\( \beta \)-Lipoprotein Quantitation in Cord Blood Spotted on Filter Paper: A Screening Test

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We describe an electroimmunodiffusion technique for measuring \( \beta \)-lipoprotein in cord blood spotted on filter paper.

A series of cord-blood samples, taken from 916 consecutive live-born infants, was spotted directly onto filter paper and assayed for \( \beta \)-lipoprotein. Eleven had above-normal \( \beta \)-lipoprotein. Of these 11, seven were tested two to six months later, along with their parents, for total cholesterol and triglyceride concentrations. Five infants had increased cholesterol values, and four parents of these infants had either increased cholesterol or triglyceride values.

We also measured \( \beta \)-lipoprotein concentrations in 63 paired samples of dried cord-blood and three-day post-delivery blood specimens, routinely collected for phenylketonuria screening. We saw a significant correlation between results for the specimens, but detected no cases of increased \( \beta \)-lipoprotein.

\( \beta \)-Lipoprotein assay in dried specimens of cord blood is simple, inexpensive, and potentially is useful in mass screening of newborns for familial type II and combined hyperlipidemia.

Additional Keyphrases: triglycerides (triacylglycerols) • cholesterol • heritable disorders • hyperlipemia • newborns • lipids • atherosclerosis • familial hyperlipoproteinemia • screening

Familial hyperlipoproteinemia type II (FH II) is a dominantly inherited disorder, characterized in both homozygotes and heterozygotes by hypercholesterolemia, tendon and subcutaneous xanthomas, and premature coronary heart disease (1). The disease is transmitted in homozygotes with an estimated frequency of 1 in 108 and in heterozygotes with a frequency from 1 in 200 to 1 in 500. Most studies of FH II have focused on heterozygous individuals because they show a higher prevalence of symptoms. Unlike children homozygous for FH II, heterozygous children do not manifest overt cardiovascular symptomatology (2); however, they often have increased plasma cholesterol concentrations, predominantly in the form of LDL-cholesterol, from birth (1).

The detection of FH II heterozygotes in childhood may offer the possibility of prophylaxis and (or) early treatment of atherosclerosis (3). Diets high in polyunsaturated fats and low in cholesterol have been shown to normalize plasma cholesterol values in FH II heterozygous infants, if instituted within the first postnatal year (4,5). In contrast, similar diets given to older children reduce plasma cholesterol by only 12–24% (2,4,5,6,7). However, it is not known how long beyond one year of age the remarkable sensitivity of plasma cholesterol concentrations to diet persists. Furthermore, the long-term advantages and (or) disadvantages of decreasing plasma cholesterol concentrations in FH II heterozygotes has yet to be determined.

Familial combined hyperlipidemia has also been described, in which members of the affected kindred have hypercholesterolemia, hypertriglyceridemia, or both. The disorder appears to be transmitted as a distinct autosomal dominant trait, with variable expression of the phenotype (8,9). Not enough is known about the children of these kindreds to determine their response to dietary treatment (9).

The measurement of total cholesterol or of LDL-cholesterol in cord plasma, coupled with family studies, has been routinely used to detect FH II heterozygotes in newborns (10–12). However, there was a high rate of false-positive results in these studies, and it has been suggested that the measurement of cholesterol in cord blood is not reliable for detecting heterozygotes (13–15). It also has been shown that cord-blood cholesterol concentrations may be transiently increased, owing to non-genetic factors (16) associated with maternal–fetal distress and fetal anoxia. A particularly significant correlation has been found between post-term delivery and hypercholesterolemia in neonates (16).

