We describe an electroimmunodiffusion technique for measuring $\alpha_1$-fetoprotein in blood spotted on chromatography paper. The system is being used as a complementary test in a neonatal mass-screening program for detection of inborn metabolic diseases in the Province of Quebec. In a series of 102 cases of neonatal hypertryosinemia, the test has proven to be highly discriminative for hereditary tyrosinemia. It has permitted early detection of eight cases of this disease, including two that would have been missed by the previously used screening procedure, tyrosine measurement only. The test not only virtually eliminates the risk of misdiagnosis or missed diagnosis, but also permits earlier diagnosis of hereditary tyrosinemia and considerably reduces the follow-up work required for newborns with transitory tyrosinemia. The AFP test is simple, fast, practical, and inexpensive. Combined with tyrosine determination, it constitutes an optimal device for mass screening of hereditary tyrosinemia.

Hereditary tyrosinemia is an autosomal recessive metabolic disease that has a relatively high prevalence in the Province of Quebec. In some parts of the province prevalence of asymptomatic carriers (heterozygotes) among the population is as high as 1:14 and the disease is manifested (homozygotes) in one of every 685 live births (1).

The provincial neonatal mass-screening program for detection of hereditary tyrosinemia and other genetic diseases in the Province of Quebec is partly based on chemical analyses performed on blood samples spotted on strips of chromatography paper (2); a first series of tests ("first test") is performed early, an average of four days after birth, and tests disclosing abnormal results are repeated about a month (average 28 days) later ("second test").

Determination of the tyrosine concentration in the paper-spotted blood, the basis of the current screening test for hereditary tyrosinemia, is not completely satisfactory because there is a high frequency of physiological "transitory tyrosinemia." In the Province of Quebec, 1% of all newborns have, at birth, blood tyrosine concentrations of 75 mg/liter (upper normal limit) or greater (1), while at the time of the "second test" blood tyrosine concentrations of "true" tyrosinemics may be within the normal range. Other biochemical abnormalities commonly associated with hereditary tyrosinemia (4) cannot be used for early detection of disease; evaluation of hypoprothrombinemia, hypermethioninemia, hyperphenylalaninemia, or hypoglycemia are either technically unusable or are inconstant or non-discriminatory features of the disease.

In 1972, one of us reported the presence of abnormally high concentrations of $\alpha_1$-fetoprotein (AFP) in the blood of all patients suffering from hereditary tyrosinemia (5). Because these values were found to be normal in transitory tyrosinemia (6) and in other inborn metabolic diseases (7), the possibility was raised that AFP determination might be used as a discriminative test for early detection of hereditary tyrosinemia.

Here, we describe the development of a simple and practical electroimmunodiffusion technique for quantitative determination of AFP in paper-spotted samples of dried blood and its use as a highly discriminative test for neonatal screening of hereditary tyrosinemia when done on samples with high blood tyrosine concentration at the first test and on all samples at the second test.

**Material**

**Anti-AFP Serum and AFP Standards**

Monospecific antisera against AFP were obtained from rabbits that had been immunized with pure antigen isolated from pooled cord blood serum by the method of Nishi (8).
AFP standards were prepared from stocks of pure antigen quantitated by electroimmunodiffusion by reference to the World Health Organization standard (kindly supplied by Dr. Ph. Sizaret, International Agency for Research on Cancer, Lyon, France); dilutions were made in phosphate-buffered saline (PO₄⁻³·0.1 mol/liter, NaCl 0.15 mol/liter, pH 7.4) containing 10 g of bovine serum albumin per liter.

Agarose: Agarose powder was obtained from Bio-Rad Laboratories, Richmond, Calif. 98404.

Buffer: Barbital buffer, 50 mmol/liter, pH 8.6.

Staining and destaining solutions: The staining solution consisted of a 9/2/9 by vol mixture of ethanol/glacial acetic acid/distilled water, containing, per liter, 5 g of Coomassie Brilliant Blue R (Sigma Chemical Co., St. Louis, Mo. 63178). The destaining solution had the same composition except for the Coomassie Blue.

