Determination of Serum Biotinidase Activity with Biotinyl Derivatives of Iodotyramines as Substrates

STAVROS A. EVANGELATOS*, SOTIRIS E. KAKABAKOS*, GREGORY P. EVANGELATOS*, AND DIONYSSIS S. ITHAKISSIOS*#x

Received April 21, 1992, from the *Institute of Radioisotopes and Radiodiagnostic Products, Radioimmunochemistry Laboratory, N.C.S.R. "Demokritos", Aghia Paraskevi Attikis, 153 10, Athens, Greece, and the #Department of Pharmacy, Pharmaceutical Technology Laboratory, University of Patras, Patras, 261 10, Greece. Accepted for publication March 11, 1993.

Abstract □ We synthesized biotinylated mono- and di-iodotyramine and their radioactive counterparts and used these substances as substrates to estimate serum biotinidase activity in a radioassay system. The K_m values determined for mono- and di-iodobiotinyl derivatives were 15.8 and 25.9 μM, respectively, whereas, the maximum velocities of the enzymatic reaction were 27.0 and 8.7 nmol · min^{-1} · mL^{-1}, respectively. Both substrates competed with biocytin for the same active site of the enzyme and the K_i values were 7.30 and 9.56 μM for the mono- and di-iodinated substrate, respectively. Higher assay sensitivity was obtained using [^{125}I]biotinyl-monoiodotyramine as substrate, and the values obtained were directly related with those determined with the well-established colorimetric method (r = 0.9377, n = 31). However, for routine use, the assay may be accomplished by diluting the radiotracer with biocytin instead of its "cold" counterpart, because it is a commercially available reagent. The values obtained in this case were very well correlated with those determined by the colorimetric assay as well (r = 0.9289, n = 31).

Biotinidase [EC 3.5.1.12] liberates biotin from biocytin (*N*-biotinyl-L-lysine) or other biotinyl peptides.[1] Biotinidase deficiency is the primary enzyme defect in late-onset form of multiple carboxylase deficiency, which is an inborn error of metabolism characterized by significant clinical findings that lead to death.[2–4] Early diagnosis of the disease can be life saving because treatment with biotin can reverse acute symptoms and prevent irreversible neurologic damage.[5,6]

We have recently developed a radioassay for biotinidase activity in human serum with a nonhomogeneous substrate, [^{125}I]biotinyl-monoiodotyramine in combination with biocytin, with avidin as a binding protein and polyethylene glycol as a separation reagent.[7] However, to gain additional information about the enzymatic behavior and to investigate possible discrepancies that could be raised in the determination of biotinidase activity with a nonhomogeneous substrate, we prepared nonradioactive mono- and di-iodobiotinyl-tyramines and we studied their characteristics as artificial substrates for the measurement of biotinidase activity.

Experimental Section

Materials—All reagents were of analytical grade. The water was doubly distilled. *N*-Hydroxysuccinimidobiotin, *p*-hydroxymercuribenzoate, tyramine, biocytin, and *N*-biotinyl-*p*-aminobenzoate (B–PABA) were all obtained from Sigma Chemical Company (St. Louis, MO). Avidin was obtained from S. C. Bio-Partners, B 6655 Bastogne, Belgium. The TLC plates (silica gel 60 without fluorescent indicator) and all the other reagents were obtained from Merck-Schuchardt, 6100 Darmstadt, Germany, except as otherwise indicated. Carrier-free Na^{125}I (17 KCi/g; radiochemical purity, 99.9%; iodates, <2%) was a product of Radiochemical Company, Atomic Energy of Canada, Ottawa, Canada.

