ESSENTIAL AMINO ACID METABOLISM IN INFECTED/NON-INFECTED, POOR, GUATEMALAN CHILDREN

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Abstract

During the recent past decades, major technological advances have been achieved in the study of amino-acid and protein metabolism. The refinement of mass spectrometry to measure very small amounts of tracers in the form of stable isotopes has also motivated refinements of the concepts of metabolism of amino acids and protein and their application to relevant health problems in both the developed and developing countries.

Traditional methods used to evaluate protein metabolism left unanswered some of the relevant questions in public health in developing countries, such as growth retardation in children. Particularly, in developing countries, infection (clinical and subclinical) and malnutrition are still relevant problems, and the most important scientific issues for the application of stable isotope tracer methods are related to the impact of infection, such as the oxidative disposal of essential amino acids in well-nourished and malnourished children. The objectives of the present proposal are:

(1) To simplify, make less expensive, less time-consuming, and less invasive, methods in clinical research on amino acid metabolism using stable-isotope tracers in children; and

(2) To assess the effects of infection (clinical and subclinical) on whole-body protein turnover in children with and without malnutrition.

The objectives involve the engineering and assessment of a portable instrument to be used in evaluations of protein oxidation in the developing world. Methodological issues such as intra- and inter-subject variability, which are of great importance for the interpretation of amino acid metabolism and protein turnover, will also be considered.

The study will be the result of the combined effort between the Centre for Studies of Sensory Impairment, Aging and Metabolism in Guatemala and the Centre for Human Nutrition, Johns Hopkins University in Baltimore, under the leadership and sponsorship of the International Atomic Energy Agency, IAEA, in Vienna, Austria.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

The advent of mass spectrometry and the reduction of biological fluids and gases to forms in which quantitative separation, detection and measurement procedures for stable isotope ratios have been applied, has provided the experimental basis for the application of non-radioactive tracers into the chemical structure of macronutrient molecules to follow their metabolism *in-vivo* in the human organism. Examples of these applications that have contributed to our thinking and learning in the production of this research proposal have come from the work undertaken by our team collaborator, Dr. Benjamin Caballero, who, working at the Massachusetts Institute of Technology (M.I.T.), studied the metabolism of phenylalanine using ^{13}C and 3,3-$^{2}H_2$ tracers, to quantify the rate of conversion of Phe to Tyr and the oxidation of Phe- and Tyr-derived carbons [1]. The antecedent groundwork for this came in the form of the work of Motil and Matthews and collaborators in St Louis and M.I.T. who developed the methodology for continuous infusion of carbon-13-labelled leucine [2] and then simultaneously measured turnover of leucine with the ^{13}C marker and lysine with a ^{15}N-label [3].

The adventure and opportunities in the program of the IAEA, as we interpret it, are:

(a) to translate the stable isotope technology from developed country settings (in which the sophistication and resources may be abundant, but the relevance to the public health problems related to protein energy deficiency and/or retarded growth is minimal) to developing country settings;

(b) to develop or modify technologies as needed;

(c) to teach investigators effective uses of isotope technologies to improve human nutrition in developing countries.

Prakash Shetty and Ismail Noor [4], from India and Malaysia, respectively, in their recent review entitled "Application of stable isotopes in human research in developing countries" state:

> "The most elegant experiments using stable isotopic tracers which are of considerable relevance to developing countries are those which have been used to prove the 'essentiality' of amino acids in the diet. Exciting results from the CNRC Houston indicate that short-term feeding of proteins uniformly labelled with ^{13}C followed by analysis of a rapidly turning over plasma protein can be used as an *in-vivo* probe of 'conditional essentiality' for individual amino acids during periods of development and growth..... Stable isotopes are emerging as safe, silent tracers which are ideal tools for nutritional research in humans. The nature of the problems that these newly emerging tools can be used to extensively investigate are of particular interest to developing countries. The use of these stable isotopic tracers is likely to throw light on nutritional problems of poorer countries if systematic efforts are made to design studies using these tracers to address issues which are a priority in third world countries."[4]

1.1. Nutrition-infection interaction

Protein-energy malnutrition (PEM), still is one of the principal public health problems in Guatemala, as it is in most developing countries. The National Survey on Maternal-Infant Health carried out in Guatemala in 1987 reported that about 58% of children suffer some degree of malnutrition [5].

