Background: Hereditary tyrosinemia type I (HT) fulfills the criteria for inclusion in neonatal screening programs, but measurement of tyrosine lacks clinical specificity and quantitative assay of succinylacetone is laborious. We developed a semiquantitative assay based on inhibition of δ-aminolevulinate dehydratase (ALA-D) by succinylacetone.

Methods: Preincubation of 3-mm discs from dried-blood spots and reaction of the enzyme with δ-aminolevulinic acid as substrate were performed in microtiter plates. After separation of the supernatant and 10 min of color reaction with modified Ehrlich reagent, the formation of porphobilinogen was measured at 550 nm in a plate reader.

Results: The detection limit for succinylacetone was 0.3 µmol/L; imprecision (CV) was <5.5% within-run and 10–16% between-run. Storage of blood spots at ambient temperature for several days led to a significant decrease of ALA-D activity. Enzyme activity was lost in filter cards at 45 °C, but remained stable at 2–37 °C. Enzyme activity was decreased in EDTA blood. The absorbance at 550 nm was 0.221 (± 0.073) in healthy neonates and 0.043–0.100 in 11 patients with HT. All neonates with increased tyrosine (above the 99.5th centile) in neonatal screening (97 of 47 000) had normal results by the new assay.

Conclusions: The spectrophotometric microassay for ALA-D is a simple and sensitive test for HT. This represents a basis for further examination of its general reliability as a confirmatory test if tyrosine is found to be increased.

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Enzymes and Protein Markers

Spectrophotometric Microassay for δ-Aminolevulinate Dehydratase in Dried-Blood Spots as Confirmation for Hereditary Tyrosinemia Type I

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The primary enzyme defect in hereditary tyrosinemia type I (HT; McKusick 276700) has been attributed to a deficiency of fumarylacetoacetase (EC 3.7.1.2) (1). Untreated HT can lead to liver and renal failure, rickets, porphyric and neurologic crises, and hepatocarcinoma. Some infants with the severe neonatal form of HT die without treatment within the first months of life. Because the initiation of treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (2) has become a favorable therapeutic option when treatment starts early (3), HT fulfills the criteria for neonatal screening (4).

Several groups have searched for optimized analytical methods to include tyrosinemia in a neonatal screening setting. Tyrosine measurement solely in dried-blood spot (DBS) specimens lacks specificity (9) because various pathologic conditions, including other disorders in tyrosine catabolism (10) and benign transient hyper tyrosinemia of the newborn (9, 10), can lead to increased tyrosine. A simple assay of immunoreactive fumarylacetocetase in erythrocytes has been developed by Laberge et al. (9), but fumarylacetocetase deficiency may not be accompanied by a decrease in immunoreactive protein (11) and may give false-negative results after blood transfusions (9).

Succinylacetone is a potent noncompetitive inhibitor of ALA dehydratase (ALA-D; EC 4.2.1.24) (12–14). Its measurement is therefore reliable for early diagnosis of HT. This method has been used in Quebec, Canada, where the prevalence of HT (1:1846) is high in the French-Canadian

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1 Nonstandard abbreviations: HT, hereditary tyrosinemia type I; ALA, δ-aminolevulinic acid; DBS, dried-blood spot; ALA-D, ALA dehydratase; TCA, trichloroacetic acid; and DTT, dithiothreitol.
population (15), but it is too laborious for general neonatal screening. Indirect detection of succinylacetone by a simple microtiter plate assay of ALA-D was recently reported by Holme and Lindstedt (16). However, this assay lacks of sensitivity because it requires visual judgment of the color reaction. We refined this assay as a semiquantitative spectrophotometric test and investigated its reliability as confirmatory test for HT in a setting of neonatal screening.

**Materials and Methods**

**Reagents and Solutions**

MES (cat. no. 6126), trichloroacetic acid (TCA; cat. no. 100807), mercuric chloride (cat. no. 4419), dimethylaminobenzaldehyde (cat. no. 3085), and perchloric acid (cat. no. 0514) were purchased from Merck. Tris (cat. no. T1503), ALA (cat. no. A3785), and succinylacetone (cat. no. D1415) were purchased from Sigma. Dithiotreitol (DTT; cat. no. 197777) was from Boehringer Mannheim, and glacial acetic acid (cat. no. 6771.1) were from ROTH. All reagents were analytical grade. Distilled water was used for the preparation of all aqueous solutions.