Radioiodination of Tyramine—The radioiodination of tyramine was performed as previously described.[8,9] Ten microliters of Na^{125}I solution (250 Ci/L in 0.25 M phosphate buffer, pH 7.5) and 5 μL of chloramine-T solution (3.55 mM in 0.25 M phosphate buffer, pH 7.5) were incubated with 10 μL of 0.73 mM tyramine solution in a 3-mL test tube for 70 s at 20 °C. The reaction was stopped by adding 5 μL of sodium metabisulfite solution (5.26 mM in 0.25 M phosphate buffer, pH 7.5). The [^{125}I]monoiodotyramine and the [^{125}I]di-iodotyramine synthesized were isolated from the reaction mixture by paper electrophoresis[10] (500 V, Whatman 3MM, 0.02 M barbital buffer, pH 8.4, 2 h, 4 °C), moving 5 and 1 cm, respectively, from the origin. The radioactivity was detected with a gamma-scanner (YEW, 3086 X-Y Recorder). The radioactive bands were cut off, eluted with ethanol:water (2:1), and dried under a stream of nitrogen, providing carrier-free radioiodinated mono- and di-iodotyramines with specific radioactivities of ~2500 and 5000 Ci/mmol, respectively.

Preparation of Mono- and Di-iodotyramines—The preparation of mono- or di-iodotyramine was accomplished by adding slowly at 20–22 °C 2 or 5 mL, respectively, of an 1 N iodine solution, into 20 mL of a tyramine solution (50 mM in 30% ammonia). The solutions were condensed under reduced pressure to almost 5 mL and they were left overnight at 0 °C. The precipitates formed were filtered and washed with ice-cold water, followed by two recrystallizations from water. Monoiodotyramine was in the form of almost colorless needles, whereas the di-iodinated tyramine needles were cream colored. The distances from the origin in paper electrophoresis (500 V; Whatman 3MM; barbital buffer, pH 8.4, 0.2 M; 2 h, 4 °C, visualization after spraying with Folin-Ciocalteu reagent) were 5 and 1 cm, respectively, and their R_f values in a TLC separation system (*n*-butanol:2N NH_4OH:ethanol, 3:1:1) were 0.46 and 0.52 for the mono- and di-iodinated products, respectively. The ^{1}H NMR spectra were recorded in CD_3OD on a Bruker AC 250 spectrometer equipped with the aspect 3000 computer, using Me_4Si as the internal reference; ^{1}H NMR for monoiodotyramine: δ 2.76 (2H, t, J = 7.5 Hz), 3.02 (2H, t, J = 7.5 Hz), 6.78 (1H, d, J = 8.3 Hz), 7.07 (1H, dd, J = 8.3, 2.1 Hz), 7.59 (1H, d, J = 2.1 Hz); ^{1}H NMR for di-iodotyramine: δ 2.71 (2H, t, J = 7.4 Hz), 3.03 (2H, t, J = 7.4 Hz), 756 (2H, s); IR spectra (Perkin-Elmer FT 1600, KBr pellets) for monoiodotyramine: 792, 819 cm^{-1} (aromatic CH bending), 1575–1615 cm^{-1} (aromatic C = C skeletal), and 3257, 3330 cm^{-1} (NH stretching); IR spectra for di-iodotyramine: 793, 1575–1615, 3257, and 3330 cm^{-1}. Mono- and di-iodotyramine decomposed at 166–167 °C and 168–169 °C, respectively, and the final yield was >70% in both cases.

Preparation of Biotinyl-tyramine Derivatives—These derivatives were synthesized by mixing solutions of iodotyramines with *N*-hydroxysuccinimidobiotin in a molar ratio of 1:9. For the radioiodinated biotinyl-tyramines, 30 μL of a 2.33 μM radiotyramine solution in 0.2 M borate buffer (pH 9.0) were added to 90 μL of a 7 μM *N*-hydroxysuccinimidobiotin solution in anhydrous dioxane, and the mixtures were incubated for 90 min at 20–22 °C. For the nonradioiodinated biotinyl-tyramines, 0.5 mL of a 0.178 M mono- or di-iodotyramine solution in 0.2 M borate buffer were added to 2.3 mL of a 0.35 M *N*-hydroxysuccinimidobiotin solution in anhydrous dioxane, and the mixtures were incubated for 90 min at 20–22 °C. The radioactive or nonradioactive products, synthesized by either procedure, were isolated by TLC with *n*-butanol:2N NH_4OH:ethanol (3:1:1) as a solvent mixture, on 0.25- or 2.0-mm thick silica gel plates, respectively. The radiolabeled biotinylated compounds were detected autoradiographically by exposing X-ray film for 2 min to the plate, whereas the nonradiolabeled compounds were detected by spraying a part of the plate with Folin-Ciocalteu reagent. The final products

