Summary of Workshop Discussions on Establishing Upper Limits for Amino Acids with Specific Attention to Available Data for the Essential Amino Acids Leucine and Tryptophan

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Abstract
The morning of the first day of the 8th Amino Acid Assessment Workshop was organized and co-sponsored by the International Council on Amino Acid Science (ICAAS) and the International Life Sciences Institute Research Foundation and was focused on the International Life Sciences Institute Research Foundation’s approach to establishing upper limits of nutrients. The remainder of d 1 and all of d 2 were focused on the safety of leucine and tryptophan, with special emphasis on determining the upper level of the safe range of intake. It was recognized that some toxicological frameworks, mainly the key-events dose response framework, might be applicable to amino acids and provide appropriate assistance to regulators in establishing upper limits for amino acids as a group of nutrients used in dietary supplements. ICAAS-funded projects for determining the upper intake limits for the essential amino acid leucine provided the main pool of leucine data discussed at the workshop. The acute clinical study suggests 500 mg/(kg \( \cdot \) d) as a possible upper limit for leucine in healthy humans, but the safety margin needed to widen this limit to the general population has not been determined. For tryptophan, the workshop participants found less ground to support a safe upper limit. Older efficacy studies suggested that tryptophan at 8–15 g/d was well tolerated, but human research was abruptly terminated in the late 1980s and no new data are available. Animal results obtained in pigs and rodents were discussed and two possible strategies for applying those outcomes to humans were described.

Introduction
The 8th Amino Acid Assessment Workshop was held as part of a continuing dialogue among experts in amino acid nutrition, metabolism, cell and molecular biology, toxicology, and regulation/policy, with the objective of establishing a framework for assessing and predicting the consequences of differing levels of amino acid intake(s) in humans under various conditions. The morning session of the first day of the workshop was devoted to general considerations and existing frameworks for setting upper limits for nutrients and contaminants. Particular attention was given to the International Life Sciences Institute Research Foundation experience with the key-events dose response framework (KEDRF), an analytical framework for systematically examining key events that occur between the initial dose of a bioactive agent and the effect of concern. In this framework, individual key events that influence the dose-response relationship and factors that underlie variability are considered. It promotes an evidence-based approach for using mechanistic data to reduce reliance on default assumptions, quantify variability, and better characterize biological thresholds.

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4 Abbreviations used: EM, eosinophilia myalgia; ICAAS, International Council on Amino Acid Science; KEDRF, key-events dose response framework.
This framework has been used to arrive at a safe upper level of intake for some food contaminants but also for vitamin A (1), which has known toxicity when taken in excess. Developed as an extension of the International Life Sciences Institute/International Program on Chemical Safety Mode of Action/Human Relevance Framework, KEDRF allows risk assessors to construct not only an understanding of how the studied chemical is imposing its toxicity but also the dose-response(s) for each key event. Discussions also touched upon the recommendations by the 2007 NRC in its report “Toxicity Testing in the 21st Century: A Vision and A Strategy” to move away from phenomenological approaches by using molecular technologies to identify the ways environmental agents perturb normal cellular pathways to produce their adverse effects (2).

The U.S. regulatory experience with setting upper limits during the last 2 decades is reflected through the various Institute of Medicine committees charged with developing safety margins for a range of nutrients. The committees utilized the risk assessment framework(s), making modifications and adjustments as dictated by the data, but recognized that a framework cannot compensate for a lack of data. A framework makes clear the decision-making that is to occur and is a highly useful tool for identifying research needs, but it cannot fix the basic problem of too little data (3).

Sessions 2 and 3 of the workshop were specifically devoted to the current experimental data available for 2 essential amino acids, leucine and tryptophan, as well as to the application of KEDRF to leucine. General discussions from both leucine and tryptophan sections as well as the final general discussion that closed the whole workshop are summarized in the rest of this report.

