How to Assess Amino Acid Requirements
(With Particular Reference to Inborn Errors of Amino Acid Metabolism)

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The 20 amino acids for which transfer RNA exists are the components from which all proteins are synthesized. If they cannot be synthesized endogenously in sufficient amounts then they are needed in the diet (1). Classically in healthy adults, nine amino acids are regarded as essential (or indispensable) (Table 1). During growth or in disease, several others are regarded as conditionally indispensable; in neonates only five of the 20 can be regarded as fully indispensable (Table 1). If amino acids are not present in the right proportions, then protein synthesis will be reduced (2,3). Furthermore, it has been shown that whole body protein breakdown is increased when less-balanced mixtures of amino acids are fed (4). The endogenous amino acids then provide the missing amino acids for protein synthesis, but net protein accretion and nitrogen balance (retention) is lower when a less than ideal mixture of amino acids is given, either as a protein or as a mixture of free amino acids (4,5).

In inborn errors of amino acid metabolism the requirement for certain amino acids may be altered. For example, in phenylketonuria (PKU), owing to the block in the hydroxylase pathway in the classic form, tyrosine formation from phenylalanine is reduced to less than 2% of normal (6). As a result, tyrosine becomes an indispensable amino acid (7). Similarly, phenylalanine needs are reduced (8).

Until recently, the requirements for the amino acids involved in inborn errors of metabolism have been estimated by plasma amino acid concentrations (8)—phenylalanine concentrations in the case of patients with PKU and branched chain amino acids (BCAA) in patients with maple syrup urine disease. However, plasma amino acid concentrations are generally recognized as insensitive indicators of amino acid requirements (2).

METHODS TO DETERMINE AMINO ACID REQUIREMENTS

Determination of amino acid requirements involves feeding graded levels of the test amino acid to the subject and looking for a clearly definable change in a relevant biologic variable. Classically, outcome variables such as nitrogen balance and growth
Adapted from Pencharz et al. (1) While this table was first developed for neonates, the amino acids listed as conditionally indispensable may become so in older age groups during times of disease.

have been used in infants and children, and nitrogen balance in adults (2,3,9). Clearly both of these variables are indirect and hence may be insensitive. Furthermore, the body urea pool may need 7 to 10 days to adapt to changes in the dietary intake of protein or amino acids (10). This becomes a problem in infants and children, who cannot be maintained on either deficient or excessive intakes for an extended time (11). Thus, alternative methods are needed.

Although carbon oxidation methods had previously been developed in animals (12-14), it was the pioneering work of Meguid and coworkers (15) which first applied carbon oxidation methods to the determination of essential amino acids in humans. They used the direct oxidation approach in which the test amino acid was also used as the tracer. Their initial studies were in the fed state only, and questions were raised as to whether this was an accurate representation of amino acid requirements on a 24-hour basis. This led them to conduct 24-hour direct oxidation amino acid balance studies (16), which provided similar estimates of leucine requirements compared with the earlier direct oxidation studies (15).

Before the development of the 24-hour balance method, a different carbon oxidation model was introduced (17) to study adult amino acid requirements—namely, indicator amino acid oxidation (IAAO). This model had first been developed and validated in growing pigs, and was shown to provide an estimate of requirement very similar to that determined by the classical techniques of nitrogen balance and growth (13,14). IAAO uses another essential amino acid such as lysine or phenylalanine (in the presence of an excess of tyrosine) as a tracer, which is independent of the changes in the levels of intake of the test amino acid (2,13,14,17-20). As the intake of the limiting amino acid increases from deficient to adequate, the indicator amino acid is partitioned between incorporation into protein and oxidation (2,3,13,14). In the early animal studies, it was shown that the IAAO method did not require adaptation to the level of the test amino acid (13,14), which is a basic requirement for using nitrogen balance to determine amino acid requirements (2,9).

When we decided to adapt the IAAO to humans, one of the first steps was to study the effects of adaptation on the requirement estimate. This was done with subjects fed either
4.2 mg/kg/d or 14 mg/kg/d of phenylalanine for two 9-day periods, during which the test levels of phenylalanine were fed on days 3, 6, and 9. There was no effect of previous adaptation to the two levels of phenylalanine on the rate of oxidation within test intakes of amino acid, and therefore no effect on the estimate of requirement (21).

