Whole body protein kinetics using Phe and Tyr tracers: an evaluation of the accuracy of approximated flux values

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Whole body protein kinetics using Phe and Tyr tracers: an evaluation of the accuracy of approximated flux values. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E1194–E1200, 1999.—Phenylalanine (Phe) kinetics are increasingly used in studies of amino acid kinetics, because the metabolic fate of Phe is limited to incorporation into protein (protein synthesis, S_p) and catabolism via hydroxylation (Q_pt) to tyrosine (Tyr). Besides an infusion of labeled Phe to measure Phe flux (Q_p), a priming dose of Tyr and an independent Tyr tracer are used to measure Tyr flux (Q_t) and Q_pt. Alternatively, Q_p, Q_pt, and S_p can be approximated by using equations, based on Phe and Tyr concentrations in body proteins, that eliminate the need for a Tyr tracer. To evaluate the accuracy of this approach, data were obtained from 12 type I diabetic patients and 24 nondiabetic control subjects who were studied with the full complement of tracers both with and without insulin infusion. S_p approximations closely matched measured values in both groups (mean difference <2%, all values <5%), but the agreement was poor for Q_pt (error range = –8 to +43%) and Q_t (error range = –22 to +41%). Insulin status had no effect on these comparisons. The lower approximation error for S_p vs. Q_pt is due to the small contribution (~10%) of Q_pt to Q_p. Approximation error for Q_t (r > 0.99) can be explained by variability in the ratio of Tyr to Phe coming from protein breakdown. The approximation equations were originally demonstrated in four newborns or young children have used the approximation equations, presumably for these reasons (4, 7, 15, 17). However, Tyr infusions have been successfully performed in premature infants (5). In many clinical conditions, such as renal and cardiac failure, however, there is obvious advantage if the volume of the infusion can be restricted.

Regardless of the reason for using the approximations, the method has yet to be adequately tested for its validity. The assumptions of the model are that Phe and Tyr appear only from protein breakdown in the fasted state and that the ratio of their appearance should resemble the proportional content of Tyr and Phe in whole body proteins. For their calculations, Thompson et al. (16) selected a Tyr-to-Phe ratio of 0.73 on the basis of animal studies (10). The validity of their approximation equations was originally demonstrated in four healthy adults (16). In this small group approximated and measured averages differed by only 1% for both Q_pt and S_p. Subsequent comparisons have been less consistent, however. In six type I diabetic patients, approximated S_p was only 5% lower than measured, but Q_pt approximations averaged 51% higher (14). Likewise, Q_pt approximations in six premature infants ranged from 11% under to 25% over the measured values (15). In contrast, less than 2% variation between the approxi-
imated and measured parameters was reported in three pediatric cancer patients (4). With the increasing use of labeled Phe in human metabolic studies, it is important that the models and equations employed are rigorously tested to determine the validity of the approximation equations. Previous reports raise the possibility that use of approximations could produce variable results. It is uncertain whether the variability in these earlier studies is due to shortcomings in the model and equations, limited sample sizes, or differences in the metabolic state of the subjects studied. The current study was performed with these concerns in mind. We have evaluated the accuracy of the indirect method for determining whole body Phe kinetics by using a much larger study population than has previously been reported. Furthermore, the data were obtained from studies of patients with type I diabetes and nondiabetic control subjects, each studied with and without administration of exogenous insulin, so that the influence of metabolic status could be assessed.

METHODS

Materials. L-[15N]Phe, L-[ring-2H5]Phe, L-[ring-2H3]Tyr, and L-[ring-2H2]Tyr were purchased from Cambridge Isotope Laboratories (Andover, MA). L-[15N]Tyr and additional L-[ring-2H4]Tyr were purchased from Isotec (Miamisburg, OH). All isotopes were 99 atom percent excess. The chemical, isotopic, and optical purities of these compounds were confirmed before use. Sterile solutions were prepared and shown to be bacteria and pyrogen free before use in human subjects.