MES buffer (50 mmol/L, pH 6.4) was prepared by dissolving 10.66 g of MES in 900 mL of distilled water, adjusting the pH to 6.4 with 2 mol/L sodium hydroxide, and bringing the volume to 1000 mL with distilled water; the prepared buffer was stored at 4 °C. For 2.5 mol/L Tris, 30.3 g of Tris was dissolved in distilled water and brought to a total volume of 100 mL with distilled water; the prepared buffer was stored at 4 °C. DTT solution (21 mmol/L) was prepared fresh by dissolving 16.2 mg of DTT in 5 mL of 50 mmol/L MES. ALA (30 mmol/L, pH 6.0) was prepared fresh by dissolving 25.2 mg of ALA in 4.5 mL of distilled water, adjusting the pH to 6.0 with 2.5 mol/L Tris, and bringing the volume to 5 mL with distilled water. TCA (125 g) and mercuric chloride (10 g) were dissolved in distilled water, brought to a volume of 1000 mL, and stored in an amber glass bottle at ambient temperature.

The modified Ehrlich reagent consisted of 1.0 g of dimethylaminobenzaldehyde dissolved in 30 mL of glacial acetic acid and 8 mL of 700 g/L perchloric acid, and brought to a volume of 50 mL with glacial acetic acid. The reagent was prepared fresh and used within 6 h.

**Preincubation**

Discs (3 mm) of DBSs on filter paper (S&S 2992; Schleicher & Schuell) were punched out into microtiter wells, and 50 μL of 21 mmol/L DTT was added to each well. Plates were gently shaken (100 rpm) on a shaker at room temperature for 15 min.

**ALA-D Reaction**

ALA-D in erythrocytes catalyzes the formation of porphobilinogen from ALA. ALA-D activity was estimated as an end-point determination. ALA (10 μL of a 30 mmol/L solution) was added to each sample. For blanks, 10 μL of 50 mmol/L MES was used instead of ALA. The enzyme was allowed to incubate for 4 h at room temperature with gentle shaking (100 rpm) during the whole incubation period. The reaction was stopped by the addition of 40 μL of the TCA–mercuric chloride mixture. After complete mixture was obtained by repeated aspiration, the liquid samples were transferred into Eppendorf cups and centrifuged at 390g for 30 s; 80 μL of each supernatant was then transferred to microtiter plates.

**Color Reaction and Measurement**

For the color reaction, 100 μL of freshly prepared modified Ehrlich reagent was added. A purple color produced by porphobilinogen appeared within 1 min and increased for another 5 min before the reaction mixture turned brown because of solubilization of hemoglobin. Blanks and samples from tyrosinemic patients remained straw-colored. The reaction was judged visually within these 5 min, but the color development reached a maximum after 8–10 min (Fig. 1). The absorbance of porphobilinogen was measured at 550 nm on a microtiter plate spectrophotometer (Multiscan; Labsystems). The color reaction time before reading was set at 10 min for all additional experiments.

**Samples**

DBS specimens from neonates from the regional neonatal screening program (Baden-Württemberg, Germany) were used. The blood collected by a heelstick on days 3–5 (median, day 4) of life was spotted on filter paper, allowed to dry, and sent via “post mail” to our laboratory. Tyrosine was measured by electrospray tandem mass spectrometry as described previously (17). All samples with tyrosine above the decision point of 384 μmol/L were used.

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Fig. 1. Color development over time after the addition of modified Ehrlich reagent plotted against absorbance at 550 nm.

Six different filter-paper samples (in duplicate) were measured with ALA in the assay (●), and six discs punched from a single DBS were measured without ALA in the assay as blanks (□). Data are the mean ± SE (error bars).
(99.5th centile of the healthy newborn population) were evaluated by the ALA-D assay.

DBS specimens from 11 patients suffering from HT were assessed by the ALA-D assay and by tandem mass spectrometry. All patients had been diagnosed recently on the basis of clinical suspicion and additional metabolic work-up. We received the neonatal screening samples (patients 1–5, 7, and 9–11) from the pediatricians caring for the infants or from the screening laboratories after informal consent of parents. These DBS specimens were measured after different storage times. The samples taken at the time of diagnosis (patients 1, 2, 6, 8, and 9) were measured immediately. This study was performed in accordance with the current revision of the Helsinki Declaration of 1975.

Statistics
Data are expressed as the mean ± SD or as the mean ± SE if indicated. Statistical significance was determined using the nonparametric Mann–Whitney test. Differences with P values < 0.01 were considered significant.