Recently it has been suggested that the quantitation of plasma pre-\( \beta \)- and \( \beta \)-lipoproteins (17)—and, more specifically, of the apoprotein B contained in these lipoproteins (18)—is more discriminating for the detection of atherosclerosis in pediatric and adult populations than are determinations of cholesterol and triglyceride. Quantitation of pre-\( \beta \)- and \( \beta \)-lipoproteins has the potential for detecting individuals with FH II (increased \( \beta \)-lipoprotein with or without increased pre-\( \beta \)-lipoprotein), familial hyperlipidemia type IV (increased pre-\( \beta \)-lipoprotein), and those with familial combined hyperlipidemia. Several immunoassays have been developed for measuring apoprotein B, including radial immunodiffusion (19), electroimmunooassay (20–22), enzyme-linked immunosorbent assay (23), and radioimmunoassay (24–27). A comparison of electroimmunoassay, radial immunodiffusion, and radioimmunoassay, showed the first to be faster, simpler, and to account for more of the total protein in low-density lipoproteins than the other two techniques (28). Nevertheless, these methods, as they were applied, are not practical for mass screening because the plasma must be separated from the whole blood.

Simple and inexpensive tests for inborn metabolic disorders have been developed for use with a single drop of whole blood, taken from a heel prick of three-day-old infants and spotted on filter paper; the best known of these is the test for phenylketonuria (29). We describe a test for the detection of increased pre-\( \beta \)- and \( \beta \)-lipoprotein concentrations by immunologically quantitating the apoprotein B in whole cord blood spotted and dried on filter paper.

Materials and Methods

Specimens

A series of 916 cord blood specimens was collected at Children’s Hospital of Buffalo by spotting single drops of cord
blood from a 5-mL pipette directly onto no. 903 filter paper (Schleicher and Schuell, Keene, NH 03431) at birth. The specimens were dried at room temperature (about 1 h) and stored at 4 °C in plastic bags for five days to four weeks. Matching three-day heel-prick specimens were spotted on filter paper from 63 of these infants. (Informed consent was obtained from the parents before blood was collected.) A second series of 296 cord blood samples was collected in ethylenediaminetetraacetate-containing tubes (Vacutainer; Becton-Dickinson, Rutherford, NJ 07070) for the purpose of determining a mean hematocrit for cord blood (see Standards).

Antisera

Rabbit anti-human β-lipoprotein serum was obtained from Cappel Laboratories, Inc., Cochranville, PA 19330. This antiserum was prepared against LDL separated from VLDL by the method of Burstein et al. (30) and tested for monospecificity by immunoelectrophoresis against whole human serum and β-lipoprotein standard (see Standards; Calbiochem-Behring Corp., La Jolla, CA 92037). A single immune precipitation arc in the β-globulin region was observed, which had the same mobility against both the whole serum and the standard. Goat anti-human IgA serum, specific for α heavy chain, was obtained from Cappel Laboratories and tested for monospecificity by immunoelectrophoresis against whole human serum. A single precipitation arc was observed in the β-γ-globulin region.

Standards

A pool of cord blood was divided into two aliquots. The aliquots were centrifuged, the plasma was discarded, and the erythrocytes were washed three times with phosphate-buffered (10 mmol/L, pH 7.4) isotonic saline. One aliquot of the cells was resuspended in an equal volume of the commercial β-lipoprotein standard solution (4.28 g/L). According to the manufacturer, this standard represents only the β-lipoprotein fraction of human serum (d = 1.050 kg/L), isolated by density-gradient ultracentrifugation and quantitated by radial immunodiffusion against antiserum prepared against apoprotein B. The concentration of 4.28 g/L therefore refers to the whole β-lipoprotein fraction. The second aliquot was resuspended in an equal volume of phosphate-buffered isotonic saline. This second suspension was used as a diluent for making twofold dilutions of the first suspension. Hence, we prepared a series of β-lipoprotein standard concentrations in a suspension that closely resembled whole cord blood. Each of these suspensions was then spotted on filter paper and stored at 4 °C for as long as a month. This standards-preparation procedure was justifiable in that the resulting hematocrit of 50 for each standard preparation closely approximated the mean hematocrit of 51.8 (SD 6.2) found in 296 consecutive cord-blood specimens.