Subjects. Data from 72 studies were obtained from a total of 36 men and women who provided informed written consent to participate in three different institutionally approved protocols (Table 1). Subjects were classified as either having type I diabetes or as being nondiabetic controls. All diabetic volunteers were studied in the insulin-treated and insulin-deprived states on separate occasions at least 3 wk apart. Results from six members of the diabetic group (referred to here as subgroup A) have already been published (12). Data from the other six diabetic subjects, referred to as subgroup B, have not previously been published. The control group was composed of 24 young healthy men and women. Control studies were performed in two phases (basal and insulin infusion) on a single day, as described below. Additional results from the control group appear elsewhere (9).

Protocol. For 3 days preceding each study, participants received a weight-maintaining diet. All subjects were admitted to the hospital 24–48 h before each investigation. No food or caloric beverages were consumed after the evening meal the night before the study. All of the type I diabetic subjects were switched from long- or intermediate-acting insulin to regular insulin 72 h before the study to avoid the carry-over effects of the longer-acting insulin (12). Their subcutaneous regular insulin injections were discontinued at 6:00 PM, and either saline or insulin was infused overnight and throughout the duration of the protein turnover measurements on the following morning. During insulin treatment, insulin dosage was regularly adjusted to maintain blood glucose within the normal range.

Isotope infusions were started at ~7:00 AM via forearm veni catheters. Members of the control group and diabetic subgroup B received primed, continuous infusions of L-[15N]Phe (3.9 µmol·kg⁻¹·h⁻¹, 3.9 µmol/kg prime) and L-[ring-2H2]Tyr (2.9 µmol·kg⁻¹·h⁻¹, 2.9 µmol/kg prime), and a priming dose of L-[15N]Tyr (1.4 µmol/kg). Diabetic subgroup A received primed, continuous infusions of L-[ring-2H4]Phe (4.6 µmol·kg⁻¹·h⁻¹, 4.6 µmol/kg prime) and L-[ring-2H3]Tyr (3.5 µmol·kg⁻¹·h⁻¹, 3.5 µmol/kg prime), and a priming dose of L-[ring-2H4]Tyr (1.5 µmol/kg) (12). Isotope infusions were maintained for up to 5 h. Arterial blood samples were collected from either brachial or femoral lines, immediately before the isotope infusion was started and then at 10- to 20-min intervals after isotopic plateau had been reached (2–3 h after start of the infusion). The protocol for controls included a second study phase on the same day. Immediately on conclusion of the basal period, insulin was infused at either 0 (saline), 0.25, 0.50, or 1.00 mU·kg⁻¹·h⁻¹ for 2.5 h (9). Six subjects were assigned to each insulin dose. Four arterial blood samples were collected at 10-min intervals beginning 2 h after the start of insulin in this group.

Sample analysis and calculations. Plasma enrichment of Phe and Tyr were determined using gas chromatography-mass spectrometry, as described (9). M-4 mass abundance of [2H4]Tyr was adjusted for m-2 mass distribution by use of a matrix correction. The reproducibility and stability of the measurements were determined for each isotope measured. Interassay coefficients of variation, determined from replicates of a representative plasma sample, were between 3.8 and 6.0%. Isotopically enriched standards were used to calculate the intra-assay coefficients of variation, which ranged from 2.7 to 6.7%.

Whole body kinetics for Phe and Tyr were calculated using the equations described by Thompson et al. (16) and outlined below. Infusion and flux rate units are micromoles per kilogram per hour. The rates of flux (Q) of Phe and Tyr (measured) were obtained from isotope dilution

\[
Q = \frac{i}{(E_{\text{infusate}} - E_{\text{plasma}})}
\]

where \(i\) is the rate of tracer infusion and \(E_{\text{infusate}}\) and \(E_{\text{plasma}}\) correspond to the enrichments of infusate and plasma amino acids, respectively. Conversion rate of Phe to Tyr (Q\(_\text{pt}\)) in the measured model was calculated as

\[
Q_{\text{pt}} = Q_i \frac{E_t}{E_p} \left( \frac{Q_o}{E_p + Q_o} \right)
\]

where \(Q_i\) and \(Q_o\) are the flux rates for Tyr ([2H4]Tyr or [2H3]Tyr) and labeled Phe, respectively, \(E_t\) and \(E_p\) are the Phe