0022-3549/93/1200-1228$02.50/0

were removed by scrapping off the spot area of the plates at $R_f = 0.67$ and eluting with an equivolume solution of ethanol and water. The specific radioactivity values of the radioiodinated mono- and di-iodobiotinyl-tyramines were ~2500 and 5000 Ci/mmol, respectively. The ^{1}H NMR for biotinyl-monoiodotyramine: δ 1.21–1.74 (6H, m), 2.14 (2H, t, J = 7.2 Hz), 2.67 (2H, t, J = 6.9 Hz) and 2.69 (1H, d, J = 12.4 Hz) partially overlapped, 2.94 (1H, dd, J = 12.4, 4.9 Hz), 3.35 (2H, t, J = 6.9 Hz), 4.27 (1H, dd, J = 7.9, 4.4 Hz), 4.50 (1H, m), 6.75 (1H, d, J = 8.2 Hz), 7.04 (1H, dd, J = 8.2, 2.0 Hz), 7.53 (1H, d, J = 2.0 Hz); ^{1}H NMR for biotinyl-di-iodotyramine: δ 1.27–1.79 (6H, m), 2.14 (2H, t, J = 7.3 Hz), 2.65 (2H, t, J = 6.9 Hz) and 2.69 (1H, d, J = 12.7 Hz) partially overlapped, 2.95 (1H, dd, J = 12.7, 5.0 Hz), 3.16 (1H, m), 3.35 (2H, t, J = 6.9 Hz), 4.28 (1H, dd, J = 7.9, 4.5), 4.50 (1H, dd, J = 7.9, 5.0), 7.57 (2H, s); IR spectra (KBr pellets) for the cream-colored crystals of biotinyl-monoiodotyramine: 806, 818, and 1665 cm^{-1} (amide C = 0) and for the flesh colored crystals of biotinyl-di-iodotyramine: 806 and 1665 cm^{-1} (the products decomposed at 216–218 °C and 200–202 °C, respectively).

Substrate Solutions—The [^{125}I]biotinyl-monoiodotyramine was diluted with 0.5 M phosphate buffer (pH 6.5) containing either 0.18 mM biotinyl-monoiodotyramine to give a final specific radioactivity of 3.7 mCi/mmol or 0.18 mM biocytin to provide a final tracer concentration of 33.2 pM. The [^{125}I]biotinyl-di-iodotyramine was handled in a similar way.

Avidin Solution—The activity of the avidin (10 g/L) used was 13 000 units/g. One unit of avidin binds 1 μg of biotin.[11]

Biotinidase Inhibitor—The biotinidase inhibitor was a 2 mM *p*-hydroxymercuribenzoate (*p*-HMB) suspension.[12]

Polyethylene Glycol (PEG) Solution—PEG 6000 (200 g) in 1 L of 50 mM phosphate buffer (pH 7.4) containing 0.1% NaN_3 was used.

Specimens—Serum was separated from freshly drawn whole blood, which was collected from apparently healthy adult volunteers, and was stored at −20 °C for up to 2 months. Pool serum material was prepared by pooling freshly prepared sera from six healthy adults and storing at −20 °C.

