffusion • LP-X • diagnosis of cholestasis*

In addition to measurements of serum total cholesterol and serum triglycerides, assessment of the serum lipoprotein pattern is necessary for the diagnosis of the various types of hyperlipoproteinemia. Consequently, simple but reliable techniques are needed to identify and, in particular cases, to quantitate normal and abnormal lipoprotein fractions. Lipoprotein electrophoresis combined with ultracentrifugation and immunological techniques have been proved useful in assessing the lipoprotein pattern. However, such techniques are very time-consuming and are too laborious and expensive for routine use in many laboratories.

The purpose of this series is to describe improved methods that are practicable for every laboratory and that provide all the information needed to evaluate a lipoprotein pattern.

Here, we present a fast and simple technique for making lipoprotein bands visible, including the abnormal lipoprotein (LP-X) characteristic of cholestasis, by polyanion precipitation in gels after electrophoresis.

Materials and Methods

Samples

Blood samples were obtained from well-classified hyperlipoproteinemics [Types I, IIa, IIb, III, IV, and V (*1, 2*)] and from patients with obstructive jaundice, whose plasma contained the abnormal plasma lipoprotein LP-X (*3, 4*). Blood was sampled after the subjects had fasted overnight, and the serum was promptly separated by low-speed centrifugation. Citrate plasma should not be used for this technique.

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Lipoprotein Electrophoresis

To determine the lipoprotein pattern we performed electrophoresis in agarose (Serva, Heidelberg, Germany) on each sample (in duplicate) by a modification (*5*) of Noble's method (*6*), with use of a barbital buffer (pH 8.6, 50 mmol/liter) and an LKB electrophoresis system (Bromma, Sweden).

After electrophoresis, one of each duplicate was stained with Oil Red O (Serva, Heidelberg, Germany). The slides were fixed for 30 min in dilute acidic acid (5 ml plus 95 ml of water), washed for at least 10 h under running tap water, and then dried in a drying oven at 90°C for 3 h. This was followed by staining (4 h) with Oil Red O at 37°C. After staining, the slides were rinsed briefly, then dried on blotting paper.

Immediately after electrophoresis the second duplicate was placed for 60 min in a bath of 0.2 molar $CaCl_2$ containing 6 g of "Sodium Dextransulfate 2000" (Pharmacia, Uppsala, Sweden) per liter, to completely precipitate all lipoprotein bands.

Lipoprotein-X Determination

To determine LP-X, we electrophoresed samples in "Bacto-Agar" (Difco, Detroit, Mich.), also in duplicate, according to Seidel (*7*). Heparin (Serva, Heidelberg, Germany) in a final concentration of 1 g/liter was added to the agar. A barbital buffer (pH 8.6, 50 mmol/liter) and an LKB electrophoresis system (Bromma, Sweden) were used.

One duplicate was treated with a specific anti-LP-X serum as previously described (*7*). After electrophoresis the rabbit antiserum was applied to the gel surface on the cathodic side to each sample and afterwards incubated for 3 to 8 h at 37°C, to allow the characteristic immunoprecipitin reaction of LP-X to develop.

The second duplicate of this series was placed for 30 min in a bath of 0.1 molar $MgCl_2$ containing, per liter, 2.5 g of heparin (Serva, Heidelberg, Germany) and 9 g of NaCl, to visualize the LP-X by precipitation with the heparin.

Electrophoresis in Polyacrylamide

Untreated native serum was electrophoresed in polyacrylamide according to the method of Davis (*8*), with use of a continuous glycine–tris(hydroxymethyl)aminomethane buffer system (pH 8.3, 200 mmol/liter). The acrylamide monomer concentration was 31.5 g/liter.

Completeness of Precipitation

To prove the completeness of precipitation of all lipoproteins by the polyanions used, we precipitated 10 ml of serum from patients with various forms of hyperlipoproteinemia by adding 5 ml of the polyanion solution used to precipitate the lipoprotein bands after electrophoresis in agarose, and the mixture was allowed to stand for 20 min. The precipitate was removed by low-speed centrifugation at 9,000 × *g*, dissolved in a sodium chloride solution (50 g/liter), and the dissolved precipitate and the supernatant fluid were separately dialyzed against a sodium chloride solution (9 g/liter).