<table>
<thead>
<tr>
<th>Table 1. Subject characteristics</th>
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<tr>
<td>Group</td>
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<tr>
<td>Controls</td>
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<tr>
<td>Diabetics</td>
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<tr>
<td>Subgroup A</td>
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<tr>
<td>Subgroup B</td>
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Values are means ± SD. BMI, body mass index. Body fat determined from dual X-ray absorptiometry. M and F, nos. of males and females, respectively.
Fig. 2. Comparison of phenylalanine hydroxylation (Qt) derived from measured (solid bars) and approximated (open bars) equations. Subject groups and treatments are the same as in Fig. 1. *Significant difference between basal and insulin in controls; †significant difference from measured value.

RESULTS

Isotopic enrichment values are shown in Table 2. All subjects studied achieved isotopic plateau for plasma amino acid enrichments during the study period (9, 12). To increase the sample size in the diabetic group, data from subgroups A and B were pooled, despite the fact that they received differentially labeled (15N or deuterated) Phe and Tyr tracers as described in METHODS. Data pooling was performed only after it was confirmed that there were no systematic differences in any of the outcome variables of interest between the two data sets. For the sake of clarity, no distinction between subgroups A and B is made in the remainder of the text. However, to demonstrate the overlap of the results, different symbols are used in Figs. 4–6 to denote the two diabetic subgroups. Agreement between [15N]Phe and [3H]Phe kinetics has been demonstrated previously by others (8).

As previously reported and shown in Fig. 1, insulin infusion decreased S_p in both the diabetic (12) and control groups (9). A slight dose-dependent effect was evident in controls receiving insulin, but this was not statistically significant. The agreement between measured and approximated S_p was not affected by insulin status (see Fig. 4 and Table 3). Correlational analysis indicated excellent agreement between measured and approximated S_p values in both the control (r = 0.99) and diabetic (r = 0.98) groups. Approximated values tended to be lower than measured S_p in the control subjects, but the average error for the S_p approximations was <2%, and the range was 4.8% below to 1.1% above the measured value (Table 3). However, Fig. 4 reveals a tendency for greater separa-
Comparison between measured and approximated values as measured values increase.

Agreement between the direct and indirect models was not as strong for $Q_{pt}$ as it was for $S_p$. Exogenous insulin reduced $Q_{pt}$ in controls but not diabetics (Fig. 2), although there was no systematic effect of insulin on the relationship between measured and approximated values (Fig. 5 and Table 3). The correlation coefficients between the measured and approximated $Q_{pt}$ values were high in each subject group and treatment ($r = 0.89$). By comparing Figs. 4 and 5, it is clear that the absolute differences between measured and approximated $Q_{pt}$ are the same as for $S_p$ ($\pm 2 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). The percent error of the approximations is an order of magnitude higher for $Q_{pt}$ than $S_p$, however. The range of $Q_{pt}$ approximation error in all subjects was $-8\%$ to $+43\%$, with all of the control data and one-half of the diabetic data higher than measured. Unlike $S_p$, the differences between measured and approximated $Q_{pt}$ were evenly distributed (Fig. 5).

Diabetics and controls both had reductions in Tyr flux in response to the insulin administration (Fig. 3). Again, there was no effect of insulin on the agreement between measured and approximated values (Fig. 6, Table 3). Correlations between approximated and measured values were lower than for $S_p$ or $Q_{pt}$ ($r < 0.77$, controls; $r < 0.45$, diabetics). The range of error was $-22\%$ to $+41\%$, and the approximated values were most likely to be overestimates of the measured values (Table 3). Mean differences between measured and approximated $Q_{pt}$ were statistically significant in controls but not diabetics.