Electrophoresis chamber: A Spinco paper electrophoresis chamber was used (Model R, Series D) combined to a Gelman power supply (Model 38201).

Method
Preparation of Samples

Test samples were prepared by eluting (2 h at room temperature) dried blood from 7.94-mm paper disks (Schleicher and Schuell No. 903c) into 0.25 ml of 0.15 mol/liter NaCl.

Preparation of Gel Plates

Attempts to run these whole blood specimens in conventional electroimmunodiffusion gel plates (9) resulted in substantial nonspecific precipitation of protein, which precluded analysis of immune precipitates. To eliminate this problem, we devised double-compartment plates such that the nonspecific precipitation is allowed to take place in an antibody-free section of the gel while AFP-anti-AFP immune precipitation takes place in a second, antibody-containing, gel section (Figure 1).

Preparation of the plates can be summarized as follows. A "non-antibody gel solution" is first prepared by dissolving, at 90 °C, 1 g of agarose and 20 mg of thimerosal (Sodium Merthiolate), a preservative, in 100 ml of barbital buffer, and pouring 16 ml of this solution on to 80 × 100 mm glass plates. After the gel has solidified, a straight cut, perpendicular and median to the smaller section of plate, is made in the gel and one of the two halves is scraped off. The antibody-containing section of the plates is then prepared by adding an optimal amount of anti-AFP serum to the basal gel solution (maintained at 58–60 °C) and pouring 8 ml of this solution onto the bare part of the plates. When the gel has solidified, plates are stored in a humidified chamber at 4 °C.

Assay

A series of wells (about 2 mm diameter, about 4 μl capacity, and about 6 mm apart) is punched at about 10 mm from the cathodic edge ("no antibody" side) of a plate. The plate is transferred to an electrophoresis chamber and a 10 mA current is immediately applied to the gel (to avoid radial diffusion of samples during the filling operation). Wells are then filled with either 3 μl of standard samples (5 to 50 mg/liter) or 9 μl (three consecutive 3-μl applications) of test samples. When this filling operation is completed, the current is increased to 50–60 mA and electrophoresis is continued for 90 min. The plate is then washed overnight in 0.15 mol/liter NaCl, dried at 100 °C for 4 h, and stained by incubation in staining solution for 5 min. Background coloration is removed by washing twice, for 5 min each, with destaining solution. As shown in Figure 1, the double-compartment plates permit one to completely separate AFP-anti-AFP precipitates from nonspecific precipitates, thus allowing adequate assessment of immune rockets.

The calibration curve is constructed by plotting AFP-anti-AFP peak height of standard samples as a function of the AFP content. Values for AFP in blood samples is obtained by multiplying the values from the curve by a "correction factor" (see Results).

Results
Method

By comparing a series of AFP values determined for dried-blood samples with those obtained for paired and matched samples of fresh serum, we found that a correction factor ranging from 12 to 16 must be introduced, to convert spotted blood AFP values into concentration in serum [this figure (12 to 16) is actually higher than the theoretical correction factor; if a 50% hematocrit is assumed, triple application eluates of spots containing 20 μl of dried-blood eluted in a total volume of 250 μl of saline yields a calculated multiplication factor of only 8.3; this discrepancy may be attributable to various factors including viscosity effects, incomplete elution of samples, and partial denaturation or nonspecific precipitation of AFP]. By using 15 as a standard correction factor for all paper-spotted blood samples, the
maximal discrepancy between serum and blood spot results is 25%. Although the accuracy index is admittedly poor, it in no way affects the practical usefulness of the system (see next section).

Screening

We included in this study 94 cases of neonatal transitory tyrosinemia and eight cases who proved to be cases of hereditary tyrosinemia on subsequent hospitalization. To be included in this series, the cases of transitory tyrosinemia had to have, on both the first and the second test, a screening form duly filled (birth date, sample collection date, birth weight, and sex), a good blood sample so that both tyrosine and AFP concentration could be measured, and a birth weight of 2.5 kg or more (the reasons for this are discussed in the next section).

