unknown. Now that a procedure is available for quantifying HDL-phospholipids in population studies, large-scale epidemiological studies are now underway in our laboratory to determine the role of various HDL components in the prediction of coronary atherosclerotic risk.

We thank Mrs. M. Lenzyk and Mr. W. Rensinghoff for excellent experimental work and technical assistance.

References

Enzyme Immunoassay of Thyroxin-Binding Globulin in Dried Blood Samples on Filter Paper
Naoshige Hata,1 Masao Ito,3 Hitoshi Mizuta,1 Osamu Nose,2 and Kiyoshi Miyai1

A double-antibody enzyme immunoassay was developed for determination of thyroxin-binding globulin in dried blood samples on filter paper. The measurable concentration range of thyroxin-binding globulin in two 3-mm blood discs was 3.3 to 52 mg/L equivalent of serum (i.e., equivalent to the concentrations in known serum standards). Thyroxin-binding globulin in dried blood samples on filter paper was stable for at least four weeks when kept dry at −20 °C, 4 °C, or room temperature. The mean coefficients of variation were 6.6% (within assay) and 5.9% (between assays). The concentrations of thyroxin-binding globulin in dried blood samples determined by this method correlated well with those in serum determined by radioimmunoassay (r = 0.95) and by enzyme immunoassay (r = 0.96). This method is applicable for detecting cases of thyroxin-binding globulin deficiency and avoids the false-positive results for neonatal hypothyroidism obtained by measuring thyroxin.

Additional Keyphrases: screening • hypothyroidism • neonates • reference intervals

Because congenital hypothyroidism, which causes irreversible mental retardation, can be prevented by early treatment (1), mass screening for this condition is carried out throughout the world. Several screening methods have been developed for this purpose, such as measurement of total thyroxin and thyrotropin concentrations in dried blood samples on filter paper (2–7). Measurement of total thyroxin is useful for detecting primary, secondary, and tertiary hypothyroidism but gives false-positive results for patients with thyroxin-binding globulin (TBG)4 deficiency (hypo-TBG), who do not need to be treated. Therefore, Dussault et al. (8, 9) measured TBG in dried blood samples on filter paper by using radiolabeled thyroxin when the total thyroxin concentration was low. However, the large scale of screening programs makes a nonisotopic method preferable. Here we describe an enzyme immunoassay (EIA) for mea-

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suring TBG concentration in dried blood samples on filter paper that is suitable for use in screening programs.

**Materials and Methods**

*Chemicals.* m-Maleimidobenzoyl N-hydroxysuccinimide ester (MBS), o-nitrophenyl-β-d-galactopyranoside, and polyoxyethylene sorbitan monoacetate (Twee® 20%) were obtained from Sigma Chemical Co., St. Louis, MO 63178; β-p-galactoside galactohydrolase (β-δ-galactosidase, EC 3.2.1.23, from Escherichia coli; 5 g/L) was from Boehringer Mannheim, Mannheim 31, F.R.G.; Sephadex G-25 was from Pharmacia Fine Chemicals Ab., Upsala, Sweden; Biogel A 5m was from Bio-Rad Laboratories, Richmond, CA 94804; mercaptoethanol and bovine serum albumin (BSA; Cohn Fraction V) were from Armour Pharmaceutical Co., Phoenix, AZ 85077; goat anti-rabbit globulin was from Eiken Immunological Laboratories, Tokyo 114, Japan; special thick filter paper prepared for mass screening was from Toyo Kagaku Sangyo Co., Tokyo 103, Japan.

**TBG.** Highly purified TBG was prepared from pooled serum from healthy humans by affinity chromatography ([10]). Radioactive thyroxin was added to the purified material and the mixture was subjected to disc electrophoresis. The single band of stained protein coincided with the single peak of radioactivity.

**Rabbit anti-TBG.** Doses of 10 μg of purified TBG with complete adjuvant were injected subcutaneously into rabbits six times, with two-week intervals between injections. One week after the final injection, blood was withdrawn and serum was separated.