Flux ratios are shown in Table 4. $Q_{pt}$ and $Q_t$ data for these calculations were obtained from the measured model with all three of the Phe and Tyr isotopes. The ratio of $Q_{pt}$ to $Q_p$ indicates the relative contribution of Phe hydroxylation to total $Q_p$. It can be seen that $Q_{pt}$ accounted for $9\%$–$11\%$ of total $Q_p$ in all cases, with no differences between groups or treatments. The ratio $(Q_t - Q_{pt})/Q_p$ in Table 4 represents the ratio of Tyr and Phe flux coming from whole body protein breakdown (16). Theoretically, this ratio should approximate 0.73, which is the value used for $P_t/P_p$ in Eqs. 3 and 4. The results show that the control group value was significantly lower than 0.73 in both study phases. Diabetic subjects were evenly distributed above and below the 0.73 value, although the average was $<0.73$ in both conditions.

When the measured $(Q_t - Q_{pt})/Q_p$ ratios were plotted against the $Q_{pt}$ approximation errors (Fig. 7), a wide range of $(Q_t - Q_{pt})/Q_p$ values (0.52–0.81) was evident, and the relationship between the two variables was...
very strong ($r > 0.99$). In Fig. 7 all 72 data points are pooled to illustrate that a single line can be used to describe the relationship between $(Q_t - Q_{pt})/Q_p$ and the $Q_{pt}$ approximation error, regardless of metabolic status. The line of best fit was slightly curvilinear, as denoted by the equation ($y = 266x^2 - 527x + 244$) in Fig. 7.

**DISCUSSION**

The purpose of the current study was to determine the accuracy of the approximation equations for whole body Phe kinetics by use of a previously developed model. Several studies in the literature have used the approximation equations (4, 6, 7, 15, 17), but to date a limited amount of data has been presented that addresses their accuracy. For the purpose of analysis, we have evaluated the approximated values by using the measured values as a benchmark, assuming that the measured values are accurate.

The results show that whole body $S_p$ approximations differed from measured values by <=2%, or <=0.75 $\mu$mol·kg$^{-1}$·h$^{-1}$, on average. The error was biased toward underapproximation in all of the control subjects. Values for subjects with diabetes, however, were equally under- and overapproximated. There was also a slight tendency for the size of error to increase with the absolute value of $S_p$. In practical terms, however, the discrepancies between measured and approximated values are negligible. Figure 1 demonstrates that the variability among subjects within groups is typically equal to or greater than that of the approximation errors. A natural concern when the indirect model of Phe kinetics is used is that agreement between the measured and approximated outcomes could change among treatments or patient groups. Our data show no effect of insulin on accuracy of approximations in either the group with type I diabetes or the group without diabetes. There was a small difference in average approximation error between the diabetic and nondiabetic groups, but, again, in practical terms this difference should not prevent the identification of physiologically important effects in similar patients. Other metabolic states or treatment conditions need to be examined, but in general these results suggest that in studies in which whole body $S_p$ is the variable of interest, the indirect model provides acceptable results.

Fig. 5. Difference between measured and approximated values for Phe-to-Tyr hydroxylation ($Q_{pt}$) plotted against measured $Q_{pt}$ value. Symbols, subjects, and treatments are the same as in Fig. 4.

Fig. 6. Difference between measured and approximated Tyr flux ($Q_t$) plotted against measured $Q_t$ value. Symbols, subjects, and treatments are the same as in Fig. 4.
EVALUATION OF MODELS FOR Phe KINETICS

Table 2. Arterial isotopic enrichments of phenylalanine and tyrosine at plateau

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<thead>
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<tbody>
<tr>
<td>Control</td>
<td>6.58 ± 0.35</td>
<td>1.35 ± 0.08</td>
<td>8.15 ± 0.39</td>
</tr>
<tr>
<td>Basal</td>
<td>8.59 ± 0.23</td>
<td>1.19 ± 0.07</td>
<td>8.15 ± 0.18</td>
</tr>
<tr>
<td>Saline</td>
<td>9.37 ± 0.24</td>
<td>1.30 ± 0.11</td>
<td>9.25 ± 0.28</td>
</tr>
<tr>
<td>0.25 Insulin</td>
<td>9.53 ± 0.28</td>
<td>1.28 ± 0.11</td>
<td>9.39 ± 0.29</td>
</tr>
<tr>
<td>1.00 Insulin</td>
<td>10.11 ± 0.47</td>
<td>1.35 ± 0.10</td>
<td>9.99 ± 0.67</td>
</tr>
<tr>
<td>Diabetic group B</td>
<td>10.93 ± 0.22</td>
<td>1.49 ± 0.11</td>
<td>10.11 ± 0.39</td>
</tr>
<tr>
<td>Insulin treated</td>
<td>9.01 ± 0.32</td>
<td>1.05 ± 0.06</td>
<td>8.43 ± 0.51</td>
</tr>
<tr>
<td>Insulin deprived</td>
<td>8.15 ± 0.26</td>
<td>1.26 ± 0.07</td>
<td>6.77 ± 0.36</td>
</tr>
</tbody>
</table>