Assay Protocol—One hundred microliters of diluted serum (1:25, in 0.1 M phosphate buffer, pH 6.5) and 50 μL of substrate solution were pipetted into a 3-mL polystyrene tube and incubated for 30 min at 37 °C. Then, the enzymatic reaction was stopped by adding 25 μL of the *p*-HMB suspension. Twenty-five microliters of avidin solution were added and incubated for 30 min at 37 °C. Then, 50 μL of rabbit or human serum were added as a protein source, followed by 2 mL of the PEG solution, and the solution was incubated for 15 min at 20 °C. After this, all samples were centrifuged at 3000 × *g* for 10 min. The radioactivity of the precipitate was measured for 1 min (Nuclear Enterprises 1600). As reference, 100 μL of diluted serum (1:25, in 0.1 M phosphate buffer, pH 6.5) with completely inactivated biotinidase at zero time were used. The same samples were also colorimetrically assayed with *N*-biotinyl-*p*-aminobenzoate as substrate.[2]

Biotinidase Activity—The enzymatic activity (B_a) using homogeneous biotinyl-monoiodotyramine or biotinyl-di-iodotyramine as substrate, was determined by the equation $B_a = A(P_r - P)/T$ nmol · min^{-1} · mL^{-1}, where the factor A is the product of the substrate concentration, the incubation time (30 min), and the amount of serum (4 μL), P_r is the mean radioactivity (cpm) measured in the precipitate of reference tubes, P is the radioactivity (cpm) measured in the precipitate of test tubes, and T is the total radioactivity (cpm) added per assay tube. When the calculations are based on the cleavage of radiotracer added per assay tube (16.6 fmol), biotinidase activity follows the same equation but it is expressed in fmol · min^{-1} · mL^{-1}.[7]

Results

The radioiodination of tyramine was performed following the chloramine-T method[13] because the radioiodine was provided in ^{125}INa form. The yield of mono- or di-iodo derivatives increased respectively by changing the molar ratio of tyramine to ^{125}INa used. Thus, when a molar ratio of 7:1 was used, 90% monoiodotyramine was obtained in terms of radioiodinated tyramine derivatives, whereas, 80% of di-iodotyramine was produced when the molar ratio was 2:1.

The nonradioactive iodination of tyramine was performed by electrophilic aromatic substitution with 1 N iodine solution, thus avoiding the usage of other than iodine agents, and consequently facilitating the isolation of the products. With a molar ratio of 1:1 for tyramine-to-iodine, >90% of the iodinated product was in the form of monoiodotyramine, whereas, using a molar ratio of 1:2.5 for tyramine-to-iodine, >95% di-iodotyramine was produced.

The iodinated tyramines were coupled with *N*-hydroxysuccinimidobiotin at room temperature (yield, almost 90%). The coupling reaction required ~1–4 h of incubation, beyond which the yield decreased slightly.

The optimum pH of the enzymatic reaction was in the range 6.0–6.8. The K_m values of pooled human serum biotinidase for the substrates prepared were determined by substrate saturation experiments (Figures 1A and 1B). The K_m values determined for mono- and di-iodobiotinyl derivatives were 15.8 and 25.9 μM, respectively, and the maximum velocities of the enzymatic reaction were 27 and 8.7 nmol · min^{-1} · mL^{-1}, respectively. Both synthesized substrates competed with biocytin for the same active site of human serum biotinidase, using the latter as inhibitor and the iodinated biotinamides as substrates, as indicated in Figures 1A and 1B. The K_i values were 7.3 and 9.56 μM for the mono- and di-iodinated substrate, respectively. Because the biotinyl-monoiodotyramine was characterized by considerably higher maximum velocity (V_{max}) and lower K_m value as the biotinidase substrate compared with the di-iodinated one, it was selected as substrate for routine use.