Based on these studies and the earlier animal work, we concluded that previous adaptation to the level of the test amino acid was not needed. It appears that the response of protein synthesis and oxidation of essential amino acids occurs very rapidly following meals providing varying intakes of one amino acid, compared with the very slow response of whole-body nitrogen pools to a similar change. We have also shown in free living subjects, over a wide range of total protein intake, that it may be necessary to standardize the level of protein intake for 2 days before conducting an indicator study (22). All of these steps are important because they enabled us to develop a minimally invasive indicator model to study amino acid requirements under clinical conditions (11).

Most recently, the concept of a 24-hour indicator amino acid balance (IAAB) has been applied to the indicator oxidation model (23). While this approach is theoretically the most satisfactory method yet developed for determining amino acid requirements, it has the drawback of requiring the subject to be detained for 24 to 29 hours for each study, which compares with the 8 to 10 hours needed for the standard fed-state IAAO study. Furthermore, careful inspection of the 24-hour indicator oxidation data shows that oxidation of the indicator amino acid in the fasted state is constant across the four levels of lysine fed (23), and hence does not alter the requirement determined from the fed oxidation data alone. In addition, the users of the 24-hour IAAB method still believe that adaptation to the level of the test amino acid is needed (23), despite the fact that estimates based on 24-hour amino acid balances, using both direct oxidation and indicator oxidation approaches, are not different from the nonadapted, short-term studies.

Users of the IAAB method have chosen leucine as their indicator amino acid. Up to now we have been reluctant to do this in our IAAO studies, because of the common degradative pathway for the three BCAAs and hence the possibility of interactions between the three. This would introduce an uncontrolled variable that might affect the linearity of the oxidation response to decreasing intakes of the limiting (test) amino acid, which in turn could alter the estimate of requirement for the test amino acid. Studies have been conducted in piglets to show that lysine and phenylalanine give comparable values (2,13,14). Similar studies have not been conducted to confirm that leucine is a satisfactory indicator amino acid.

Our group also introduced a two-phase linear crossover regression analysis for determining amino acid requirements (17,21). This approach provides an objective statistical estimate of the mean requirement and an estimate of the population variance that can be used to calculate a safe dietary intake for the particular test amino acid. Before the introduction of this approach, and indeed for many subsequent experiments, the number of levels of the test amino acid studied had been inadequate for proper statistical derivation of the requirement. For the IAAO and IAAB studies, the subjects are fed amino acid–based diets, which are unpalatable. For the IAAO studies using the minimally invasive model, the subjects are on the test diet only on the
day of the oxidation study (7,11). Conversely, for the IAAB study, depending on the length of adaptation, the subjects are on the amino acid based test diet for 8 days for each test level (23). The two-phase method needs each subject to be studied at six or seven levels of the test amino acid. The reason for this large number of levels is to ensure that at least three data points are used for determination of the slope of the line. In practical terms, this means that subjects need to take the study diet for 6 or 7 days using the IAAO model, and for between 54 and 63 days for the IAAB model, as presently used. Clearly the IAAB method is much more demanding and probably totally impractical in children.

The change in the plasma level of the test amino acid has also been used to define essential amino acid requirements, most successfully for tryptophan (24); however, on balance it is an insensitive method and has been supplanted by the carbon oxidation method (9,25–27). Clinically, the blood levels of essential amino acids are used to monitor the control of the diet in patients with PKU or maple syrup urine disease (8). We have found that in children with PKU, the blood concentration of phenylalanine does not change in a quantitative way in response to graded levels of dietary phenylalanine (28). Conversely, the change in blood phenylalanine (fed minus fasting) responds in a linear fashion to phenylalanine intake (28). The possible implication of these data is that clinical monitoring of patients with PKU could be improved by comparing the fed to fasted levels.