On the other hand, infectious diseases are also within the leading causes of morbidity and mortality in children under five years old in Guatemala. Poverty, lack of both education and sanitary conditions, and limited access to health services, mediate the development of infectious diseases.

Early studies in Guatemala in the 1950's by N.W. Scrimshaw [6], highlighted the interaction between malnutrition and infection in children living in developing countries. In underpreviledged populations, living in unsanitary conditions, infectious diseases may represent a powerful factor modulating the nutritional status. In addition, it has been described that children from these populations usually suffer from overt infectious diseases several times a year, which is believed to further impair their already marginal nutritional status.

1.2. Protein metabolism and infection

In acute and subacute infections, the loss of body protein is proportionally much higher than the energy deficit [7]. The traditional approach for documenting this N loss is the N balance technique, which measures urinary and faecal N output relative to dietary N intake. The N balance, however, is not sensitive enough to identify early alterations in the rate of protein catabolism. On the other hand, the rate at which free amino acids are released from body proteins (breakdown), and the rate of their incorporation into proteins (synthesis) can be now quantified using amino acids labelled with non-radioactive (stable) isotopes. One approach is to administer a dose of ^{15}N-glycine, and assess its appearance into the urinary urea and ammonia pool [8,9]. Although other methods are more precise, they require constant intravenous infusions of isotopes and repeated blood sampling, making them not appropriate for use in children. Like all the tracer methods, the ^{15}N-glycine approach involves some assumptions on the way that the tracer equilibrates with the free amino acid pool. The ^{15}N-glycine method has been used to assess body protein turnover in malnourished children with or without infection [10].

1.3. Related work already performed or in progress at Institute

Recent field studies have characterized the linear growth of more than 500 children in a community setting. Current and past projects have focused on *Shigella dysentery* (ADDR, Harvard Institute for International Development); acute watery diarrhoea (CeSSIAM and University of California at Davis and sponsored by ADDR, Harvard Institute for International Development); and cytokines as markers of catabolic influences on growth (in collaboration with University of California, Davis and sponsored by Nestlé Foundation). A brief summary of the findings of these studies is presented below.

In 1991 and 1992, a survey was conducted to describe the growth pattern of a poor peri-urban neighbourhood of Guatemala City ("Peronia"). A total of 2714 children were evaluated individually one time. A cohort of 541 children were followed during one

year. Based on Z-score for height-for-age index, the mean for the whole population was 1.77. Most children could be classified as having linear growth retardation compared to NCHS reference. Based on longitudinal data, it was documented that the growth failure occurred principally during the first two years of life in peri-urban Guatemalan children [11] (Figs. 1 and 2). This study confirms previous reports by Martorell & Habicht [12] in other developing populations. Although we have documented linear growth retardation, very little is known about the mechanisms. Because protein accretion is central to growth, we are particularly interested in how amino acid metabolism and rates of protein turnover and/or oxidation are changed and the roles of these changes in interrupting linear growth.

