

FOREWORD

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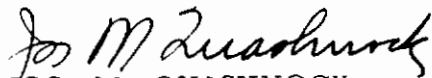
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ABSTRACT

The practical and fundamental shortcomings of the nitrogen-balance method as a means of ascertaining protein and specific amino acid requirements of humans find ample support in the literature, and emphasize the need for a more flexible, rapid, and economical procedure. A familiarity with factors involved in the mechanism and route through which proteins and amino acids are metabolized would undoubtedly help in evaluating protein and amino acid needs of man under various physiological and pathological conditions. Inasmuch as the literature indicates that blood amino acids reflect the size and qualitative characteristics of the amino acid pool, the available chemical, radiochemical, and chromatographic procedures for determining amino acids in body fluids were carefully considered. Of these, the quantitation of paper chromatographic measurements of amino acids has proved in our hands to be the most practical in terms of sample size, rapidity, and convenience of analysis. The report indicates that procedures developed under this contract are suitable for metabolic studies concerned with determination of nutritional individuality.

PUBLICATION REVIEW

This technical documentary report is approved.


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I. INTRODUCTION

The problem of protein needs is the most complex in the field of nutrition. However, it is equally the most interesting from scientific and practical aspects, not only for nutritionists, biochemists, and clinicians, but also for a multitude of national and international health agencies. These diverse attributes of protein nutrition are but a reflection of the indispensable role of proteins for life. Without exception, proteins as structural elements, or as biocatalysts, participate in every biological process at every level of biochemical organization. Consequently, definitions of the need for calories, carbohydrates, fats, vitamins, or minerals are meaningless without indications of the level and character of protein in the diet. For example, it is grossly misleading to report that the calcium needs of population groups in underdeveloped areas are low, without noting the fact that in these areas the average protein intake is also low, both in quantity and in quality. Of course, the converse is equally true; protein needs may be altered significantly by limitations of other components of the diet. It is well known that with diets low in calories, the need for proteins is increased by virtue of their use as energy sources by the organism.

Because of these interrelationships, it is necessary that careful attention be given to the composition of experimental diets. Excessive artificiality may lead to results of limited practical usefulness. A case in point is the attempt by many investigators to resolve the problem of protein requirements by the use of synthetic diets containing mixtures of only essential amino acids as the principal source of nitrogen. Under such conditions, nitrogen equilibrium has been maintained for a short time on a daily intake of 3.5 gm or less of total nitrogen in diets providing some 4000 calories. This low nitrogen value approximates the endogenous nitrogen minimum for man reported by workers at the turn of the century and, therefore, represents a survival minimum rather than a physiological requirement. The lack of nonessential amino acids appears to be reflected in a need for a calorie intake approximately 50 per cent greater than that of diets containing all of the amino acids as derived from whole, or digests of, animal proteins. Accordingly, it would seem unreasonable if not hazardous to draw conclusions from such measurements regarding patterns of amino acid needs of man in his customary nutritional environment. Recent ill-advised attempts to do so have led to questionable conclusions which have confused rather than clarified the problem.

In addition to the complexities of diet formulation, investigators are also confronted with the task of selecting adequate analytical procedures. To this end, it seemed worthwhile to review in some detail the latest chemical and chromatographic methods for the measurement of nitrogen by- and end-products of protein metabolism which may be employed in the biochemical approach to problems of protein nutrition. In order to clarify the scope and limitations of these procedures, their application to various aspects of human protein nutrition are discussed with demonstrations of results and interpretations.