**TBG-β-δ-galactosidase complex.** The TBG-β-δ-galactosidase complex was prepared as described in detail previously ([11]). The purified TBG was mixed with MBS and applied to a Sephadex G-25 column. The eluted solution of TBG-MBS complex was added to β-δ-galactosidase solution and applied to a column containing Biogel A 5m. Fractions in the peak of enzyme activity were pooled and stored at 4°C.

**TBG standards.** TBG-free serum was prepared by thyroxin-affinity column chromatography. The TBG concentration in this serum was less than 0.025 mg/L, as determined by a sensitive radioimmunoassay (RIA). Various amounts of purified TBG were then dissolved in the TBG-free serum and the mixtures were used as standard TBG sera. Heparinized pooled blood from normal subjects was centrifuged at 1000 × g for 10 min, and the packed cells were washed four times with phosphate-buffered saline (0.15 mol of sodium chloride in 1 L of 10 mmol/L sodium phosphate buffer, pH 7.0); the washed, packed cells were then mixed with an equal volume of the TBG serum standards. When 30 μL of the mixture was spotted on the special thick filter paper and allowed to dry at room temperature, the blood spot formed was about 9 mm in diameter. TBG concentrations in the dried blood samples were expressed as equivalent to the concentrations of the TBG serum standards added to the packed cells.

**Subjects.** Venous blood was withdrawn from 10 normal adult subjects (five men and five women, ages 18 to 32 years); 10 normal neonates (five boys and five girls, ages three to four weeks); 10 normal pregnant women (ages 25 to 29 years, at 31–35 weeks of gestation); and 10 neonatal patients with hypo-TBG (five boys and five girls, ages three to four weeks). Serum and dried blood spots were prepared and kept at −20°C, except for those in the study on the stability of TBG in blood spots on filter paper.

**RIA for TBG in serum.** For comparison, we measured the TBG concentration in the serum by a double-antibody RIA with a kit obtained from Eiken Immunochernical Laboratories.

**EIA for TBG in serum.** We also measured the TBG concentration in the serum by a double-antibody EIA as previously reported ([11]).

**TBG measurement in dried blood samples.** The reagents were diluted with phosphate buffer containing, per liter, 1 mmol each of MgCl₂ and mercaptoethanol and 1 g of BSA (diluent). Two 3-mm-diameter blood discs, which contained about 2.7 μL of blood each, were punched out from standard or test blood spots on filter paper. We soaked the discs in 200 μL of anti-TBG serum containing normal rabbit serum (anti-TBG serum diluted 720-fold and normal rabbit serum diluted 60-fold with diluent, final dilutions) and incubated this for 30 min at room temperature (about 25°C). We then added 100 μL of solution of TBG-β-δ-galactosidase complex (diluted 25-fold), and incubated for 20 h at room temperature. After adding 100 μL of 14-fold diluted goat antiserum to rabbit globulin, we incubated the mixtures further for 30 min, added 3 mL of washing solution (Twee 20, 1 g/L in phosphate-buffered saline), and centrifuged the mixtures at 1000 × g for 30 min. The resulting precipitates were washed twice with 3 mL of washing solution and suspended in 1 mL of 1 g/L o-nitrophenyl-β-d-galactopyranoside solution containing 50 mL of methanol per liter. After incubating the suspensions for 2 h at 37°C, we mixed them with 1 mL of 30 g/L sodium carbonate solution and measured the absorbance at 405 nm.

**Results**

**Calibration curve.** Figure 1 shows a typical calibration curve obtained with anti-TBG serum diluted 720-fold. From this calibration curve the minimum detectable concentration, as estimated from six replicate determinations of TBG-standard blood spots (0, 1.6, 3.25 mg/L), was 3.3 mg/L (equivalent to 8.9 ng of TBG per assay tube), the point where the 95% confidence limit of the response at zero concentration intersects the calibration curve. We could measure TBG at concentrations of 3.3 to 52 mg/L. The absorbance of the blanks, mixtures with normal rabbit serum substituted for anti-TBG serum, was 0.22 (SD 0.04) at 405 nm.