**Determining an Upper Limit for the Amino Acid Leucine**

The review of the existing literature that was presented at the workshop pointed out that the biochemical features of the essential amino acid leucine make it attractive as a supplement in a diverse range of circumstances and that it is well tolerated up to high levels of intake when given together with valine and isoleucine (4). Although rat studies have attempted to identify tolerable upper limits of the individual BCAA, the relevance of these studies to human situations (5) has been questioned. Although humans appear to tolerate BCAA mixtures up to high levels of intake, data on the upper limit of leucine individually or as a balanced mixture of BCAA has not yet been reported in the peer-reviewed literature. The first presentation at the workshop and consequent discussion predicted that: 1) tolerance of all 3 BCAA would be higher than that of leucine alone, because of its influence on valine and isoleucine concentrations; and 2) tolerance would increase with time because of adaptation of the oxidative capacity.

A week-long animal study was presented (6), showing that leucine intake of up to 8% triggers no adverse effects when moderate or high amounts of protein are consumed. In a low-protein (6%) condition, a gene-marker panel suggested that dietary leucine supplementation of 2% (wt:wt) might be the no-observed adverse effect level dose in rats. Integrated transcriptomics and metabolomics analyses were highlighted as a powerful tool for evaluating the influence of a large intake of a single amino acid. In the presented acute human study, leucine oxidation was measured using \[^{13}\text{C} \text{O}_2\] and the appearance of \[^{13}\text{C} \text{O}_2\] in breath (8). No gut intolerance was seen and blood glucose fell progressively but remained within normal values, without any changes in plasma insulin. Maximal leucine oxidation levels occurred at an intake of 550 mg leucine/(kg · d), after which plasma leucine progressively increased and plasma ammonia also rose in response to leucine intakes >500 mg/(kg · d). After analyzing the experimental data vis-a-vis key events for leucine leading to toxicity (using the KEDRF approach), it became evident that the rate of leucine oxidation was the determining event.

There was extensive discussion of both rodent and human studies, especially on the liver weight changes in rats fed low protein supplemented with leucine (6). No such change was seen in humans and it was questioned whether the major trigger in rodents was low protein or leucine in combination with a low-protein diet. Discussion was thereafter extended to the species comparisons (rodents vs. pigs vs. humans), highlighting that both the requirements and upper limits may differ among species, especially during the times of rapid growth when the protein content of mother’s milk vastly differs among species. This point led to considerations on scaling factors that may “translate” animal results to human conditions. Currently used scaling factors are invariably based on energy intakes and thus appear inadequate for rodent-human comparisons. Two main suggestions were made in that respect: 1) to use scaling of amino acid (i.e., leucine) intake to protein intake, especially based on previous work done in piglets. In that sense, the rodent data presented at the workshop (6) were conducted in relatively low-protein situations, because even the 10–12% content of protein is a borderline situation and a diet with protein content below this threshold (7) stimulates integrated stress responses that might have caused the liver pathologies observed and described above; and 2) to use scaling of amino acid (i.e., leucine) intake on a requirement basis for either the amino acid lysine or dietary protein. Previous experience (e.g., 12) was discussed that indicated that an upper limit might be comparable among mammals if expressed as a multiple of the nutritional requirement for lysine with respect to protein. In the presented experiment (6), protein intake was not sufficiently controlled to allow for an appropriate scaling of the results to human conditions.

Whether the toxicity observed in rodents fed a low-protein diet is a direct consequence of leucine or a secondary outcome of amino acid imbalance was addressed by several discussants, especially due to the fact that the low-protein diet used in the experiment was not fortified with other essential amino acids. Such an imbalance in humans (Yoshiji Shimomura, Nagoya University, Japan, personal communication), especially a rapid drop of both isoleucine and valine triggered by increased doses of leucine, was presented during the workshop discussion and shown to correspond well to the rodent observations.

Another important issue raised during the discussion was the possibility that toxicity (if any) of a high dose of leucine might be related to age and that the elderly might have both a higher requirement and higher tolerance for this specific amino acid. Indeed, age-related muscle wasting (sarcopenia) is accompanied by a loss of strength that can compromise the functional abilities of the elderly. Resistance exercise combined with BCAA (especially leucine) ingestion elicits the greatest anabolic response and may assist the elderly in producing a “youthful” muscle protein synthetic response provided sufficient protein is ingested following exercise (9).