Electrophoresis

We used a modification of Laurell’s “rocket” technique (electroimmunoassay) to quantitate β-lipoprotein in dried cord blood samples (31). Glass plates (8.3 x 10.2 cm; Eastman Kodak Co., Rochester, NY 14650) were precoated with a 50 g/L solution of agarose in distilled water, air dried, and 12 mL of an 8.5 g/L solution of agarose (cat. no. A-6877; Sigma Chemical Co., St. Louis, MO 63178) in barbital-HCl buffer (50 mmol/L, pH 8.8, Electro HR Buffer; Helena Laboratories, Beaumont, TX 77704) containing 40 µL of rabbit anti-β-lipoprotein serum (final concentration, 3.3 mL/L) was poured onto each plate. Discs 3 mm in diameter were punched from the filter paper containing the spots of dried cord blood and were applied, in duplicate, directly onto the surface of the agarose gel, 2.5 cm from the lower edge of the plate. Similar rocket heights were obtained from discs punched from different areas of the blood spot. Discs from each of four concentrations of β-lipoprotein standard were also applied to each plate, resulting in a total of 16 samples per plate. Electrophoresis was at 6 V/cm for 24 h, at room temperature, in the barbital buffer, with use of a zone-electrophoresis chamber (Helena). The plates were then washed in 2 L of isotonic saline for 24 h at 4 °C, blotted on filter paper, and air-dried. The β-lipoprotein–antibody precipitation peaks were made visible by staining for 2 h with Sudan Black B (2.5 g/L) in a 600 mL/L mixture of ethanol and NaOH solution (final concentration, 1 g/L). The plates were then destained for 15–30 s in a 600 mL/L solution of ethanol. Previous experiments showed that this lipid stain showed the peaks more clearly than a protein stain. We plotted a standard curve on linear graph paper for each plate, using the peak heights (in mm) of the four dilutions of β-lipoprotein standards vs their concentrations. This plot was linear under the conditions just described. The height of the peaks formed after electrophoresis were taken to represent the β-lipoprotein concentration of the standard. The actual immune reactivity in β-lipoprotein consists predominantly of apoprotein B, because this antigen comprises 98% of the protein content of β-lipoprotein (32).

We tested all specimens with a β-lipoprotein concentration exceeding the mean ±2 SD for maternal blood contamination, using a similar electroimmunoassay for IgA. IgA does not cross the placenta and normally is not present in the infant’s peripheral blood or cord blood at birth (33). The immune precipitates on these plates were made visible by staining with Coomassie Blue 250 (1 g/L) in acetic acid/ethanol/water (10/45/45 by vol).

Stability of Cholesterol and β-Lipoprotein

To determine the stability of β-lipoprotein and cholesterol in wet cord blood stored for prolonged periods of time at 4 °C, we measured total cholesterol daily for 15 days, in aliquots of pooled, wet blood, using an enzymic method (Calbiochem-Behring). Aliquots were also spotted on filter paper daily and the β-lipoprotein concentration was determined by electroimmunoassay on the following day.

We also determined the concentration of β-lipoprotein in a pool of cord blood and stored on filter paper, dried, and stored at room temperature at 4 °C for as long as 32 days, to determine whether β-lipoprotein was more stable in wet or dried blood and whether the temperature of storage could influence the results.

Follow-up Studies

Of the 916 neonates who were tested at the time of birth for β-lipoprotein, seven of the 11 infants found to have increased β-lipoprotein in their dried cord blood samples were again tested between two and six months of age, along with their parents, for total cholesterol and triglyceride concentrations. Cholesterol was determined enzymatically with use of cholesterol esterase, cholesterol oxidase, and phenoazone peroxidase to hydrolyze cholesterol esters (Biodynamics/bmc, Indianapolis, IN 46250). The resulting reaction product was made visible at 520 nm in a centrifugal analyzer. Triglycerides were also measured enzymatically by the action of lipase, glycerol kinase, and pyruvate kinase (SmithKline, Sunnyvale, CA 94086).