**Reproducibility.** Blood samples were obtained from two

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**Fig. 1. Typical calibration curve obtained with anti-TBG serum diluted 720-fold**

The dotted line indicates the value for the blank.
normal subjects every week for four weeks and the dried blood spots on filter paper were stored at -20°C, 4°C, room temperature, or 37°C, with or without drying. As shown in Figure 2, when the blood spots were stored in dry conditions, the maximum change in TBG values was about 5% at -20°C or 4°C, and 15% at room temperature. The TBG values decreased significantly (by about half) when the samples were not kept dry on storage at 37°C or room temperature. As shown in Table 1, the within-assay coefficients of variation (CVs) were 4.0 to 9.9 (mean 6.6%) and the between-assay CVs were 4.8 to 6.8 (5.9%) for samples stored dried at -20°C.

Comparison with EIA and RIA. The TBG concentrations in dried blood samples determined by the present EIA correlated well with those in serum determined by RIA (Figure 3a, r = 0.95, y = 0.96x - 2.05, p < 0.01) and by EIA (Figure 3b, r = 0.96, y = 0.81x - 1.01, p < 0.01).

TBG concentrations in various subjects. The mean TBG concentrations in dried blood samples determined by the present EIA were as follows (mean ± SD): 10 normal adult subjects, 23.9 ± 6.8 mg/L; 10 normal neonates, 26.5 ± 4.2 mg/L; 10 normal pregnant women, 50.0 ± 11.2 mg/L; 10 patients with hypo-TBG, less than the minimum detectable concentration (<3.3 mg/L).

Discussion
We previously described a double-antibody EIA for determining TBG in serum. Here, we have used this method for determining TBG in dried blood samples on filter paper. The TBG in dried blood samples on filter paper was stable for at least four weeks when the samples were stored at low temperature (-20°C or 4°C), or at room temperature when dried. Therefore, samples in mass screening programs can safely be sent by mail to the laboratory for testing. The reproducibility of our method for determining TBG in dried blood samples on filter paper—within-assay CV 6.6%, between-assay CV 5.9%—is comparable with that of RIA.

Table 1. Precision of the Assay

<table>
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<th>SD</th>
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<td></td>
</tr>
<tr>
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<tr>
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<td>6.6</td>
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<tr>
<td>Between assays</td>
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<td></td>
<td></td>
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<tr>
<td>D</td>
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<td>0.7</td>
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<tr>
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</tr>
<tr>
<td>Mean</td>
<td></td>
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* mg/L equivalent serum: see text

Fig. 2. Stability of TBG in dried blood samples on storage at -20°C

Fig. 3. Correlation between TBG values in blood spots determined by EIA and those in serum determined by RIA (top) and by EIA (bottom) x - axis (both figures): TBG, mg/L; serum equivalent.
within-assay CVs 3.4–5.0%, between-assay CVs 3.1–8.2% (12). The reliability of the proposed method was satisfactory as shown by the correlation of the TBG values in dried blood samples determined by this method with those in serum determined by RIA and EIA. The discrepancy between TBG values for serum and blood spots would have to be explained by different hematocrit values between standard and test blood spots. Several mass screening programs for congenital hypothyroidism have been organized, but measurement of total thyroxin by either RIA or EIA gives false-positive results in persons with hypo-TBG. The incidence of hypo-TBG in the general population has been reported to be 1 in 5400 in Europe (13) and 1 in 9000 in America (8), incidences comparable with that of congenital hypothyroidism. Patients with hypo-TBG can be detected by measurement of total thyroxin and TBG in dried blood samples with radiolabeled thyroxin (8); more recently, Mizuta et al. (14) reported an RIA for measuring free thyroxin in dried blood samples on filter paper. Patients with hypo-TBG did not give false-positive results for hypothyroidism with these methods. However, a nonisotopic assay is preferable in screening programs. The combined measurements by EIA of total thyroxin and TBG in dried blood samples may be helpful for detecting hypothyroidism and for reducing the number of false-positive results.

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