II. NITROGEN BALANCE

A. Basic Concepts

The chemical changes of proteins in living organisms have aroused the curiosity and challenged the ingenuity of investigators for more than a hundred years (ref. 1). The earliest method for studying these changes is based on the time-honored concept that nitrogen entering the body of mammals as food is ultimately stored in the form of body protein or eliminated, chiefly through urine and feces, as urea, ammonia, creatinine, uric acid, amino acids, and other known nitrogenous substances. Nitrogen loss occurs also through perspiration. In comprehensive reviews of the literature, it has been reported by Cuthbertson and Guthrie (ref. 2), Mitchell and Hamilton (ref. 3) and, more recently, by Mitchell and Edman (ref. 4), that dermal nitrogen losses are quite appreciable, ranging from 21 to 130 mg/100 ml of sweat. Mitchell and Hamilton (ref. 3) have observed that on a daily intake of 98 gm of protein, the nitrogen excretion in sweat, of men sweating profusely, averaged 152 mg/hour, which was approximately 22.5 per cent of the total daily output. These authors also observed that during normal or minimal sweating conditions, the nitrogen loss in sweat averaged 15 mg/hour, equivalent to 2.7 per cent of the total daily output. In general these dermal losses have been ignored in most of the nitrogen equilibrium studies reported in the literature (ref. 5).

Nitrogen balance, then, is the difference between nitrogen intake and nitrogen excreted, and may be defined mathematically as follows:

$$NB = NI - (UN + FN)$$

where NB = nitrogen balance, NI = nitrogen intake, UN = urinary nitrogen, and FN = fecal nitrogen (ref. 6). If the nitrogen intake is greater

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than the nitrogen excreted, the body is gaining in nitrogen and is in positive balance. If the nitrogen intake is just equal to the nitrogen excreted, the body is maintaining nitrogen and is said to be in equilibrium. If the nitrogen intake is less than the nitrogen excreted, then the body is losing nitrogen and is in negative balance.

Although the basic principle of the method remains unchanged, there have been many refinements with regard to procedure and interpretation of results. The nitrogen output is now most conveniently and accurately determined by one of the many available modifications of the Kjeldahl technique. In our hands, the boric acid version of the micro adaptation of Scales and Harrison (ref. 7), and of Vickery (ref. 8), described on page 35, has proved dependable and rapid for more than a score of years. The nitrogen content of thymol-preserved 24-hour urine collections is determined simply from 1-cc aliquots. The nitrogen content of the feces is determined from dry- or wet-ashed specimens accumulated for each balance period. The wet-ash sampling is now the technique of choice.

Because of the difficulties of collection and handling, it has become common practice to estimate the fecal nitrogen from the total urinary nitrogen. This simplification has become possible because of the existing body of evidence which indicates that under normal circumstances the fecal nitrogen is approximately 10 per cent of the total nitrogen content of a 24-hour urine specimen (ref. 9). Since nitrogen balance differences as small as 10 per cent are rarely considered significant in such studies, it is obvious that even an error as great as ± 100 per cent in the fecal nitrogen estimation would not greatly affect the results or conclusions (ref. 10).

Kjeldahl determination of nitrogen content of the food intake during balance studies is a simple matter when synthetic or semisynthetic diets are employed, but cumbersome when free choice of natural foods is permitted. The quality of the nitrogen content can be estimated by the use of tables (ref. 11). When standardized portions of food are fed, tables may also be employed for estimating the nitrogen content of the diet (ref. 12, 13). Under these conditions the calculated nitrogen content should be checked by periodic analysis of aliquots of the food ingested daily.

Studies employing synthetic diets with complete analysis of the excreta will obviously yield balances of the highest accuracy. Such procedures were used by Albanese (Ref. 14, 15), Rose (ref. 16), and Leverton (ref. 17), who found considerable variation in the amino

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acid requirements of young male and female adults. In the light of the concept of nutritional individuality (ref. 18), it is doubtful if the precision attained by elaborate techniques is meaningful or useful in resolving problems of practical nutrition. The results of studies with natural foods offered on a self-selection basis are of course less accurate, but they do not suffer from inferences needed to equate synthetic diets to regimens of normal and conventionally prepared meals.

The optimal duration of balance periods has long been a point of discussion. Some investigators have claimed adequacy for periods as short as 1 day (ref. 19), while others have urged the need for test periods of 3-4 weeks (ref. 20). The difference of opinion arises from the fact that most test organisms can attain nitrogen equilibrium at nearly all levels of intake above the minimum (ref. 21).