Over the past 15 years, since the original direct oxidation studies, there has been controversy over how best to determine essential amino acid requirements and what the correct values are for adults (2,3,9,25–27). In general, the older values, based on nitrogen balance, were two to three times lower than some of the new values derived by carbon oxidation techniques. To aid the reader, a comparison of the methods is shown in Table 2, with a listing of strengths and weaknesses. Strong support for the estimates obtained by direct oxidation of the test amino acid was provided by the introduction of indicator amino acid oxidation (2,17), which is based on different assumptions. Finally, it was realized that part of the problem with the older nitrogen balance estimates was that most of the studies did not examine each individual subject at a series of graded levels. This is important because we have shown in every one of our requirement studies, using repeated measurements on individuals, that the subject is always a highly significant variable. This source of variability can only be accounted for if the subjects are studied at several levels. Reanalysis of one study—in which nitrogen balances were performed in women at different levels of lysine intake, using regression analysis and taking into account miscellaneous nitrogen losses—resulted in a lysine requirement estimate of about 30 mg/kg/d (29). This value is comparable with the estimates obtained using direct oxidation (30), indicator oxidation (17), 24-hour lysine balance (31), and, most recently, 24-hour IAAB (23). Given the convergence of these different methods the carbon oxidation methods are now regarded as having replaced the traditional nitrogen balance technique, at least for studies in adults.

We have thus moved to make the IAAO model as noninvasive as possible (11) so that it can be used in a wide variety of age groups, physiologic states, and clinical conditions. We have shown that the tracer can be given orally (rather than intra-
**TABLE 2. Comparison of methods used to determine amino acid requirements**

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths and limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth</td>
<td>• Useful only in rapidly growing young animals.</td>
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<tr>
<td>Nitrogen balance</td>
<td>• Classical method, but it needs great care to make sure the balances are as precise as possible and miscellaneous losses must also be measured or estimated from literature values.</td>
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<td></td>
<td>• Requires 5 to 7 days adaptation to each level of amino acid for equilibration of the body nitrogen pools.</td>
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<tr>
<td>Direct oxidation</td>
<td>• Unclear as to whether, or how much, previous adaptation is needed—indeed Zello (21) suggests that at most only 2 days of adaptation are needed.</td>
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<tr>
<td></td>
<td>• Can only be used to determine the requirements of amino acids whose carboxyl group is directly released to the bicarbonate pool and so will appear in breath (lysine, phenylalanine, leucine, isoleucine, valine, and possibly threonine).</td>
</tr>
<tr>
<td></td>
<td>• Fed state studies appear to provide similar requirement estimates to (24 h) balances.</td>
</tr>
<tr>
<td>24-h Direct oxidation</td>
<td>• Unclear as to whether previous adaptation to the level of the test amino acid is needed (see direct oxidation above), but adaptation to the experimental diet is needed for at least 4 before starting the study.</td>
</tr>
<tr>
<td></td>
<td>• Can only be used to determine the requirements of amino acids whose carboxyl group is directly released to the bicarbonate pool and so will appear in breath (see above).</td>
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<tr>
<td>Indicator oxidation</td>
<td>• There is still some debate as to the best indicator to use and to the present phenylalanine (in the presence of an excess of tyrosine), lysine and leucine (for 24-h indicator balance studies see below) have been used; the only other possible choices are valine and isoleucine.</td>
</tr>
<tr>
<td></td>
<td>• The evidence (discussed in the text) suggests that previous adaptation to the level of the test amino acid is not needed.</td>
</tr>
<tr>
<td></td>
<td>• The requirements of any essential amino acid or conditionally indispensable amino acids can also be studied with indicator oxidation.</td>
</tr>
<tr>
<td>24-h Indicator balance</td>
<td>• This is a combination of the features of 24-h direct balance with the indicator oxidation approach and so has many theoretical advantages. So far its users have made its use more limited by insisting on adaptation to the level of the test amino acid for 8 days. Leucine was used as the indicator, which has some potential problems that are discussed in the text.</td>
</tr>
<tr>
<td></td>
<td>• As discussed in the text, it is more invasive and time-consuming for the subjects and costly for the investigative team. As such, unless it is modified its application will be limited to studies in healthy adult volunteers and exceptionally well-funded scientists.</td>
</tr>
<tr>
<td></td>
<td>• The requirements of any essential amino acid or conditionally indispensable amino acids can also be studied with 24-h indicator oxidation and balance.</td>
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</table>

venously); that there is no need for previous adaptation to the level of the test amino acid [although a 2-day adaptation to the level of the protein fed may be necessary (22)]; and finally, that the subjects do not have to be adapted to the experimental diet, although if they are not, this extends the length of the study from about 5 hours to about 9 hours. The additional 4 hours are needed to allow for equilibration of the
background $^{13}\text{CO}_2$ enrichment in the breath, in addition to the 5 hours needed for the oxidation study itself (11). Children with inborn errors of amino acid metabolism are already on standardized dietary protein intakes; therefore, in these subjects even adaptation to the level of protein intake is not needed.