All seven infants tested had uncomplicated births and their cord bloods were spotted directly onto filter papers at birth, dried, and stored at 4 °C. All but two specimens were analyzed within one week and the remaining two within 20 days of collection.

The remaining four infants were not followed up at this time.
because of complications or stress at birth: prematurity, resuscitation, meconium staining, increased bilirubin, and fetal bradycardia. We believed that these factors might artificially increase the β-lipoprotein and therefore could have introduced bias in this study, as observed in other studies (16). However, these four infants will be followed up later, to determine if perinatal stress does indeed affect β-lipoprotein as well as cholesterol concentrations at birth.

**Results**

The mean concentration of β-lipoprotein in the dried cord blood samples collected was 1.07 (SD 0.46) g/L. Values were arbitrarily considered abnormal when they were >3 SD above the mean—i.e., if they exceeded 1.99 g/L. A typical electrophoretic plate is shown in Figure 1. The height of the peaks for each dilution of a given batch of standard varied by 10–13% from plate to plate. Therefore, we considered it necessary to include a set of standards on each plate.

Figure 2 shows some results of assay of 14 specimens that had β-lipoprotein concentrations >1.99 g/L and were therefore also tested for the presence of IgA. Those specimens with detectable IgA were considered to be contaminated with maternal blood and were discarded from the study as false positives.

The cholesterol concentration remained relatively constant in a pooled specimen of liquid cord blood stored at 4 °C and spotted daily for up to two weeks, whereas the β-lipoprotein concentration decreased steadily during the same period (Figure 3). When blood from the pool was spotted immediately and stored on filter paper at 4 °C, the β-lipoprotein concentration remained almost unchanged for as long as a month (Figure 4), but storage for longer periods led to variable results. Hence, new filter paper preparations of β-lipoprotein standards in cord blood were prepared each month. The β-lipoprotein in the same samples spotted on filter paper and stored at room temperature instead of 4 °C appeared to lose its antigenicity after five days as determined by the appear-

**Follow-up Studies**

Table 1 presents the results of a follow-up study of seven of the 11 infants with increased β-lipoprotein at birth, and of their parents. Of the seven babies studied, five had cholesterol concentrations on follow-up that exceeded our reference interval (determined in the Clinical Chemistry Laboratory of the Children's Hospital of Buffalo) for infants to one year old, 0.50–1.20 g/L.

One parent in each of four families studied had abnormal concentrations of either cholesterol or triglyceride. Four of the seven families had a known family history of heart
Table 1. Family and Follow-up Study of Infants with Above-Normal β-Lipoprotein at Birth

<table>
<thead>
<tr>
<th>Family no.</th>
<th>Cholesterol a,b</th>
<th>Triglycerides c,d</th>
<th>Neonatal β-lipoprotein</th>
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<tbody>
<tr>
<td></td>
<td>Conc. g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>1.88</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1.79</td>
<td>1.69</td>
<td></td>
</tr>
<tr>
<td>Infant (5 mos)</td>
<td>1.38 f</td>
<td>0.92</td>
<td>2.29</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>1.49</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1.89</td>
<td>1.54</td>
<td></td>
</tr>
<tr>
<td>Infant (4 mos)</td>
<td>1.64 f</td>
<td>0.84</td>
<td>&gt;2.50</td>
</tr>
<tr>
<td>3</td>
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<td></td>
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<tr>
<td>Mother</td>
<td>1.35</td>
<td>0.53</td>
<td></td>
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<tr>
<td>Father</td>
<td>2.56 f</td>
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<td></td>
</tr>
<tr>
<td>Infant (1 mos)</td>
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<td>0.60</td>
<td>2.27</td>
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<tr>
<td>4</td>
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<tr>
<td>Mother</td>
<td>2.38 f</td>
<td>1.63</td>
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<tr>
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<td>2.00</td>
<td>0.82</td>
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<tr>
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<tr>
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<td>1.76</td>
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<td></td>
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<tr>
<td>Infant (6 mos)</td>
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<td>0.96</td>
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<tr>
<td>Mother</td>
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<td>1.28</td>
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<tr>
<td>Father</td>
<td>1.11</td>
<td>0.69</td>
<td>2.28</td>
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<tr>
<td>Infant (4 mos)</td>
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<td></td>
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<tr>
<td>Mother</td>
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<td>1.40</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1.61</td>
<td>0.78</td>
<td>2.32</td>
</tr>
</tbody>
</table>