Another project, carried out in 1992, was related to the determination of the prevalence of apparent and inapparent infection in preschool children in the same peri-urban Guatemala community ("Peronia") [13]. A total of 111 children were evaluated by history, physical exam and laboratory tests (acute phase reactants), including white blood cell counting, erythrocyte sedimentation rate and C reactive protein. The children could be classified as infected or non-infected based on clinical findings, with or without abnormal laboratory tests. From the total group, 41.5% (46/111) were classified as infected based on clinical findings. On the other hand, from the group of children clinically classified as non-infected, 15.3% (17/111) had abnormal laboratory tests, therefore they were considered to have inapparent infection. In conclusion, in addition to the high prevalence of overt infection documented in this community, the high prevalence of inapparent infection indicates that based only in clinical information, it would provide an underestimation of the real prevalence of infection in this poor population. It is known that the acute-phase reaction to an infection is expressed as an increase in a series of hormone-like substances (Interleukines) with documented properties on protein metabolism. Therefore, infection whether overt and covert might divert amino acid metabolism from normality, toward a diminution in the synthesis and/or increment in the catabolism. This might explain in part the high prevalence of growth retardation in developing countries.

Given the importance of the determination of infection status at the level of population when evaluating nutritional status, we have been developing a method to determine cytokines in the urine as a indicative of the infection status. This study was supported by Nestlé Foundation and involved three phases:

(a) Infected children to correlate cytokines in plasma and urine in children with severe infectious diseases;

(b) To evaluate if cytokines are detectable in urine after immunological stimulation under controlled conditions (vaccination with DPT); and

(c) To determine both the feasibility of using the technique at the level of field and the prevalence of infection in children. Preliminary results suggest the usefulness of this method in the characterization of the infection status, therefore we are proposing to use it in the present study.

The findings mentioned above provide the bases to the present proposal to study protein metabolism in children with and without infection, and with and without malnutrition. This is an issue not yet investigated and of great importance in clinical medicine and public health.

In addition to the obvious methodological questions involved in this proposal, some of the most important scientific issues are:

(1) Is there a correlation between the changes in amino acid/protein metabolism and cytokine levels?

(2) What is the role of cytokines in exerting the catabolic drive?

(3) Is chronic subclinical infection in undernourished children an important mechanism by which protein is diverted from whole body anabolism?

(4) Is the effect different in well-nourished children?

(5) What is the time-course in the catabolic drive triggered by infection?

(6) Can early nutritional intervention ameliorate/suppress the catabolic response? And if so, is this reflected in the clinical course of the infection?

1.4. Goals of project

(1) To simplify, make less expensive, less time-consuming, and less invasive, methods in clinical research on amino acid metabolism using stable-isotope tracers in children.

(2) To assess the effects of infection (clinical and subclinical) on whole body protein metabolism in children with and without malnutrition.

1.5. Specific objectives

(1) Adaptation of a dose administration procedure and sample collection to field cconditions and in young children for amino acid turnover studies (Leucine; Glycine) using stable-isotope-labelled substrates.

(2) Assessment of the intrasubject stability and intersubject variability of amino acid turnover in young children using simplified, field collection procedures.

(3) Impact of clinical and subclinical infection on amino acid metabolism in relationship to growth and markers of the acute-phase response.

2. METHODS

Note: We were interested in developing simplified methodologies, starting with validated methods developed for use in more sophisticated facilities, to study protein metabolism. However, as a result of discussions held in Boston last year (October 1993), a simplified procedure was developed, namely, "Generic Protocol", which will be adopted entirely. This generic protocol is presented elsewhere in the report of the RCM.

3. PLANS FOR WORK IN YEAR 1

3.1. Phase I (first year)

Adaptation of a dose administration procedure and sample collection to field conditions and in young children for amino acid turnover studies (Leucine; Glycine) using stable-isotope-labelled substrates.

3.2. Detailed explanation of goals and strategy

The goal will be to assess the feasibility of the application of the "Generic Protocol" using isotopically-labelled amino acids to study protein metabolism in children under field conditions. The efforts will be aimed to minimize the invasiveness and inconvenience to the subject while maximizing the acceptability for application in the community but maintaining the best reliability and correspondence with what could be considered a "gold standard" laboratory in-patient procedure and aiming toward combined administration of two labelled amino acids, leucine and glycine.

The adopted generic protocol requires collection of breath, urine and blood samples; therefore, we will assess the feasibility and reliability of these procedures before we begin collecting data on children with infections.