Although we developed the minimally invasive model to permit the study of children with PKU, we have more recently been able to use the model to determine total BCAAs in 6- to 10-year-old healthy children (32). The way is now open to a more general application of this modified IAAO model to the determination of amino acid requirements in vulnerable groups such as infants, children, adolescents, and pregnant and lactating women (3,7,11).

**DETERMINATION OF TYROSINE AND PHENYLALANINE REQUIREMENTS IN CHILDREN WITH PKU**

PKU is a disorder of aromatic amino acid metabolism in which phenylalanine cannot be converted to tyrosine, or only to a very limited extent (6). Thus, tyrosine is an indispensable amino acid in PKU because it is not supplied endogenously through phenylalanine hydroxylation (33), or only to a very limited degree (6).

The aim of dietary treatment of PKU is to maintain plasma concentrations of phenylalanine, tyrosine, and other amino acids within the normal range, thereby allowing optimal growth and brain development. Treatment consists of supplying adequate energy, other amino acids, and nutrients, while phenylalanine intake is restricted and tyrosine intake is supplemented (8). At present, the dietary management of patients with PKU is empirical because it is based on monitoring plasma amino acid concentrations, blood urea nitrogen, and growth indices, and not on direct measures of tyrosine and phenylalanine requirements.

The recommended daily aromatic amino acid intakes (phenylalanine plus tyrosine) in healthy infants and children (34), and the currently recommended levels for phenylalanine and tyrosine for children with PKU (8), are shown in Table 3 for three age groups. In PKU, the supplemental tyrosine intake when added to the phenylalanine intake far exceeds the recommendations for aromatic amino acid intake in the general, healthy population (8,34). In fact, the median recommended tyrosine intake across the different age groups represents five to seven times the corresponding phenylalanine intake. This suggests that, of the total of aromatic amino acid requirement, phenylalanine contributes around 20% and tyrosine around 80%. These proportions are significantly different from the relative balance of phenylalanine and tyrosine—and their respective contributions to meeting aromatic amino acid requirements—in animals (35–39) and healthy humans (40), in whom dietary tyrosine was shown to spare 40% to 50% of the phenylalanine requirement. This almost-equivalent contribution of phenylalanine and tyrosine to total aromatic amino acid intake is consistent with the plasma (33,41) and mixed body protein (42) ratio of phenylalanine to tyrosine. Therefore, we hypothesized that the recommended phenylalanine and tyrosine intakes for children with PKU were incorrect.

We determined tyrosine requirements using lysine as the indicator amino acid (7). Based on the ratios of phenylalanine to tyrosine described above, we hypothesized
that the tyrosine requirement in PKU would account for approximately 45% of the total aromatic amino acid requirement. When tyrosine intake was below the requirement level, lysine oxidation was high. As the tyrosine intake was increased, lysine oxidation fell until a constant low level of oxidation was reached. These data were analyzed by two-phase linear crossover analysis and a breakpoint was defined as the point where lysine oxidation was first minimized. The mean dietary requirement was determined to be between 16.3 and 19.2 mg/kg/d, depending upon whether lysine oxidation or label appearance (F$^{13}$CO$_2$) was used as the endpoint. These values would represent 40.4% and 44.4% of the total aromatic intake in children with PKU, respectively. The current recommendations for tyrosine intake in patients with PKU are thus overestimated by a factor of around 5. The findings of this study have significant implications for the dietary treatment of individuals with PKU.

Dietary phenylalanine restriction has been the mainstay of the treatment of PKU for more than 40 years (8). Its main aim is to maintain a phenylalanine intake that will allow optimum growth and brain development, by supplying adequate energy, protein, and other nutrients while restricting phenylalanine. Implementation of such a diet soon after birth usually prevents most of the overt clinical manifestations of PKU. Nevertheless, there is evidence that neuropsychologic and cognitive function is not entirely normalized in individuals with PKU receiving current treatment regimens (43–46). Present estimates of phenylalanine requirements are based on plasma phenylalanine levels and growth rate in relation to dietary intake, and not on direct and sensitive measurements of amino acid metabolism.