As shown in Figure 2, the reaction velocity was constant up

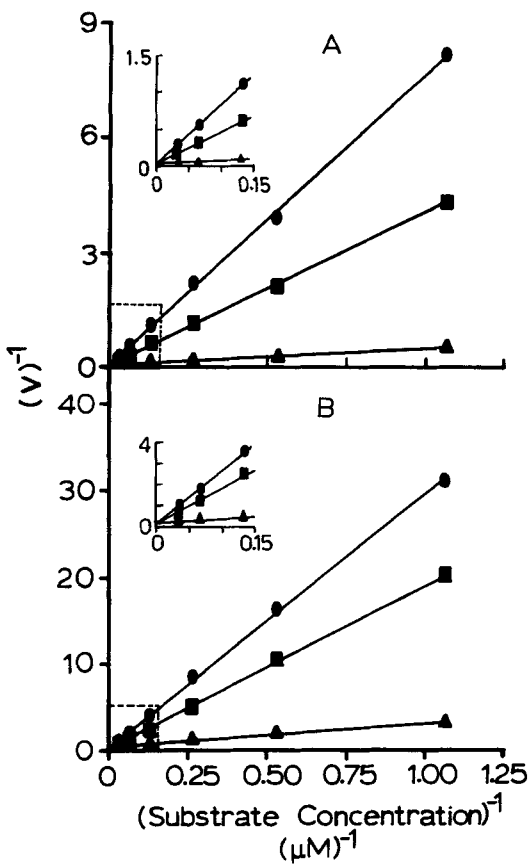

Figure 1—Lineweaver–Burk plots of human serum biotinidase in the presence of 0 (●), 30 (■), and 60 (▲) μM of biocytin with either biotinyl-monoiodotyramine (**A**) or biotinyl-di-iodotyramine (**B**) as substrate. Each point represents the mean value of the enzymatic velocity obtained by a full time study experiment. The conditions of the assay were as described under the *Experimental Section.* In the inserts are presented the data included in the areas determined by dashes with enlarged scales.

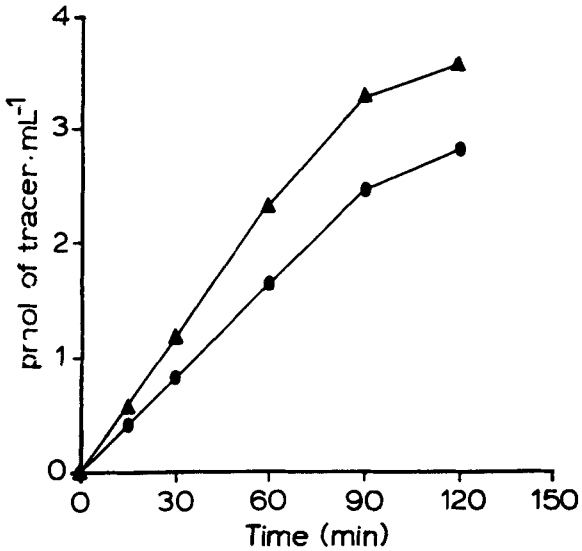

Figure 2—Cleavage by serum biotinidase of [^{125}I]biotinyl-monoiodotyramine tracer diluted either with cold biotinylmonoiodotyramine (▲) or biocytin (●) in a period of up to 2 h.

to 90 min at 37 °C in 100 μL of 1:25 diluted serum sample with [^{125}I]biotinyl-monoiodotyramine tracer diluted with either biotinyl-monoiodotyramine or biocytin. However, with homogeneous substrate, the amount of tracer cleaved per milliliter of serum per minute was almost 40% more than that cleaved with the heterogeneous substrate.

The biotinidase activity values of 31 serum samples were simultaneously measured by the homogeneous biotinyl-monoiodo-tyramine radioassay or the colorimetric assay; linear regression analysis (Figure 3) indicated that both assays measure biotinidase activity in a directly related way: $B_a = 1.936B_{color} + 2.592$, where B_{color} is the enzymatic activity determined colorimetrically; the correlation coefficient (r) was 0.9377 [critical value of 0.01 significance level, $r_{0.01(30)} = 0.449$]. The same samples measured with [^{125}I]biotinyl-monoiodotyramine diluted with biocytin as substrate provided biotinidase activity values (B'_a) directly related to those obtained with homogeneous substrate, $B'_a = 1.344B_a - 0.898$, r = 0.958 (Figure 4).