3.3. Detailed outline of procedures and experiments

The main objectives are to develop breath collection procedures and conduct a pilot study of the generic protocol.

3.3.1. Development of breath collection/CO_2 analysis

Procedures will be adapted to young children in the community setting in development of the instrument and appliances. The concept is a hood that can be presented as a space helmet in a "space rangers" game or an "undersea diver" game in which the presence of the hood in place for collection activates a videogame or video cartoon console to entertain and distract the subject. When the subject asks for the hood to be lifted, the entertainment turns off; when the hood is lowered, the entertainment resumes. The stages are:

(1) the design, engineering, construction and technical testing for accuracy of carbon dioxide analysis of the hood/analyzer system;

(2) the acceptability and duration-of-tolerance testing, including the role of prior familiarity and presentation strategies to maximize the generalizability (within population) of acceptance and the duration (within the individuals) of tolerance of the chamber.

Children aged 2 to 4 years from both middle-class and poor, peri-urban homes will be enroled as needed to follow algorithmic decision-tree approaches to maximizing acceptability and tolerance.

3.3.2. Pilot study

The Generic Protocol will be applied to a small group of children. Eight healthy children of the target age of the future biological studies, i.e. 2 to 4 years, will be recruited from families and friends of CeSSIAM staff. Each child will be studied on only one occasion. Complete collection of urine during 24 h under supervision will be done at established intervals for tracer determinations as well as for total N, creatinine, and 3-methylhistidine excretion (an index of skeletal muscle protein breakdown). Expired air gas collection in the post-dose phase will be conducted with the young-child-appropriate, portable instrument developed and built in Phase 1 (and possibly by blowing up a collection balloon or Ambu mask, or Douglas bag, at specified intervals while still under the hood).

Breath collection procedures

Samples of breath will be collected into Douglas bag (or similar method). Samples of gas will then be transferred into evacuated vacutainer tubes via a syringe and kept frozen at -80°C until IRMS analysis.

Urine collection procedures

The urine from each void will be collected and frozen immediately, using dry ice if no freezer is available. Prior to analysis, the whole lot will be pooled, the total volume recorded, and an aliquot combusted and analyzed for total nitrogen, and another for ^{15}N urea, and $^{15}NH_3$.

Blood collections

Blood will be drawn at 8, 8.5 and 9h from the start of the generic isotope protocol, frozen at -20°C for derivative preparation and GCMS analysis.

Diet

A study diet should be consumed for 48 h preceding the study for purposes of dietary and nitrogen equilibration. The diet will be provided by the study for 48 h pre-isotope administration. It will be based on the locally available foods, cereal (maize) and legume (black beans) with other beverages, foods and condiments and will provide 80-90 kcal/kg, and be 10% protein, 25% fat, and 65% CHO. The fibre content of the Guatemalan diet is relatively high [18], nonetheless the generic protocol recommends a low fibre content.

3.3.3. Analyses of samples and data

Collected and processed in Guatemala

Collected, processed and preserved for future chemical and isotope/ratio analyses in Guatemala will be: expired air samples; plasma samples; urine samples.

Recorded and analyzed in Guatemala

Total/partial carbon dioxide excretion volumes and partial and total urine volumes;

Recorded and analyzed in USA (Baltimore/Boston)

Analyses for total and individual plasma amino acids, for urinary phenylalanine and tyrosine and the isotope/ratios in gas and fluid samples will be conducted in the developed-country partnership laboratories.

4. PLANS FOR WORK IN YEAR 2

4.1. Phase II (second year)

Assessment of the intrasubject stability and intersubject variability of amino acid turnover in young children using simplified, field collection procedures.

4.2. Goals

Due to the nature of this phase, it will involve a very small number of subjects. A simplified protocol (Generic Protocol) to study protein turnover will be applied to a group of 8 healthy children of 3-4 years old, from the middle class families of Guatemala City. It will require children with an adequate nutritional status, defined as a weight-for-height greater than 90% according to NCHS curves. The protocol will be applied two times, one week apart, to the same child.