Because patients with classical PKU have a negligible or minimal capacity to oxidize phenylalanine (6), we reasoned that their dietary phenylalanine requirements would be lower than those in healthy children by an amount equal to the obligatory losses of phenylalanine. Dietary requirements for phenylalanine in healthy children or in those with PKU have yet to be defined by direct measurements of amino acid metabolism. We therefore referred to our previous study using indicator amino oxidation, which showed that the mean tyrosine requirement of children with PKU was 19.2 mg/kg/d (7). Next we used a ratio of 55:45 between phenylalanine and tyrosine in the tissues of humans and animals (18,21,35,36,47), which, when factored to the mean tyrosine re-
requirements, predicts a mean phenylalanine requirement for healthy children of approximately 23.5 mg/kg/d. The requirement for phenylalanine or any other indispensable amino acid is the sum total of that needed for protein synthesis plus irreversible losses (10). Because there are no data in children, we had to turn to a study of phenylalanine requirements in adult male subjects (21), in whom obligatory oxidation was estimated to be approximately 26%. Using the predicted phenylalanine requirement for children, and the estimated obligatory oxidation for phenylalanine, the obligatory loss was calculated to be approximately 6.1 mg/kg/d. Subtracting the estimate for obligatory loss from the predicted mean requirement of 23.5 mg/kg/d, the resulting value of 17.4 mg/kg/d became the mean phenylalanine requirement predicted for children with PKU.

The objectives of this study were to determine the phenylalanine requirement of children with classical PKU using the technique of indicator amino acid oxidation, and to compare the results with our previous estimate of the tyrosine requirement obtained using the same technique. This study has only been presented in abstract form (28), but the mean estimate of phenylalanine needs is 14 mg/kg/d. This compares with the tyrosine estimate obtained using the same technique of label oxidation ($^{13}$CO$_2$) of 19.2 mg/kg/d. Therefore in children with PKU we estimate that the total mean aromatic amino acid requirement is about 33 mg/kg/d, and that the proportions of phenylalanine and tyrosine are 42% and 58%, respectively. This is the reverse of the relative proportions in the tissues of normal individuals (47).

DETERMINATION OF AMINO ACID REQUIREMENTS IN OTHER INBORN ERRORS OF AMINO ACID METABOLISM

Clearly from the success of the minimally invasive indicator oxidation method (11) in defining tyrosine (11) and more recently phenylalanine needs (28) in children, the way is open to using this technique in studying the next most prevalent inborn error of amino acid metabolism, maple syrup urine disease (8). Before attempting to study patients with this condition, we felt it necessary to determine total branched chain amino acid needs in healthy children (32) and adults (48). Previous work by others had only determined requirements for leucine and valine (26), and has merely estimated isoleucine requirements. We reasoned that, because of the common degradative pathway for the three BCAAs and the known interactions between them, it is better to study them collectively, in a balance approximating that in egg protein, using the indicator amino acid oxidation model.

Now that we have control data, we are poised to study patients with maple syrup urine disease. Again our reasoning is similar to that already outlined for the study of phenylalanine requirements in children with PKU—namely, to try and estimate obligatory oxidation of the three BCAAs and subtract that value from the requirement estimates made in healthy children and adults. This theoretical exercise is important because it allows the estimation of levels of intake above and below the predicted requirement breakpoint. The statistical definition of the breakpoint is optimized when the levels are evenly distributed so that three are above it and three below.
SUMMARY AND CONCLUSIONS

The advent of the minimally invasive indicator amino acid oxidation model has opened the way to objective and sensitive determination of amino acid requirements in patients with inborn errors of amino acid metabolism. Requirements for tyrosine and phenylalanine have been determined for children with PKU. From these estimates, it is possible to state that published estimates of tyrosine requirements in PKU are a five-fold overestimate. Using our minimally invasive IAAO method, it is now possible to extend quantitative studies of amino acid requirements into vulnerable groups such as infants, children, pregnant women, and patients with diseases.