The existence of nitrogen equilibrium does not necessarily mean that adequate dietary protein is being fed. More correctly, it means that for a time nitrogen equilibrium has been established by adaptation to the intake. If the nitrogen intake or quality is too low to maintain equilibrium, the individual will go into negative balance; protein stores will be depleted until a smaller amount of dietary nitrogen will maintain equilibrium, albeit in a relatively depleted state. Similarly, if the intake is high, positive balance will be produced and the stores will be filled to the point where the high dietary intake will become essential to maintain those stores. The larger the protein stores, the higher the metabolic activity, and the more dietary nitrogen is needed to maintain equilibrium (ref. 6).

The effects of immobilization were investigated by Deitrick and his associates (ref. 22) on 4 healthy, normal males who were maintained on an isonitrogenous and isocaloric diet throughout the period of study. The protein intake was set at slightly less than 1.5 gm/kg body weight, and was more than adequate to maintain nitrogen equilibrium during the control periods. Nitrogen excretion began to increase on the 5th to 6th day of immobilization, and reached its peak during the first half of the second week. The total nitrogen losses for the 6-7 weeks of immobilization ranged from 29.8 to 83.6 gm and averaged 53.6 gm. During recovery from immobilization (4-6 weeks) there was retention of nitrogen, calcium, phosphorus, sulfur, and potassium. The recovery or return to control levels of metabolic functions was slow. Retention of nitrogen and phosphorus continued for 6 weeks. Restabilization of calcium metabolism appeared to require more than 6 weeks. These observations and those of Howard and associates (ref. 23) suggest that physical activity constitutes an important

factor in the utilization of proteins. Hence, any evaluation of biological value of proteins should take into account the level of physical activity prevailing throughout the period of study.

Available evidence suggests that with few exceptions the interval in which balance is achieved at new levels, either below or above initial levels of intake, is primarily a function of physiological state of the organism (ref. 24) and nutritional quality of the test protein. In general, the greater the biological activity of the organism, the shorter the interval; the poorer the protein, the longer the interval. Thus, whereas 3- and 4- day balances are adequate test periods for normally growing infants and young children, assays of 1-2, and even 3 weeks duration may be required for normal healthy adults. Adjustment of nitrogen equilibrium to different proteins such as those contained in eggs, meat, and milk, proceeds very rapidly. With cereals poor in lysine or tryptophan, or other foods poor in one or more individual amino acids, the adjustment in nitrogen equilibrium is never achieved.

B. Interpretations

In studies of protein metabolism, nutritional adequacy of the nitrogen component of the diet is commonly measured in terms of weight change and nitrogen balance. It is generally assumed that these two biological functions should vary in parallel and direct proportion. Such a view fails to acknowledge the obvious possibility that tissue water and fat depots can be mobilized independently of body proteins. This possibility is amply supported by observations that weight gain in infants and children is not in accord with Rubner's Law, which states that 1 gm of nitrogen is equivalent to 33 gm of body substance (ref. 25). From data reported by Albanese and his associates (ref. 26), it will be observed (Table I) that tissue deposition, estimated as

$$\frac{\text{grams daily weight change}}{\text{grams daily N retention} \times 33} \times 100$$

yields approximately 59 per cent as a mean value for all diets tested.

This problem has also been examined by Wallace (ref. 27) who was unable to reconcile the divergences of nitrogen retention and body weight changes on the basis of shifts in tissue fat or water of young children. He attributed the discrepancies to unassessable errors of the nitrogen balance method. Costa (ref. 28) investigated the subject very carefully in dogs, rats, and mice, and concluded that the excess