This phase will determine the stability of the tests in several of the physiological variables of interest. In addition to knowing the intra- and between subject variability of the tests, this will be the baseline for the calculation of sample size for the investigation proposed as the last phase.

5. PLANS FOR WORK YEAR 3 and 4

5.1. Third Phase (third and fourth year)

Impact of clinical and subclinical infection on amino acid metabolism in relationship to growth and markers of the acute-phase response

5.2. Goals

The third phase involves the most important biological questions of this proposal. In this phase, two groups of children matched for age and nutritional status will be studied. One group will be in the acute state of infection, and the other will be free of infection. Until we know the variability of some of the variables of protein turnover in healthy children, we propose to study a tentative number of 20 children for this phase. The generic protocol will be applied in the same fashion as in the previous phase.

Infection status will be characterized by using acute phase reactants, including cytokines IL1, IL4, IL6 and TNF. Children will be recruited from the peri-urban areas of the City of Guatemala, where other CeSSIAM projects are ongoing.

6. CONCLUSIONS

This project will involve three aspects:

(1) Development of a mini-metabolic-cart: the custom-designed portable mini-metabolic cart designed to combine the entertainment and distraction for the child with the tolerance of the gas-collection breathing hood to set the bases for turnover studies in ambulatory populations in a community/field setting;

(2) The adoption and assessment of the IAEA-Generic Protocol to study amino acid metabolism in children at the level of field setting;

(3) The application of the assessed IAEA-Generic protocol in children with subclinical and clinical infection, to study their impact in terms of amino acids metabolism.

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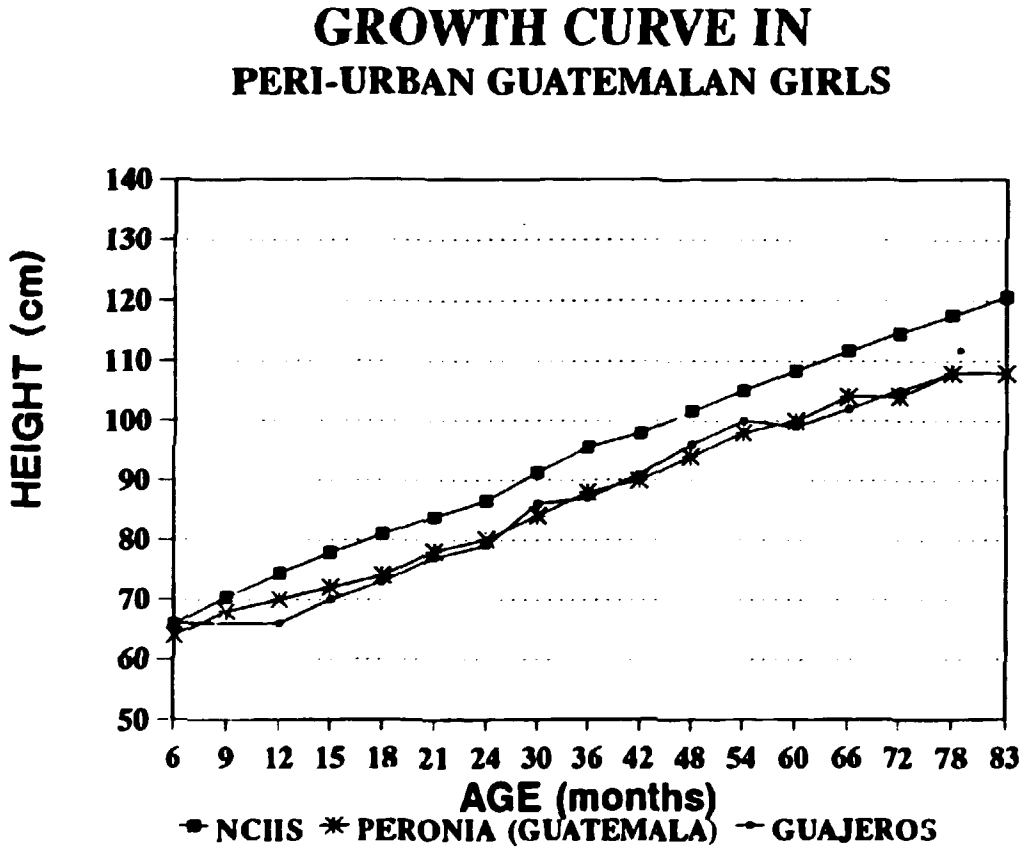