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REFERENCES

**DISCUSSION**

**Dr. Koletzko:** You said that adaptation occurs within a few hours. We know from many other examples that the organism has several ways to adapt to variations in substrate intake. I wonder how good the arguments are that this adaptation really occurs within a few hours, and there is no effect of interpretation on the measurements.

**Dr. Pencharz:** Well the estimates obtained for lysine—which has been studied the most—show that no adaptation versus adaptations for as long as 40 days gives you the same estimate, so in practical terms it looks as though the minimally invasive model with 6 hours of adaptation is sufficient. For phenylalanine, we've shown that it makes no difference whether you adapt for no time, 2 days, 6 days, or 9 days. So whereas with nitrogen balance it makes a very big difference, the adaptation of amino acid metabolism is very rapid, a matter of hours.

**Dr. Endres:** I think the motivation behind ranges of tyrosine intake in infants or children with PKU is quite variable, and some people give additional tyrosine (1). When you determined the breakpoint, as you call it, does that really answer the question of how much tyrosine is needed by a PKU patient? It's not the tyrosine that matters, it's the dopamine that's formed. Could you comment on that? I think your answer is perhaps a little too easy for this disease.

**Dr. Pencharz:** Your comment is well taken and I apologize if I wasn't sufficiently clear in my caveats. I said that the indicator would suggest how much is needed for protein synthesis; it won't necessarily measure the other pathways. I did mention, however, that Joe Clarke, who is a co-investigator in our PKU studies, had been involved in a study where the endpoint was neurologic behavior, and was unable to show any benefit in a double-blind, placebo-controlled trial with 100 mg of tyrosine (2). I am aware that there are other people who don’t agree, and we could argue at length about the validity of various data. I think there are different endpoints, and I remain open to using others. But in terms of whether you need additional tyrosine to deal with competition from large neutral amino acids affecting uptake into cells and then the synthesis of dopamine—or other brain endpoints—your point is well taken. I accept that’s not the end of the story.

**Dr. Bachmann:** Could you also clarify what protein synthesis we are talking about? In an adult, there is about 15 kg of muscle mass, 1.5 kg of liver, a similar amount of brain, and the kidneys. Are there not some functionally important protein synthesizing organs that might be completely ignored if we look only at the total sum? I'm not criticizing your approach, but perhaps it won't show up the weakest points in the process of protein synthesis in tissues that have a rapid turnover. We have only averages, which are dominated by muscle.

**Dr. Pencharz:** I agree that although we talk about, let’s say, lysine being 7% or 8% of mixed body protein, the amount of lysine in different proteins in the body varies quite markedly, and this applies to other amino acids. All available methods ultimately reflect what's happening at a whole body level. None of the studies that I've referred to looks at something occurring across skeletal muscle or across the kidney. These techniques are analogous to resting metabolic rate for energy metabolism. We are not trying to determine the tyrosine requirement of the kidney; rather, we are attempting to determine the tyrosine requirement for the whole body. From a dietary point of view, we are really saying how much the diet should contain. In energy expenditure studies, we can only say how much energy the whole body needs, not what is the energy expenditure of...
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the heart or liver. These protein studies are very analogous. Whether they involve nitrogen balance, direct oxidation, indicator techniques, or whatever, they are still reflections of whole body metabolism in an integrated fashion. However, the minimally invasive technique can be applied, for example, to patients with chronic renal disease. Dr. Scott talked about the sulfur amino acids earlier, and I would say that all methionine requirements are reduced if you have chronic renal disease, because the enzymes and pathways for sulfur amino acid metabolism exist in the kidney as well as in the liver. Therefore, at a dietary level you can use this technique to define whether methionine requirements are indeed reduced if you have chronic renal insufficiency.

Dr. Micheli: Eighty percent of very preterm babies receive antenatal corticosteroids. By giving these, we modify genetic programming not only of the lungs but also of the circulation in the heart and brain. Can you tell us how this interferes with amino acid metabolism and requirements?

Dr. Pencharz: You raise a good point. Stephanie Atkinson, who works down the road from me in Hamilton, has shown very clearly that antenatal steroids have adverse affects on skeletal muscle. This is likely to be detrimental in such infants, in whom skeletal muscle is already rather a small component of total body protein. I don’t know of any good studies that show what this does to the leucine turnover versus phenylalanine, and so on. It is a problem in examining such effects that we have very few data on requirements for essential amino acids in children—only three studies in fact (3), which are the sum total of worldwide studies in this area.