TABLE I
EFFECT OF VARIOUS PROTEIN DIGESTS ON THE NITROGEN ECONOMY OF THE INFANTS^{a,b}

| Initial, age, and weight of subject | Diet ^c | Average period weight (gm) | Daily weight gain (gm) | Intake/kg | | Total N | | Nitrogen output (gm) | | N retention (mg/kg) | Tissue deposition (%) |
|-------------------------------------|-------------------|----------------------------|------------------------|-------------|---------------|-------------|-------|----------------------|-------|---------------------|-----------------------|
| | | | | Volume (cc) | Nitrogen (gm) | intake (gm) | Urine | Feces | Total | | |
| A. J. 11 mo 9618 gm | Evaporated milk | 9707 | 25.6 | 112 | 0.63 | 6.12 | 3.68 | 0.58 | 4.26 | 193 | 41.5 |
| | Amigen | 9896 | 28.3 | 95 | 0.62 | 6.08 | 3.15 | 0.97 | 4.12 | 197 | 44.0 |
| | Edamin | 10,094 | 28.3 | 95 | 0.61 | 6.09 | 3.50 | 0.80 | 4.30 | 178 | 40.8 |
| | CTH | 10,262 | 19.9 | 103 | 0.65 | 6.65 | 4.43 | 0.88 | 5.31 | 130 | 44.8 |
| P. D. 6 mo 6484 gm | Evaporated milk | 6584 | 28.4 | 152 | 0.68 | 4.47 | 2.52 | 0.53 | 3.05 | 217 | 60.0 |
| | Amigen | 6789 | 30.1 | 103 | 0.63 | 4.30 | 2.50 | 0.52 | 3.02 | 189 | 71.4 |
| | Edamin | 6694 | 28.4 | 104 | 0.64 | 4.25 | 2.60 | 0.46 | 3.06 | 180 | 71.4 |
| | CTH | 7136 | 12.0 | 100 | 0.69 | 4.96 | 3.46 | 0.47 | 3.93 | 140 | 36.0 |
| D. R. 5 mo 6620 gm | Evaporated milk | 6721 | 29.0 | 140 | 0.63 | 4.22 | 2.32 | 0.55 | 2.87 | 200 | 65.1 |
| | Amigen | 6923 | 28.9 | 100 | 0.60 | 4.15 | 2.53 | 0.31 | 2.84 | 189 | 66.8 |
| | Edamin | 7120 | 27.2 | 100 | 0.60 | 4.29 | 2.28 | 0.63 | 2.81 | 208 | 55.3 |
| | CTH | 7281 | 16.0 | 102 | 0.69 | 5.01 | 3.33 | 0.63 | 3.96 | 144 | 48.0 |

a From Albanese et al. (ref. 26)

b Results given as daily averages

c Protein Preparations Used: Amigen, an enzymatic casein digest (Mead Johnson); Edamin, an enzymatic lactalbumin digest (Sheffield Farms); CTH, acid hydrolysate of casein supplemented with 1.5% of l-tryptophan and 1.0% of l-cystine.

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in nitrogen retention not represented by gain in weight does not appear to be due to a technical artifact. This investigator hypothesized that some of the nitrogen fed in excess is eliminated through unmeasured and hitherto unrecognized pathways--probably respiration.

Conversely, on the basis of N¹⁵ isotope studies, Volskii (ref. 29) expressed the opinion that some animal organisms and higher plants are capable of assimilating nitrogen not only from food but from the air as well. His studies show that the total quantity of nitrogen in chick embryos is 3-4 per cent higher than the nitrogen content in unincubated eggs. Since nitrogen can reach the egg during incubation only by diffusion from the air, he concluded that the chick embryo assimilates gaseous nitrogen during its development. Analogous results were obtained in studying the nitrogen content of honeybee pupae in different stages of their development. With apparent justification he stated: "It seems to us that the time has arrived for a re-examination of the postulate proposed by Lavoisier more than 150 years ago, that the living organisms cannot assimilate nitrogen of the air, and also to re-examine Voit's law which concerns the nitrogen balance of living organisms."