Values are means ± SE. Units of measurement are molar percent excess.

In contrast, the approximation equations provided unacceptably variable results for Qpt and Qt. As shown in Table 3, the error rates for these two parameters were 10-fold higher than for Sp. The indirect model produced Qpt values that were significantly higher than the measured approach, which in turn caused the underapproximation of Sp. Sp was calculated as the difference between Qp and Qpt, so any error associated with approximating Qpt will be reflected in the Sp value. However, Qpt was only 10% of the total Qp, so it makes only a minor contribution in the final Sp calculation. Other authors tend to report higher hydroxylation rates, i.e., 15–25% of Qp (2–4, 13–16), so there is potential for Qpt approximation error to make a greater impact on Sp, but the contribution should still remain fairly small. Approximations of Qt, on the other hand, give the same relative error as Qpt because of the method of calculation. In absolute terms, Qt approximations were as much as 8 μmol·kg⁻¹·min⁻¹ above to 11 μmol·kg⁻¹·min⁻¹ below the measured values in these subjects, which is too high to be of use in nearly any study.

A closer look at the Qpt approximation error showed that it was clearly related to the ratio of Qp and Qpt, coming from protein breakdown, (Qp − Qpt)/Qp (Fig. 7). When Thompson et al. (16) developed the equation for estimating Phe hydroxylation (Eq. 3), it was assumed that Tyr and Phe appeared from catabolism in the same ratio as their relative distributions in whole body proteins (Pp/Pp = 0.73). The data in Fig. 7 show that this ratio did not accurately fit the subjects in this study, particularly the control group, because the majority of values were lower than 0.73.

To derive the 0.73 Pp/Pp value, Thompson et al. (16) used the whole body protein amino acid composition from a hen (Pp/Pp = 0.68) and extrapolated from multiple tissue analyses in rat (0.75) and pig (0.75), which were published in the monograph of Munro and Fleck (10). There is a lack of similar data for humans, so most subsequent authors have opted to use the 0.73 ratio in their calculations of Phe hydroxylation. However, for their study on Phe kinetics in infants, Kilani et al. (7) used a ratio of 0.71 to estimate Phe hydroxylation, citing previous work on the human fetus (18). On closer examination, however, the ratio appears to have been miscalculated by those authors (7). The original report of Widdowson et al. (18) presented the amino acid content of fetal bodies in relative mass units (g/g N), which would yield a Tyr-to-Phe mass ratio of 0.71. The number that should be used in the flux equations should be the Tyr-to-Phe molar ratio, which would be 0.64 when the data of Widdowson et al. (18) are used.

In light of the different ratios available, two important points about using them in the approximation