The starting serum, the dissolved precipitate, and the supernatant fluid were then tested by double immunodiffusion in agar (10 g/liter). The plates were incubated for 48 h at 37°C. Rabbit antisera to α-lipoproteins (Behring Werke, Marburg, Lahn, Germany) were used as antibodies. Antisera to β-lipoproteins (apo-B) and to apo-C (containing antibodies to the CI, CII, and CIII subunit) were prepared in our laboratory as previously described (*3, 4*).

Results

Figure 1 shows the results of the more laborious way to stain lipoprotein fractions with a lipid dye such as Oil Red O in comparison to the herein-described quicker and simpler precipitation technique. Without exception, all different types of hyperlipoproteinemia revealed the same pattern by both techniques.

Figure 2 indicates by immunological means that Dextransulfate 2000 in combination with $CaCl_2$, as used in this polyanion precipitation procedure, precipitates all of the principal and diagnostically important lipoprotein fractions. While both whole serum and the dissolved precipitate reacted with antibodies to apo-A, apo-B, and apo-C, the supernatant fluid showed no reaction against apo-B and apo-C, and only in some cases a weak immunoprecipitin band with antibodies to apo-A, in agreement with findings of Burstein et al. (*9*). By using a radial immunodiffusion technique, we estimated that less than 5% of the α-lipoproteins were not precipitated by Dextransulfate 2000 and $CaCl_2$.

Figure 3 demonstrates that the abnormal lipoprotein (LP-X), characteristic of cholestasis, can be demonstrated by polyanion precipitation with the same precision as with the use of a specific antiserum, but with less work involved and within 30 min after electrophoresis. Because it migrates toward the cathode on agar-electrophoresis (*3*), LP-X can be distinguished from all other plasma lipoproteins, which migrate toward the anode. It is important to recognize LP-X as a unique band having a sharp margin that may, however, vary in its intensity and to some degree in its distance from the starting well, depending on the concentration of LP-X in the patient's serum (see Figure 3, III). A foggy trailing toward the cathode indicates an alteration of the investigated plasma and should not be interpreted as LP-X.

Although lipoprotein electrophoresis in polyacrylamide has not yet been fully satisfactory in the classification of hyperlipoproteinemia, it seems noteworthy to indicate that the lipoprotein bands can also be determined by polyanion precipitation in this medium.

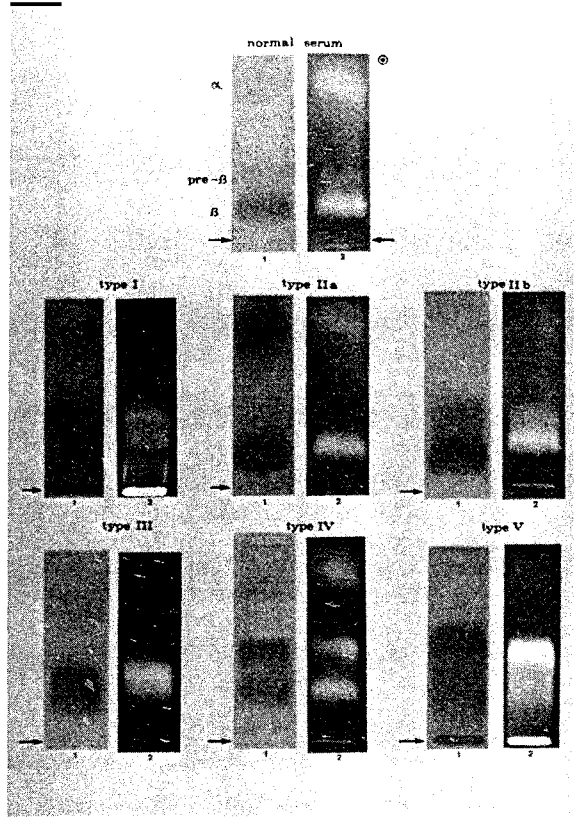

Fig. 1. Lipoprotein pattern of the various phenotypes of hyperlipoproteinemia

Agarose (10 g/liter); *1*, Oil Red O stain; *2*, polyanion precipitation (Sodium Dextransulfate 2000 and $CaCl_2$)