The biotinidase activity values, determined in serum samples from 31 apparently healthy adult volunteers with the homogeneous biotinyl-monoiodotyramine radioassay, were 13.5–25.3 nmol of monoiodotyramine liberated · min^{-1} · mL^{-1}, with a mean of 19.4 nmol · min^{-1} · mL^{-1}. The same samples analyzed with [^{125}I]biotinyl-monoiodotyramine in combination with biocytin as substrate provided values of tracer cleaved of 17.17–31.7 fmol · min^{-1} · mL^{-1}, with a mean of 25.234 fmol · min^{-1} · mL^{-1}. The colorimetric assay provided values of *p*-aminobenzoate liberated of 5.6–10.4 nmol · min^{-1} · mL^{-1}, with a mean of 8.55 nmol · min^{-1} · mL^{-1}.

For the determination of intra- and interassay precision, we used six normal serum samples with biotinidase activities ranging between 14.1 and 23.8 nmol · min^{-1} · mL^{-1} with homogeneous biotinyl-monoiodotyramine substrate. The intra-assay precision was determined from five measurements in triplicate over 5 days and the CVs ranged between 5.6 and 2.7% (mean value, 4.3%) over the range of the serum samples tested. The interassay CVs, determined from 10 measurements of the same samples run in duplicate over a period of 6 weeks, were between 9.3 and 5.5% (mean value, 7.6%) over the range of the serum samples tested. The corresponding intra- and interassay CVs, determined with the heterogeneous substrate, ranged between 7.2 and 4.8% (mean value, 5.6%) and 10.3 and 7.3% (mean value, 8.9%), respectively.

Discussion

We have reported a biotinidase radioassay with a [^{125}I]biotinyl-monoiodotyramine tracer diluted with biocytin, the natural substrate of the enzyme, as a heterogeneous substrate.[7] The purpose of the present work was to investigate the properties of the biotinyl-iodotyramine as substrate for the measurement of biotinidase activity as well as the enzyme behavior with homogeneous substrate consisting of "cold" and radioiodinated monoiodobiotinyltyramine or heterogeneous substrate (i.e., a mixture of radioiodinated [^{125}I]biotinyl-monoiodotyramine and biocytin).

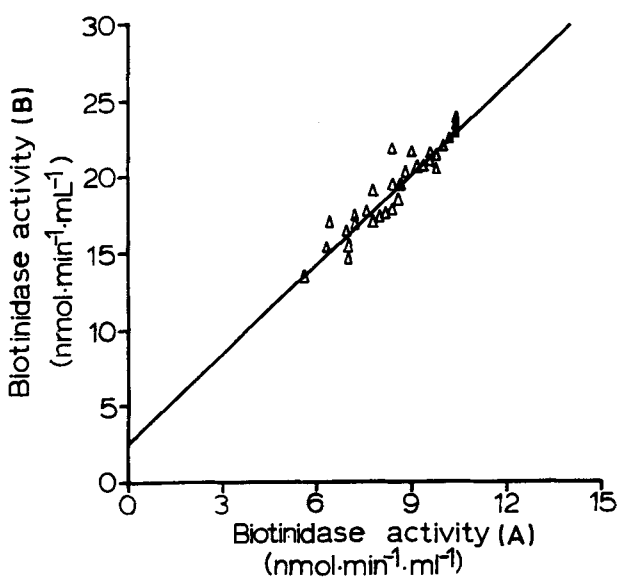

Figure 3—Correlation between biotinidase activity values with the colorimetric assay (horizontal axis, **A**, nmol of B-PABA cleaved · min^{-1} · mL^{-1}) and the radioassay with homogeneous [^{125}I]biotinyl-monoiodotyramine substrate (vertical axis, **B**, nmol · min^{-1} · mL^{-1}).