Figure 1. Linear growth pattern in peri-urban Guatemalan girls. Median length or height (cm) per age (mo) is compared to the reference curve from NCHS. Data obtained by a cross-sectional study.

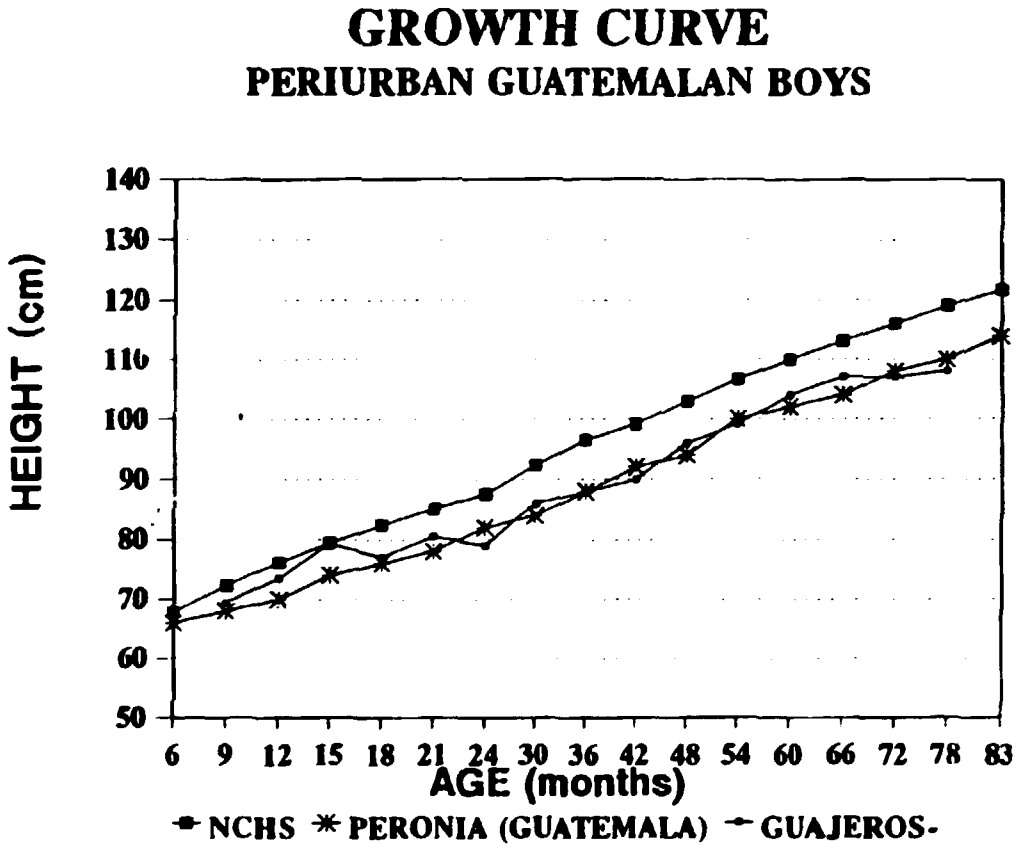

Figure 2. Linear growth per year in peri-urban Guatemalan boys. Filled bars show the linear growth per year expressed as percent of the reference population (NCHS) (blank bars), for each specific age group. Data obtained by a longitudinal study.