Dr. Bier: I’d like to comment on Dr. Micheli’s question. I don’t believe anybody has systematically studied amino acid kinetics in newborn infants as a function of maternal steroid administration. We’ve studied amino acid kinetics in a lot of neonates and preterm infants for other reasons, and without systematic analysis, we’ve been unable to distinguish between the mothers who had received antenatal steroids and those who had not on the basis of kinetic events measured in those neonates.

Dr. Scott: Dr. Pencharz, your model predicts that the rate of protein synthesis is relatively unchanged over the periods of measurement. That surprises me a bit because I would have thought the intake of other nutrients—carbohydrates and so on—would change the levels of hormones such as insulin and glucagon, which would have catabolic or anabolic effects. Do you control for other macronutrients during the study periods? I think you would expect some fluctuation in protein synthesis.

Dr. Pencharz: You are quite right. Experimentally what one does is to feed the subjects at hourly intervals, which minimizes fluctuation. This subject was explored in a recent article in the American Journal Clinical Nutrition (4). There are differences in the rates of oxidation of the indicator amino acid (the investigators used leucine in that paper) in the fasted state versus the fed state. However, they tried to keep as many variables constant as possible to come up with the kinds of answers they did.

Dr. Baerlocher: Did you mention the phenylalanine concentration in your patients with PKU when you did your study with tyrosine? High levels of phenylalanine could influence tyrosine metabolism.

Dr. Pencharz: The subjects were all maintained in what, in Toronto, is regarded as good control—that is, plasma phenylalanine in the range of 400 to 600 μmol/l. We did a post hoc correlation analysis and couldn’t find any effect of phenylalanine concentration.

Dr. Bier: You went rather quickly through the fact that lysine oxidation went up in the phenylalanine study once you got above the requirement level. Would you like to discuss that? It shouldn’t happen theoretically.

Dr. Pencharz: It was a big surprise to us. The way we are presently interpreting this is as follows. The indicator study is dependent on the concept that the indicator amino acid is partitioned between on one hand, incorporation into protein and, on the other hand, oxidation. Because oxidation increased statistically, that implies that something is happening within the whole body
of those children as you give phenylalanine intakes above 14 mg/kg/d, and that the utilization of the indicator—that is lysine—for protein synthesis is less and hence there is more oxidation. This is really saying that whole body protein synthesis is being inhibited. Why should that be? Well, we are saying in effect that the availability of all 20 amino acids at the point of polyribo-somes for protein synthesis is somehow being affected. Being more specific is impossible based on our data. From other published reports, you can hypothesize that as you increase plasma phenylalanine, you may affect the uptake of tryptophan, for example. Tryptophan may then become a limiting factor intracellularly in terms of protein synthesis. This is all speculation.

Dr. Kern: You gave very precise recommendations for intake of amino acids based on hourly feeding. In practice, patients with PKU take their powder supplements two or three times a day. Does it matter if they take their amino acid mixture in this way and not every hour?

Dr. Pencharz: This has been looked at by various people, including Vernon Young. The answer is that it probably doesn’t matter. The model that we’re using, where we try to keep everything else as constant as possible by feeding hourly, still seems to reflect the requirements even when you switch to meal feeding.

Dr. Superti-Furga: I think your observation that protein synthesis goes down when you increased the phenylalanine intake is very interesting and may be a clue to the pathogenesis of the disorder. Did you cause these controlled subjects to lose their metabolic control by giving the higher dosage?

Dr. Pencharz: No, they didn’t go out of clinical control. We figured out the highest quantity we could safely use. We didn’t see any metabolic consequences, any change in acid–base status, electrolytes, and so on.

Dr. Superti-Furga: I think this is very important because here in Switzerland we’re still struggling to find the optimal target for phenylalanine blood values. Knowing that increasing the phenylalanine intake, even if this is not reflected at blood level, may have adverse effects is very significant. Thank you.