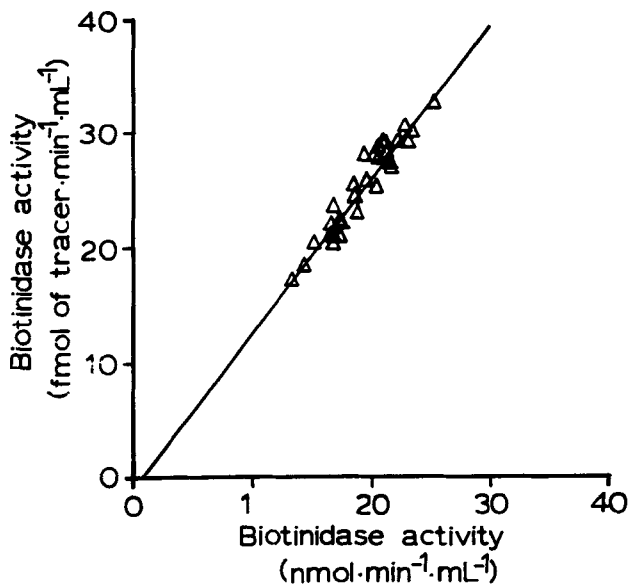

Figure 4—Correlation between biotinidase activity values determined with the radioassay with homogeneous [^{125}I]biotinyl-monoiodotyramine substrate (horizontal axis, nmol · min^{-1} · mL^{-1}) and the radioassay with [^{125}I]biotinyl-monoiodotyramine tracer diluted with biocytin (vertical axis, fmol of tracer · min^{-1} · mL^{-1} in the presence of 9 nmol of biocytin per tube).

Our studies indicate that both mono- and di-iodobiotinyl derivatives synthesized competed with biocytin for the same active site of biotinidase. However, with biotinyl-diiodotyramine, we determined an almost threefold decrease of V_{max} and a significant increase of K_m compared with the values obtained with biotinylmonoiodotyramine as substrate. Thus, the addition of one more iodine atom in the phenol ring of biotinyl-monoiodotyramine could considerably affect the enzyme kinetics.

The biotinidase activity values estimated with homogeneous [^{125}I]biotinyl-monoiodotyramine substrate were almost twofold higher than those estimated with the colorimetric method with *N*-biotinyl-*p*-aminobenzoate as substrate. Ebrahim and Dakshinamurti,[14] using biocytin as substrate in their fluorimetric assay of dialyzed serum samples, also determined 1.7-fold higher biotinidase activity values than those obtained with the colorimetric assay. They ascribed this difference, at least in part, to the different pHs of the assay buffer used in each case (5.5 for the fluorimetric, and 6.0 for the colorimetric assay). Because the optimum pH for biotinidase activity with either biotinyl-monoiodotyramine or *N*-biotinyl-*p*-aminobenzoate ranged between 6.0 and 7.0, it is difficult to ascribe the significant differences observed in biotinidase activity between our method and the colorimetric assay to the pH. On the other hand, Wastell et al.,[15] with biotinyl-6-amino quinoline as substrate, determined 1.6-fold lower activity values compared with those of the colorimetric assay. We postulate that the enzymatic activity obtained with artificial biotinyl substrates with aromatic leaving groups increases by increasing the distance of the aromatic ring from the amide bond, because the amino group in *p*-aminobenzoate and in 6-aminoquinoline is directly connected to the aromatic ring, whereas, in iodotyramine a two-carbon atom chain is interposed. In addition, data obtained in the present work with iodinated substrate analogues indicate that the aromatic substitution may affect the enzymatic activity as well.