The results show that, at the first test, all eight true tyrosinemics had serum AFP concentrations exceeding 150 mg/liter, and four of them had values greater than 300 mg/liter. By contrast, 66% of the cases of transitory tyrosinemia had AFP concentrations of less than 150 mg/liter and only 2% exceeded 300 mg/liter. Thus, even at the first test, this AFP determination is shown to have a very significant discriminating capacity for hereditary tyrosinemia.

At the second test, the AFP values become fully discriminative. Only four cases of transitory tyrosinemia had detectable (30 mg/liter) AFP values; the highest value recorded in this group was 50 mg/liter. By contrast, all true tyrosinemics had AFP concentrations exceeding 144 mg/liter (range 144–600 mg/liter).

We emphasize here that, of the eight patients with hereditary tyrosinemia, two had normal blood tyrosine concentrations (35 and 56 mg/liter, see Figure 2) at the second test and so would have been missed by the usual screening procedure. Therefore, in contrast with tyrosine determination, both discriminating and detecting capacities of AFP determination at the second test have been maximal in this series.

Discussion

Our results clearly demonstrate the detecting and discriminating efficiency of the AFP test when used in addition to blood tyrosine determination in screening for hereditary tyrosinemia. Actually, it may be concluded that in the context of the screening program, by the time the second test is performed, abnormal serum AFP concentrations are virtually diagnostic of hereditary tyrosinemia. Indeed, the association of an increase in both tyrosine and AFP values in other potentially AFP-inducing neonatal conditions, namely hepatitis and biliary atresia (10–12), will eventually be encountered, but will easily be distinguished from hereditary tyrosinemia on many clinical and biochemical grounds, including lower AFP values (11, 12). Because all tyrosinemics we have studied to this time have had abnormal AFP values and only one case out of 34 was reported to have been missed by our program because he had a tyrosine concentration of less than our 75 mg/liter threshold value at the first test (it was 74 mg/liter), we infer that the combination of tyrosine determination as the first-line screening test and AFP measurement as the complementary test virtually eliminates the risk of misdiagnosis or missed diagnosis. Indeed, the added AFP measurement allows us to lower our tyrosine first-test threshold, thus permitting us to have a more reliable system without increasing follow-up work.

Another important practical advantage emerges from this study. As mentioned above, 66% of transient tyrosinemics in this group weighing 2.5 kg or more at birth had an AFP concentration in their serum of less than 150 mg/liter (the lowest true tyrosinemic value) at the first test (see Figure 2). By keeping an empirical security margin of 50 mg/liter, use of the AFP test would eliminate the need to follow up hypertyrosinemics of less than 100 mg/liter, which is to say one-half of the transitory cases of this series. By including premature babies in the AFP screening (who represent 50% of neonatal hypertyrosinemics), we figure that, on the basis of this 100 mg/liter threshold, the total follow up will be reduced by 40 to 50%.

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3 The reported findings of high serum AFP concentrations in Indian childhood cirrhosis (13) and in cystic fibrosis (14) have not been confirmed (15–19).
At this point, we would like to explain that this study has been restricted to the high-weight group (2.5 kg or more at birth) because all of our 34 cases of hereditary tyrosinemia cited above were in that group. Because some true tyrosinemics should be among the low-weight group, we now measure AFP in every hypertyrosinemic at the first test. Preliminary results indicate that the AFP values in the low-weight group are about the same at birth as in the high-weight group.

Another advantage of this AFP test is that it permits earlier diagnosis, and thus treatment can be begun earlier in patients with hereditary tyrosinemia. For those having AFP concentration of 300 mg/liter or more at their first test, we have introduced a special follow-up system. Since the data presented here were collected, two more true tyrosinemics were detected; they had a serum AFP concentration greater than 300 mg/liter at four days of age. Adding them to the series of eight presented before and extrapolating, our system should detect 60% of all cases of hereditary tyrosinemia within the first two weeks of postnatal life.

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