The discussion was concluded with several comments on using the presented human data (8) to propose a specific recommendation for the leucine upper limit [indicated as 500 mg/(kg · d)]. The discussants recognized that the International Council on Amino Acid Science (ICAAS)-sponsored studies provided substantial
data that may assist authorities in determining an upper limit, if that was deemed necessary. A comment was made about how such a limit should be specified and whether relating the leucine limit to body weight was appropriate in light of the growing obesity epidemic worldwide. The discussants also focused on differences between acute and chronic dosing and variations of individual tolerance to leucine. Statistical deviations of ~12% from the mean upper limit were considered as appropriate to accommodate all age groups and nutritional patterns. Such broad variations were justified by extensive supplementation with leucine in bodybuilders and some other athletes who are taking supplements in addition to dietary protein intakes, which are already high. Although no direct recommendation was made at the end of the discussion, the participants recognized that the presented data make a solid, if not complete, basis for establishing an upper limit of leucine in healthy adults.

**Determining an Upper Limit for the Amino Acid Tryptophan**

The essential amino acid tryptophan has been used by humans over the past several decades for non-nutritional purposes such as elevating mood and improving sleep. The first presentation of the workshop (10) devoted to this amino acid summarized human efficacy studies and attempted to infer the safe upper limits from these. Beginning in the early 1960s, tryptophan was studied for possible use in treating depression. The rationale derived from animal studies that showed that tryptophan injections raised brain serotonin content, an important mechanism of action for antidepressants. The oral doses used were as high as 15 g/d [200 mg/(kg · d)] for a 70-kg individual, with typical doses at 5–8 g/d [70–100 mg/(kg · d)]. Of interest in the workshop’s context was that side effects, when reported, were generally mild or absent. When present, they included euphoria, drowsiness, light-headedness, dry mouth, nausea, blurred vision, and nystagmus and appeared to be dose related. For sleep, oral tryptophan dosing was once daily before bedtime using doses of 1–10 g/d. Few side effects were reported (occasional nausea at higher doses).

As experience using tryptophan accumulated, it was generally regarded as a relatively benign treatment. However, in 1989, reports began appearing that over-the-counter tryptophan use was associated with a serious condition, eosinophilia myalgia (EM). This condition could be debilitating and life threatening. Over a period of several months, the CDC identified 1531 EM cases in the U.S. population; given the seriousness of the condition, the FDA banned over-the-counter tryptophan. Once the ban was in place, EM occurrence dropped to zero. EM symptoms were a high peripheral eosinophil count and disabling myalgias (note that the side effect profile associated with tryptophan use prior to 1989 does not overlap with these symptoms.) It subsequently became evident that almost all cases were traceable to the tryptophan produced by a single company. Cases of EM were not associated with the use of tryptophan from other manufacturers (10). A contaminant in the tryptophan, rather than tryptophan itself, was therefore considered the likely cause. Multiple contaminants were found in the suspected lot of tryptophan (11), but none faithfully reproduced EM in animals. The specifications for tryptophan have been tightened in both the European and US Pharmacopeia since then.

Discussion highlighted the audience’s doubts about what might constitute side effects appropriate for designating upper limits of tryptophan use. Those associated with EM were recognized as inappropriate, because they probably derived from a contaminant originating in manufacturing. Some subset of effects identified in sleep and depression studies might be appropriate, although none appear serious in nature and presumably would be self-limiting. It was mentioned that some of the side effects are desirable and the discussants did not come to a clear conclusion about whether to consider them as toxico logically relevant. It was noted, however, that because tryptophan raises brain serotonin production, its ingestion with drugs that stimulate serotonin function (e.g., certain antidepressants, monoamine oxidase inhibitors) can produce a medically important condition termed the “serotonin syndrome,” for which medical attention is required.