Results and Discussion
Method validation (analytical variables)
Linearity and limit of detection. Succinylacetone [final concentrations, 0.001–100 μmol/L (Fig. 2A) and 0.1–2.0 μmol/L (Fig. 2B)] was added to whole blood from a healthy volunteer; the blood was then spotted on filter paper. Nearly complete inhibition of ALA-D activity occurred at 10 μmol/L succinylacetone (Fig. 2A). The limit of detection for succinylacetone was calculated as the mean absorbance minus 3 SD (0.205–0.035) for blood without added succinylacetone. The resulting absorbance of 0.170 corresponded to 0.3 mmol/L succinylacetone (Fig. 2B). ALA-D activity decreased markedly but nonlinearly at succinylacetone concentrations between 0.1 and 1.5 μmol/L (Fig. 2B). DBS specimens enriched with 0.1–2.0 μmol/L succinylacetone and stored at −70 °C were used for the daily calibration curve. The addition of succinylacetone only at the start of the color reaction revealed no interference at 0.001–100 μmol/L succinylacetone (data not shown).

Imprecision. We assessed the within-run imprecision (CV) of the whole method, using eight discs of a single DBS. The imprecision was estimated for samples and blanks (i.e., DBS measurements with or without ALA in the assay, respectively). The between-run CV was calculated using discs from six different DBS specimens assayed in duplicate on 8 consecutive days. The within-run CV for the sample (mean absorbance ± SD, 0.303 ± 0.016) and blank (0.040 ± 0.002) was < 5.5%. The between-run CV was 16% (mean absorbance ± SD, 0.167 ± 0.027) and 10% (0.045 ± 0.005) for the sample and blank, respectively. The CV of the sample was high and necessitated the use of daily calibration curves.

Effect of storage time on ALA-D activity in DBSs. The ALA-D activity in DBSs declined when the filter paper was stored at ambient temperature for several days (Fig. 3). This observation points to the instability of the enzyme in dried blood. The absorbance decreased logarithmically to 0.130 after ~7 weeks. After that time, residual enzyme activity could still be clearly distinguished from complete enzyme inhibition (absorbance ~ 0.075). DBS specimens from neonates sent via post mail usually reached the screening laboratory after 1–2 days and were assayed for ALA-D 2 days later, if the measured tyrosine concentration was high. Therefore, we used samples from healthy newborn as controls, which were stored for 4 days (Table 1). In cases of older samples, control values adopted for the respective storage time were used.

Effect of temperature on ALA-D stability in DBSs. Filter cards transported via post mail may be exposed to variable temperatures. We therefore investigated the stability of
the ALA-D enzymatic activity by exposing DBSs from a healthy volunteer to various temperatures over a 24-h period before measurement. The mean absorbance (± SD) of 12 measurements was 0.244 ± 0.029, 0.269 ± 0.034, 0.246 ± 0.059, 0.237 ± 0.022, and 0.190 ± 0.015 for samples stored at 2, 18, 26, 37, and 45 °C, respectively. The decrease in absorbance at 45 °C was significant (P < 0.001) and pointed to the loss of enzyme activity at higher temperatures, whereas the enzyme remained stable at 2–37 °C.

**Effect of blood additives.** We estimated the influence of common blood additives on the ALA-D assay. Sample tubes coated with EDTA or citrate additive were filled with 1 mL of blood from a healthy volunteer and gently shaken. The blood was spotted on filter paper and assayed for ALA-D. The mean absorbance (± SD) of six experiments was compared with the DBS results obtained for blood from the volunteer, that was not pretreated and for blanks. Citrate additive (mean absorbance ± SD, 0.173 ± 0.007) showed no obvious influence on the ALA-D assay compared with the control (0.189 ± 0.011), whereas the absorbance of the EDTA samples (mean absorbance ± SD, 0.081 ± 0.015) was significantly decreased and close to that of the blanks (0.078 ± 0.015). Thus, ALA-D activity is decreased in EDTA blood.

**ALA-D assay in controls and in patients with HT**

Typical values for neonates are shown in Table 1. The interpretation strongly depends on the time interval between blood sampling and measurement.

Children at the age of 2 years had a significantly lower ALA-D activity compared with neonates matched for the time of sample storage (Table 1). The increased activity in the neonatal population probably reflects an increased frequency of young red blood cells, which have higher ALA-D activity (18, 19).

The ALA-D activity in neonates with tyrosine concentrations above the 99.5th centile in the neonatal screening was not different from the activity in neonates with below the 99.5th centile tyrosine (Table 1). Both groups were matched for age and time of sample storage.

DBS specimens from 11 patients with HT were investigated for ALA-D activity and tyrosine concentration. All specimens were positive in the assay. For samples matched for age and storage time, the absorbance obtained for the HT samples in the ALA-D assay was always markedly below the reference values (Table 1). Retrospective investigation of neonatal screening specimens from patients 1, 3–5, 7, and 9–11 would have led to a correct diagnosis possible at this time. Only for patient 2, whose neonatal screening card was 2.5 years old at the time of the assay, were we unable to discriminate and make a diagnosis because of the complete loss of ALA-D activity in controls after that time (Table 1).