The assay developed with homogeneous [^{125}I]biotinyl-monoiodotyramine substrate was well correlated with the established colorimetric method and considerably more sensitive, because 100 μL of 1:400 diluted human serum could be used instead of the 100 μL of undiluted serum needed for the colorimetric assay (data not shown). However, for routine use, a 1:25 dilution of serum sample was included in the assay protocol to increase the discrimination ability, to improve precision, and to decrease the incubation time. The assay developed had excellent correlation with that using a mixture of [^{125}I]biotinyl-monoiodotyramine and biocytin as substrate, although the homogeneous substrate provided higher values of V_{max}. On the other hand, because the assay with homogeneous [^{125}I]biotinyl-monoiodotyramine requires a substantial amount of biotinyl-monoiodotyramine, which is not commercially available, the utilization of [^{125}I]biotinyl-monoiodotyramine tracer in combination with biocytin may be the substrate of choice for routine clinical use.

We propose that the high specific radioactivity, carrier-free [^{125}I]biotinyl-monoiodotyramine substrate described could be also utilized to significantly facilitate in vitro and/or in vivo biochemical studies when nonsaturating conditions of substrate are needed. Additionally, the radioactive leaving group, [^{125}I]monoiodotyramine, could prove to be a very useful tool for the study of other amidohydrolases.

References and Notes

1. Pispa, J. *Ann. Med. Exp. Biol. Fenn.* **1965**, *43*, 5–39.
2. Wolf, B.; Grier, R. E.; Allen, P. J.; Goodman, S. I.; Kien, C. L. *Clin. Chim. Acta* **1983**, *131*, 273–281.
3. Wolf, B.; Heard, G. S.; McVoy, J. S.; Grier, R. E. *Ann. N.Y. Acad. Sci.* **1985**, *447*, 252–262.
4. Baumgartner, E. R.; Suormala, T.; Wick, H.; Bausch, J.; Bonjour, J. P. *J. Inher. Metab. Dis.* **1985**, *8*, 59–64.
5. Wolf, B.; Grier, R. E.; McVoy, J. R.; Heard, G. S. *J. Inher. Metab. Dis.* **1985**, *8*, 53–58.
6. Wolf, B.; Heard, G. S.; Weissbecker, K. A.; McVoy, J. S.; Grier, R. E.; Leshner, R. T. *Ann. Neurol.* **1985**, *18*, 614–617.
7. Evangelatos, S. A.; Livaniou, E.; Kakabakos, S. E.; Evangelatos, G. P.; Ithakissios, D. S. *Anal. Biochem.* **1991**, *196*, 385–389.
8. Livaniou, E.; Evangelatos, G. P.; Ithakissios, D. S. *J. Nucl. Med.* **1987**, *28*, 1430–1434.
9. Livaniou, E.; Evangelatos, G. P.; Ithakissios, D. S. *Clin. Chem.* **1987**, *33*, 1983–1988.
10. Kakabakos, S. E.; Livaniou, E.; Evangelatos, S. A.; Evangelatos, G. P.; Ithakissios, D. S. *Eur. J. Nucl. Med.* **1991**, *18*, 952–954.
11. Green, N. M. *Biochem. J.* **1965**, *94*, 23–40.
12. Koivusalo, M.; Pispa, J. *Acta Physiol. Scand.* **1963**, *58*, 13–19.
13. Greenwood, F. C.; Hunter, W. M.; Glover, I. S. *Anal. Biochem.* **1963**, *89*, 114–123.
14. Ebrahim, H.; Dakshinamurti, K. *Anal. Biochem.* **1986**, *154*, 282–286.
15. Wastell, H.; Dale, G.; Bartlett, K. *Anal. Biochem.* **1984**, *140*, 69–73.

Acknowledgments

We are thankful to Dr. M. Pelecanou for the NMR studies and to Dr. E. Livaniou for her helpful suggestions during the experimental procedure and the preparation of this manuscript.