a Adult cholesterol, 95th percentile: white women 20–24 yrs, 2.16 g/L and 25–29 yrs, 2.22 g/L; white men 20–24 yrs, 2.18 g/L and 25–29 yrs, 2.44 g/L (ref. 35).
b Infants to 1 yr: normal cholesterol range 0.50–1.20 g/L (Children’s Hospital of Buffalo). c Adult triglyceride, 95th percentile: white women 25–29 yrs, 1.44 g/L; white men 25–29 yrs, 2.49 g/L (ref. 35). d Infants to 6 mos: normal triglyceride range 0.40–0.92 g/L (ref. 37). e Family history of heart disease. f Above the normal range.

There was no correlation between neonatal β-lipoprotein concentrations and the concentrations of total cholesterol measured at follow-up, but it was certain that five of seven neonates who were hyperbetalipoproteinemic at birth were also hypercholesterolemic at follow-up.

Discussion

A specific assay is needed for the early detection of FH II, because of the high proportion of false-positive results when the detection is based on cholesterol determination (13–15). In particular, a recent study of LDL-cholesterol in the cord blood of 2000 infants from Australia demonstrated that only 0.05% of them with increased LDL-cholesterol at birth still had hypercholesterolemia at follow-up testing (15). Another recent study (18) showed that lipid values lose their discriminating power with increasing age of the individuals being tested, but lipoproteins are still reliable for the oldest decade tested. The conclusion was that the "protein part of lipoproteins is clearly more important than the lipid component in discriminating between atherosclerotic patients and controls" (18).

A novel application of the rocket immunoelectrophoresis technique for measurement of β-lipoprotein in whole dried cord blood has been presented, which may prove applicable to mass screening for FH II and familial combined hyperlipidemia. The mean concentration of β-lipoprotein in dried cord blood in our study, 1.07 (SD 0.48) g/L, was comparable with the β-lipoprotein concentration reported by Andersen and Friis-Hansen: 1.23 (SD 0.37) g/L (36). These investigators selectively measured the low-density lipoprotein (d = 1.025–1.05 kg/L) in cord plasma by radial immunodiffusion (36). The same commercial β-lipoprotein standard from Calbiochem-Behring was used in their study as in our study because an international standard for this substance is still unavailable. Thus, in this case, the results of β-lipoprotein quantitation by radial immunodiffusion of plasma or electroimmunoeassay of dried blood were comparable whereas the same source of standard was used. However, it has been shown in one study that radial immunodiffusion is not generally as reliable as electroimmunoassay for the quantitation of apoprotein B (28).

Our results did not agree with those of Berenson et al. (37), who found a mean β-lipoprotein value of 0.65 (SD 0.22) g/L in the cord blood of 412 infants, but these authors used heparin and Ca++ precipitation to quantitate pre-β and β-lipoproteins. Until an international β-lipoprotein standard is available, the efficacy of any of the methods for β-lipoprotein quantitation involving various β-lipoprotein standards depends on the determination of a mean and SD for the β-lipoprotein values in the sampling studied and cannot be easily compared. Our test is offered primarily as a rapid, inexpensive method for detecting neonates with increased β-lipoprotein concentrations. A more detailed analysis of the infants found to be positive during a mass screening can be done by follow-up testing with use of conventional methods.