Dr. Pencharz: Clearly there will be the adverse effects that we are all aware of in terms of the central nervous system, but we were just trying to see whether we could define at a biochemical or physiologic level what the requirements are. They appear to be lower than previously reported, which I think is worth knowing, but now we have to decide how to apply the knowledge. There’s work still to be done before we can apply it to a PKU population.

Dr. Borum: I understand that the experimental design tightly controls carbohydrate and lipid intake and involves hourly feeding, but what is the impact of carbohydrate and lipid intake on protein metabolism and protein requirements in particular situations such as childhood obesity? If a child has an excessive energy intake, is there an altered amino acid requirement? And, what about other situations such as ketogenic diets for seizures or diets for various inborn errors of metabolism?

Dr. Pencharz: I can answer that in various ways. We directly studied this in parenterally fed neonates where we held amino acids constant and varied the nonprotein energy, either giving glucose only or replacing 33% of the glucose energy by fat. In that particular model, we were unable to show any differences in protein turnover. We weren’t defining requirements, merely glycine and leucine turnover. Therefore, we weren’t able to show any differences. When we looked at the literature on dietary requirements, the main question seemed to be what is the minimum carbohydrate intake, because we know that the carbon skeletons of protein are necessary for gluconeogenesis. Providing you give enough carbohydrate to meet the minimum necessary for glucose production, that seems to be all that is required. The balance between fat and carbohydrate doesn’t appear to matter.

Dr. Wahba: In a pregnant woman with PKU who is on a phenylalanine-restricted diet, what is the prognosis for the baby with regard to intellectual development?
**Dr. Pencharz:** It has been well established that if you don’t treat the mother the baby will be brain damaged. These babies do not grow as well, and their brains are abnormal. If the woman is on a properly controlled phenylalanine intake during pregnancy, the outcome appears to be normal.

**Unidentified participant:** You show that a safe level of phenylalanine in patients with PKU is around 17 to 20 mg/kg/d. Have you studied this “safe” level in terms of neurologic outcome?

**Dr. Pencharz:** You are asking me whether, having defined this breakpoint, we have then gone on and done long-term studies while feeding children at that level. The answer is no, and that’s what I meant when I said that more work needs to be done. The phenylalanine data have not yet been published in a peer-reviewed journal, but we are presently thinking about doing studies with tighter control.

**Dr. Fowler:** I’m sure we still need to measure blood phenylalanine levels to control our patients and not just rely on this worked out level of minimum requirements. I have a question: can you extrapolate this new level of phenylalanine that you’ve elegantly determined to a minimum level of protein requirement, as we discussed in the previous talk?

**Dr. Pencharz:** Not really. Protein requirements come down to the issue of how much you need of the other 19 amino acids.

**Dr. Fowler:** Perhaps my approach is naïve, but if I take your 20 mg of phenylalanine as a minimum requirement, and if we assume that 4% of protein is phenylalanine, then you come up to level of 500 mg/kg as the minimum protein requirement, which is not far off Hélène Ogier’s value of 600 mg.

**Dr. Pencharz:** Well, that assumes 100% utilization. Peter Reeds and I reviewed the world literature on this fairly thoroughly, and maintenance—that is, no growth at all—is about 680 mg/kg. Whether you actually need 1.5 g isn’t clear, and there is also the issue of activity. Exercise probably doesn’t make a big difference, but it may increase your value to around 800 mg/kg.

**Dr. Koletzko:** We need to be cautious about implying that the proportions of amino acids in proteins determine the metabolic requirements in the organism. Also, in the real world, at least in infants, we not only have the true protein nitrogen intake, but we also have quite a lot of non-protein nitrogen, and we don’t really know to what extent that is utilized by the organism.

**Dr. Bier:** I want to advise caution about the use of the term “requirement.” We use it to mean a variety of things. For example, there’s the maintenance requirement; there’s the minimum requirement for something or other, which might include growth; there’s an estimated average requirement; and there’s a recommended dietary allowance. They’re all remarkably different, and we have used the word “requirement” very casually throughout the discussions.

**Dr. Pencharz:** We’re talking about studies involving means for a population. When you talk about going from a mean to a requirement for a population, you usually imply the mean plus two standard deviations. The value of 0.68 g/kg which I just gave, which rounds up to 0.7, becomes 1.1 g when you add two standard deviations, so this is the population requirement rather than the mean estimate for an individual.

**REFERENCES**