With respect to the impact of neonatal screening for HT, it is of interest to contemplate the individual cases investigated to date. Patient 1 was diagnosed during a life-threatening event what could have been prevented by earlier diagnosis. Patients 2 and 8 presented with impaired liver function tests (transaminases and coagulation patterns). A girl (patient 8) was detected only in the context of family studies after her younger brother (patient 9) was found to have HT. This premature boy was detected because of prolonged clotting time and phosphaturia. His condition, after timely initiation of treatment, was excellent at the time of publication. Patients 6 and 7 suffered from the severe neonatal form of HT with liver failure and bleeding tendency, which manifested shortly after birth. In these patients, an earlier diagnosis by means of neonatal screening for tyrosinemia could probably have prevented the severe clinical course. In patients 3 and 9, increased tyrosine was observed by tandem mass spectrometry screening. However, these infants were not monitored further because of the screening policies of the respective screening laboratories, which were based on the lack of specificity of increased tyrosine.

**Preliminary results of the ALA-D assay in neonatal screening**

In 97 of 47 000 neonates in the regional screening program in Baden-Württemberg, Germany, investigation of tyrosine revealed a concentration above the decision limit of 384 μmol/L (99.5th centile of the healthy newborn population). Tyrosine concentrations were 397–1521 μmol/L (median, 517 μmol/L) in these 97 neonates. The results obtained in the ALA-D assay were interpreted visually for the first 40 samples and measured spectrophotometrically in the latter 57 samples. Measurements revealed no result suspicious for HT (Table 1). The missing of any affected newborn is not surprising because of the low prevalence.
of HT: ~1 in 100,000 neonates in the German population. On the basis of this prevalence and a cutoff based on the 99.5th centile, an additional 500 ALA-D tests would be required for each true positive.

The use of daily calibration curves for succinylacetone inhibition, as shown in Fig. 2B, improved the evaluation of ALA-D test results. Actually, we are using 200 μmol/L tyrosine (92nd centile) as a cutoff for performing the ALA-D test, and an absorbance cutoff for the ALA-D assay corresponding to the inhibition of ALA-D by 0.75 μmol/L succinylacetone. Since the original study, an additional 722 DBS specimens from a total of 38,200 neonates have been tested with the ALA-D assay. A total of 18 tests were false positive, producing a recall rate of 0.05%. To date, no true-positive infant has been found. Our experience has been too limited for a final assessment of the sensitivity of the “second tier” screening strategy with the ALA-D assay, but the 11 tyrosinemic patients measured to date would all have been detected. The sensitivity was 100% in all of the patients whose neonatal DBS specimens could be assayed (8 of 11).

In conclusion, the results obtained in this study of the spectrophotometric microassay for ALA-D represent a basis for further examination of its general reliability as a confirmatory test if increased tyrosine is found in neonatal mass screening throughout the world. Measurement of the inhibition of ALA-D by succinylacetone in DBSs is a simple method easy to apply in a setting for neonatal screening laboratories. The sensitivity of the assay was improved by spectrophotometric quantification of the color development and overcomes the previous disadvantage (16). In the original assay, the need for visual judgment after 5 min, when the color reaction had not yet reached its maximum, restricted the sensitivity in the critical span between healthy and affected infants. That is important because serum succinylacetone concentrations down to 2 μmol/L were found in neonates with HT (20), whereas a stable-isotope dilution assay measured succinylacetone concentrations of 0.005–0.163 μmol/L (mean, 0.044 μmol/L) in plasma of healthy controls (21). The availability of the ALA-D assay allows a second-tier strategy in neonatal screening for HT. As a first-line screening strategy, tyrosine should be measured, e.g., by tandem mass spectrometry, which is used by an increasing number of screening laboratories. If the tyrosine concentration is increased, the enzyme assay should be performed. The cutoff point for tyrosine has to