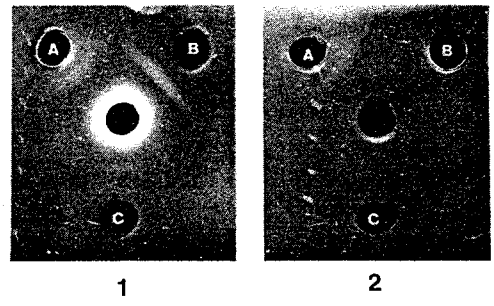

Fig. 2. Immunodiffusion pattern in agar (10 g/liter)

1, dissolved precipitate (Sodium Dextransulfate 2000 and $CaCl_2$); *2*, supernatant fluid after precipitation; well *A*, anti-LP-A serum; *B*, anti-LP-B serum; *C*, anti-LP-C serum

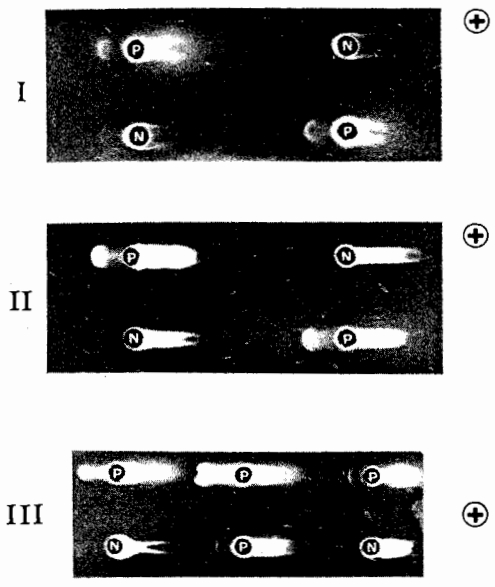

Fig. 3. Lipoprotein pattern of whole serum in agar (10 g/liter) for the determination of LP-X with its characteristic mobility toward the cathode

P, LP-X positive (patients with cholestasis); *N*, LP-X negative (patient without cholestasis; I, made visible by use of specific anti-LP-X serum, or (II and III) by poiyanion precipitation (heparin 2.5 g/liter, NaCl 9 g/liter, $MgCl_2$ 0.1 mol/liter)

Discussion

Because the classification system introduced by Fredrickson et al. (*1, 2*) has been shown to be very useful in the differential diagnosis of hyperlipoproteinemia and is now generally accepted, semiquantitative lipoprotein electrophoresis is an important requirement to determine the lipoprotein patterns of the various phenotypes. However, all lipoprotein electrophoresis systems used so far were very laborious and time-consuming.

It has been known for many years that plasma lipoproteins can be precipitated by the use of polyanions and divalent cations (*9–12*). However, so far, no attempts were made to utilize this phenomenon for determination of lipoproteins in gels after they had been separated by electrophoresis. In this study, we demonstrate this possibility for the first time. The important advantage of this new technique is its simplicity, reliability, and the fact that the pattern can be obtained only 60 min after electrophoresis.

Because of a rather complete precipitation of all lipoproteins, it may be assumed that the lipoprotein bands shown by the herein-described procedure reflect the actual concentration of each lipoprotein fraction much better than do the bands obtained by various lipid-staining procedures, which are known to depend not only on the concentration of each fraction but are also affected by a dissimilar composition. It therefore seems worthwhile to study the possibility of a quantitative determination of these precipitin bands by means of densitometric scanning. This is now under investigation in our laboratory.

The demonstration of LP-X has been proved to be the most specific chemical parameter to indicate or exclude cholestasis (*13, 14*). However, the use of a specific antiserum required for this test obviously was a limiting factor for routine work. The new technique of precipiting LP-X by polyanions overcomes this difficulty and has the important advantage of providing the answer 30 min after the electrophoresis has been performed. Most of the LP-X positive samples will show the characteristic precipitin reaction within 1–2 min of incubation, and only for those with very low LP-X concentration is the extended incubation time of 30 min needed.

After precipitation of the lipoproteins by polyanions in gels, oblique light is the best way to read the agarose or agar plates. It is possible to store the plates after they have been dried under blotting paper or in a drying oven.

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