Our findings concerning specimen storage suggest that it is essential to spot the cord blood on filter paper promptly at birth—and to store it at 4 °C, because prolonged refrigeration of blood before spotting resulted in a progressive decrease in the antigenicity of the apoprotein B in the β-lipoprotein (Figure 3). It is also necessary to air-dry the spotted specimen thoroughly at room temperature before refrigeration, otherwise the immune precipitation peaks streak during electroimmunodiffusion, which impairs proper measurement. Peaks also streak if dried specimens are stored at 4 °C for longer than one month. This artifact could lead to the misreading of normal values as false positives. If it is necessary to mail the spotted, dried specimens to another laboratory, they may remain at room temperature for three or four days maximum during transit but should be tested promptly thereafter.

The reliability of the β-lipoprotein immunoassay on dried cord blood is demonstrated in the follow-up testing on seven infants who were hyperbetalipoproteinemic at birth with five of these infants hypercholesterolemic at follow-up testing. Furthermore, the cholesterol concentrations of four of these five infants were 2 SD above the mean found in the Bogalusa study of Berenson et al. (37) for the age group from birth to six months. In comparison with another study of bimonthly cholesterol quantitation from birth to seven months (38), four of our hypercholesterolemic infants were over the reported 80th percentile for their age and, of these, one was over the 90th percentile and one was over the 95th percentile.

It is apparent from our follow-up study that at least kindreds three and four (Table 1) are carriers for familial hyperlipoproteinemia, probably type II. The cholesterol and triglyceride concentrations in kindreds one through five will be determined again when the infants are one year old. If any or all of these infants remain hypercholesterolemic at one postnatal year, the frequency of FH II in our sampling of 916 infants, using the dried blood immunoassay for detection, will be within the reported (1) frequency range of 0.2–0.5%.

The clinical value of our test is also demonstrated by its ability to eliminate false positives resulting from contamination of the cord blood with maternal blood; the dried specimens are screened for HbA content by the same immunoelectrophoretic technique.

We hope that, in a larger sampling, the three-day heel-prick specimen of infant blood will prove reliable for screening purposes, because this specimen is already routinely collected for phenylketonuria determination and other tests. We intend to study, in parallel, the cord blood and three-day specimens
of infants born to families genetically at risk for FH II, to determine if the two sorts of specimens are interchangeable for heterozygote detection.

An electromunoassay has been reported for measurement of α-fetoprotein in dried whole blood (39). However, the technique requires pre-elution of the blood specimens and the use of complicated double-compartment assay plates. To our knowledge, the procedure we describe here is the first attempt to detect FH II heterozygotes from dried blood specimens. The test does not require sample elution nor is it necessary to cut wells into the gel for sample insertion. The use of dried specimens eliminates many storage and handling problems associated with the collection of large liquid samples. The test itself could be easily adapted for mass screening by using larger electrophoretic plates and a punch index machine to rapidly deposit filter paper discs on the surface of the gel. The cost of the test, currently about 15 to 20¢ per sample when 12 samples are run per plate, would be even less when performed on a larger scale. Our test does not differentiate between pre-β- (VLDL) and β-lipoprotein (LDL). However, the test must primarily measure LDL in the newborn because very little, if any, VLDL is present at birth (40). The fact that some parents in our follow-up study had increased cholesterol and (or) triglyceride concentrations may indicate that we are also detecting families at risk for mild combined hyperlipidemia as well as FH II. A differentiation among disorders involving these two classes of lipids can be performed at follow-up by use of techniques such as electrophoresis and ultracentrifugation.

Although the treatment of infants heterozygous for FH II is still controversial, the fact remains that the disease is often not detected until adulthood, when overt cardiovascular symptoms become apparent, and by then it is very difficult to reverse the existing atherosclerosis. Mass screening by use of previously existing methods has been too expensive and detection rates low. When performed on a large scale for screening purposes, the present procedure may prove to be the method of choice for detecting FH II and related familial hyperlipidemias at birth.

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