### Table 1. ALA-D assay in blood spots of controls, neonates with increased tyrosine, and patients with HT.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age, mean ± SD</th>
<th>Sample storage time, mean ± SD</th>
<th>n</th>
<th>Tyrosine (mean ± SD), μmol/L</th>
<th>ALA-D (mean ± SD) absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy neonates, 5 days of age (groups matched for sample storage time)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (4 days)</td>
<td>4.5 ± 0.8 days</td>
<td>3.8 ± 0.9 days</td>
<td>82</td>
<td>132 ± 45</td>
<td>0.221 ± 0.073</td>
</tr>
<tr>
<td>Group 2 (10 days)</td>
<td>4.9 ± 0.4 days</td>
<td>10.1 ± 4.5 days</td>
<td>18</td>
<td>NM</td>
<td>0.215 ± 0.044</td>
</tr>
<tr>
<td>Group 3 (18 days)</td>
<td>4.9 ± 1.5 days</td>
<td>17.5 ± 1.4 days</td>
<td>57</td>
<td>136 ± 54</td>
<td>0.173 ± 0.036</td>
</tr>
<tr>
<td>Group 4 (1 year)</td>
<td>5.0 days</td>
<td>1 year</td>
<td>24</td>
<td>94 ± 48</td>
<td>0.099 ± 0.018</td>
</tr>
<tr>
<td>Group 5 (2 years)</td>
<td>5.0 days</td>
<td>2 years</td>
<td>24</td>
<td>70 ± 30</td>
<td>0.051 ± 0.010</td>
</tr>
<tr>
<td>Healthy children, 2 years of age</td>
<td>2.1 ± 0.5 years</td>
<td>10.3 ± 4.3 days</td>
<td>18</td>
<td>99 ± 28</td>
<td>0.161 ± 0.022^b</td>
</tr>
<tr>
<td>Neonates with increased tyrosine</td>
<td>5.0 ± 2.3 days</td>
<td>17.8 ± 6.9 days</td>
<td>57</td>
<td>574 ± 191 (406–1521)^c</td>
<td>0.158 ± 0.045^c</td>
</tr>
<tr>
<td>Patients with HT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>Neonatal screening</td>
<td>5 days</td>
<td>3 months</td>
<td>403</td>
<td>0.057</td>
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<tr>
<td></td>
<td>Time of diagnosis</td>
<td>25 days</td>
<td>7 days</td>
<td>2325</td>
<td>0.070</td>
</tr>
<tr>
<td>Patient 2</td>
<td>Neonatal screening</td>
<td>5 days</td>
<td>2.5 years</td>
<td>733</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Time of diagnosis</td>
<td>2.5 years</td>
<td>14 days</td>
<td>451</td>
<td>0.043</td>
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<tr>
<td>Patient 3</td>
<td>Neonatal screening</td>
<td>4 days</td>
<td>8 months</td>
<td>891</td>
<td>0.045</td>
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<tr>
<td></td>
<td>Neonatal screening</td>
<td>5 days</td>
<td>10 months</td>
<td>409</td>
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<td>Patient 4</td>
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<td>6 days</td>
<td>9 months</td>
<td>626</td>
<td>0.044</td>
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<td></td>
<td>Neonatal screening</td>
<td>4 days</td>
<td>18 days</td>
<td>19 days</td>
<td>630</td>
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<tr>
<td>Patient 5</td>
<td>Neonatal screening</td>
<td>3 days</td>
<td>27 days</td>
<td>229</td>
<td>0.044</td>
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<td>Patient 6</td>
<td>Neonatal screening</td>
<td>1.7 years</td>
<td>9 days</td>
<td>543</td>
<td>0.062</td>
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<td>Patient 7</td>
<td>Neonatal screening</td>
<td>4 days</td>
<td>19 days</td>
<td>320</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>Time of diagnosis</td>
<td>13 days</td>
<td>4 days</td>
<td>548</td>
<td>0.053</td>
</tr>
<tr>
<td>Patient 8</td>
<td>Neonatal screening</td>
<td>5 days</td>
<td>11 months</td>
<td>231</td>
<td>0.047</td>
</tr>
<tr>
<td>Patient 9</td>
<td>Neonatal screening</td>
<td>2 days</td>
<td>2 months</td>
<td>574</td>
<td>0.100</td>
</tr>
</tbody>
</table>

^a NM, not measured.  
^b P < 0.001 vs group 2, matched for sample size and sample storage time.  
^c Range.  
^d No significant differences vs group 3, matched for age, sample size, and sample storage time.
be set between the 90th and 95th percentiles because neonates with HT may present with only mildly increased tyrosine concentrations (9). False-positive results for the enzyme assay may be expected if DBSs are exposed to high temperatures, in cases of hereditary ALA-D deficiency (only six cases have been reported to date), or theoretically in cases of lead exposure via cord blood or enrichment in water used for formula feeding (22, 23). The second-tier strategy may lead to an optimal sensitivity, concomitantly omitting a high false-positive rate, thus avoiding the emotional and economic costs incurred by follow-up of high numbers of unaffected infants.

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