The second (12) and third (13) presentations focused on animal (rat and pig) studies with tryptophan. Symptoms of excess tryptophan intake in pigs included reduced food intake and growth rate, which were described as manifestations of amino acid imbalance. In growing animals, it appears that tryptophan intakes of >10 times the requirement are necessary before there are detrimental effects on growth performance. Symptoms of tryptophan excess may include development of fatty liver and fibrotic changes in muscles, lung, and pancreas and the serotonin syndrome (in rats: tremors, hyper-reactivity, hyper-tonicity). Similar to leucine (6), the effects of tryptophan excess are attenuated by higher protein intake. Although the dietary tryptophan requirement appears to vary widely across species when compared on the basis of g/kg body weight or g/kg of diet, the ratio of tryptophan:lysine requirement is fairly similar in many mono gastric species. The small differences among species are more likely due to differences in techniques and outcome measurements than a true difference in the ratio of tryptophan:lysine in body proteins. The participants focused on possibilities of comparing the metabolic effects of excess tryptophan intake across species by expressing these as a ratio to the lysine requirement.

The discussion ended with the recognition that humans used to take tryptophan supplements for several weeks up to 8 g/d prior to the EM disaster. Moreover, the participants admitted that animal studies may help in determining the upper safety limit of tryptophan even though the differences in tryptophan metabolism among the 2 studied species (rats and pigs) and humans are not fully elucidated. Two major thoughts related to animal data were discussed. First, the i.p. median lethal dose of tryptophan was determined to be 1600 mg/kg body weight in rats but was drastically decreased to a median lethal dose of 11–25 mg/kg body weight when corticosteroid production was inhibited (12). These observations suggested that the rate of tryptophan catabolism is a factor in the excess dietary intake that results in adverse effects. Thus, the upper limit of tryptophan catabolism may be a possible marker of the intake above which increasing intake increases the risk of adverse effects. Second, another option focused on a breakpoint in urinary excretion of tryptophan and its catabolites. In the rat experiment (12), the urinary excretory ratio of anthranilic acid:kynurenic acid was specified as a possible marker of the intake above which increasing intake increases the risk of adverse effects.

**General Discussion on the Scientific and Regulatory Ramifications of Upper Limits on Amino Acids**

The discussion started with some considerations on the benefit and risk ratio of amino acid supplementation. Although the benefit of single amino acids is often debated and controversies remain, the purpose of the workshop and the efforts of the organizers to establish upper limits for the commercially significant...
amino acids were recognized as important guidance to supplement companies and regulators alike.

Discussion also focused on the health effects of amino acids and how to distinguish them from true side effects. Dealing with an amino acid supplement calls for considerations that are not purely nutritional, thus reaching beyond the ramifications of the dietary reference intake literature. The upper levels that were the subject of the workshop, and the surrounding approaches and data needs, seemed to be related to approaches currently in place for nutritional substances. So, the discussion emphasized that stakeholders should be thinking about nutritional benefits rather than pursuing drug-like benefits in the context of the workshop.

Although the workshop was concerned mainly with 2 essential amino acids, leucine and tryptophan, it was acknowledged that the 20 amino acids making up protein can be roughly divided into 2 groups in terms of safety: those with clearly defined toxicological endpoints and those that are well tolerated without toxicological endpoints at high doses. It was acknowledged that the KEDRF might be a bridging point applicable to all amino acids and that perhaps blood ammonia or comparable variables could be used as the “key events” as indicated by Pencharz and Russell (14).

Finally, participants recognized that the workshop was a major milestone in the decade-long history of amino acid assessment workshops organized and sponsored by ICAAS. Discussions on leucine safety determined a possible specific upper limit applicable to healthy humans and the tryptophan session reviewed human and animal studies and pointed out tangible endpoints. It was, however, emphasized that: 1) the metabolism of each amino acid is different and generalization is difficult; 2) the true toxicological limit should not be confused with a higher metabolic limit in order to avoid a false sense of security when establishing upper limits; and 3) specific populations (elderly, intensive athletes) might have different dietary requirements and upper limits due to both intrinsic physiological factors and dietary protein intakes.

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Literature Cited