Table 3. Percent error of approximated values relative to measured values

<table>
<thead>
<tr>
<th>Group</th>
<th>Spt</th>
<th>Qpt</th>
<th>Qt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.9 ± 0.2</td>
<td>16.3 ± 1.9*</td>
<td>14.7 ± 1.9*</td>
</tr>
<tr>
<td>Basal</td>
<td>1.6 ± 0.3</td>
<td>15.9 ± 2.7*</td>
<td>14.5 ± 2.6*</td>
</tr>
<tr>
<td>Saline</td>
<td>1.8 ± 0.4</td>
<td>18.6 ± 4.9*</td>
<td>17.0 ± 4.9*</td>
</tr>
<tr>
<td>0.25 Insulin</td>
<td>1.6 ± 0.2</td>
<td>17.0 ± 2.3*</td>
<td>15.5 ± 2.3*</td>
</tr>
<tr>
<td>0.50 Insulin</td>
<td>1.5 ± 0.3</td>
<td>16.1 ± 3.8*</td>
<td>14.5 ± 3.8*</td>
</tr>
<tr>
<td>1.00 Insulin</td>
<td>1.0 ± 0.5</td>
<td>8.2 ± 3.8</td>
<td>−16. +3.6</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.6 ± 0.5</td>
<td>4.9 ± 3.9</td>
<td>−36. ±3.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Spt, Phe incorporation into protein (protein synthesis); Qpt, Phe hydroxylation to Tyr; Qt, Tyr flux. Error was calculated as (approximated − measured)/measured and converted to a percentage. *Significant difference between approximated and measured values (P < 0.05).

Values are means ± SE. Qpt, Phe flux values adjusted for Sp, (Qp − Qpt)/Qp. Data have been pooled from all subjects and treatments, and line of best fit is shown with formula. Dotted lines highlight the ratio of whole body protein contents of Tyr and Phe (Pp/Pp = 0.73, y-axis) used in equation to estimate Qpt and level of zero estimation error (y-axis).

Fig. 7. Distribution of error for approximated Phe hydroxylation (Qpt) in relation to Tyr and Phe flux values adjusted for Sp, (Qp − Qpt)/Qp. Data have been pooled from all subjects and treatments, and line of best fit is shown with formula. Dotted lines highlight the ratio of whole body protein contents of Tyr and Phe (Pp/Pp = 0.73, y-axis) used in equation to estimate Qpt and level of zero estimation error (y-axis).
equations should be made clear. First, the ratio of amino acids in whole body proteins (P_t/P_p) is not likely to match the ratio of those amino acids coming from whole body protein breakdown (Q_t – Q_p)/Q_p. This discrepancy stems from the fact that there are multiple protein pools in the body that have different amino acid ratios (10), different pool sizes, and different turnover rates (11, 12). For example, skin and muscle have a high P_t/P_p and large protein pools but slow turnover, whereas liver and gut have a low P_t/P_p, smaller pool size, and high turnover. The P_t/P_p estimate greatly oversimplifies the complexity of factors determining (Q_t – Q_p)/Q_p. To further complicate matters, changing physiological conditions could affect the (Q_t – Q_p)/Q_p if some protein pools respond differently than others.

Second, in view of the current results, selecting a “better” ratio to use in the approximation equations is not really possible. The main reason is the high variability of (Q_t – Q_p)/Q_p among subjects (range = 0.52–0.81). The cause of this variability is not apparent. Given the high degree of precision for measuring isotopic enrichment in plasma, the variability is more likely physiological than methodological. Whatever the explanation, this variability among patient groups and studies is common. Pacy and colleagues (13, 14) reported average values between 0.52 and 0.59 irrespective of nutritional state or diabetic status. In three pediatric cancer patients (4) and four healthy adults (16), the average ratio was 0.76, whereas in nine healthy infants, the average was 0.88 (2). These differences help explain why previous attempts to validate the approximation equations have met with mixed success. In the studies of young cancer patients and healthy adults, (Q_t – Q_p)/Q_p averaged 0.76, and estimate error for Phe hydroxylation was <2% (4, 16). However, in six type I diabetics, the average ratio was 0.53 under both insulin-treated and insulin-deprived states, leading to an average hydroxylation estimation error of 51% (14). These results are compatible with those shown in Fig. 7.

In conclusion, use of the approximation equations for Phe kinetics can provide reasonably accurate rates of whole body protein synthesis, but approximation of Phe hydroxylation and Tyr flux is associated with unacceptably high and variable levels of error. We have demonstrated that the approximation errors are closely related to the ratio of Tyr to Phe coming from protein breakdown and that this parameter varies widely among subjects. It is unclear why that ratio is so different among subjects, but it underscores the need to use the full measured model in all studies, if possible.

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