Associated with the above enigma is the matter of "protein stores" or "reserves." Unlike carbohydrates and fats, proteins or the amino acids derived from them cannot be stored as such by the adult animal organism, except to a very limited extent. By this is meant that no considerable deposits of protein or amino acids are formed in the animal body comparable to the glycogen granules or fat globules. However, Whipple and his associates (ref. 30) established the concept of dispensable and indispensable stores of protein in the animal body from their work on hemoglobin and blood plasma protein regeneration. The form in which these protein stores exist is not known. While their location is probably quite general, the primary organ of storage seems to be the liver (ref. 31). From tracer studies, Rittenberg (ref. 32) postulated that the "protein reserves" exist as a "metabolic pool" which functions primarily as a mechanism for the transfer of amino acids from one tissue to another. Flodin (ref. 33) has suggested that the "protein reserves" exist in extracellular-extravascular plasma as albumins, polypeptides, and smaller peptides. Working with dogs, Allison (ref. 24) has shown that high-quality protein in the diet is more effective in maintaining optimum protein stores than is low-quality protein.

Adolescence, pregnancy, lactation, and convalescence are states of anabolic stress in which the inadequacies of low-quality

protein diets become visible. This is particularly serious in pregnancy which has been described by Macy (ref. 34) as a physiological state of high nitrogen retention. The great excess of maternal storage over the requirements of the fetus is illustrated in Fig. 1. This storage is much greater than the nitrogen involved in construction of the soft tissues and in preparation for the losses and physiological changes incident to parturition. The excess in reserves at full term have been estimated at 100-200 gm of nitrogen which, when multiplied by the Rubner factor of 33, yields the equivalent of 3.3-6.6 kg of unaccountable tissue approximately twice the weight of the average new-born infant.

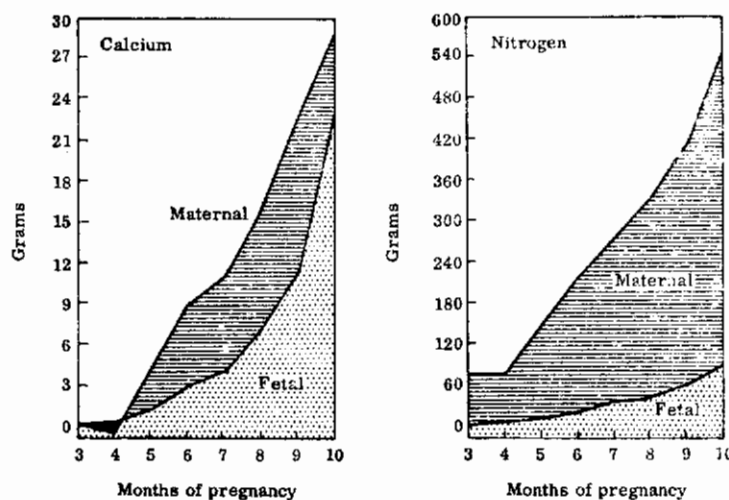


Figure 1. Maternal and fetal storage of nitrogen and calcium from the third to the tenth month of pregnancy (from ref. 34).

Recently, a revealing and novel approach to this problem has been reported by Shapiro and Fisher (ref. 35). A series of nitrogen balance experiments was carried out to determine the relationship of the essential and nonessential amino acid portions of the nitrogen requirement to the protein reserve status of the adult rooster. It was observed that dietary nonessential amino acids are adequate in the formation of protein reserves provided the maintenance requirement of

essential amino acids is satisfied. Similar studies in mammals and, ultimately, in humans in health and disease are needed.

C. Indices of Nitrogen Utilization

The relationship of nitrogen intake to nitrogen balance was analyzed and formulated mathematically by Martin and Robison (ref. 36). The term protein utilization is used to indicate the percentage of the ingested food nitrogen actually assimilated. Thus

$$\text{approximate percentage nitrogen utilization} = \frac{(\text{food N} - \text{feces N}) \times 100}{\text{food N}}$$

1. Biological Value

Inasmuch as fecal nitrogen does not originate entirely from the food, a correction is usually made for metabolic or endogenous nitrogen in more exact work (ref. 37). To overcome this problem, Mitchell (ref. 38) developed the concept and procedures which would include consideration of the endogenous nitrogen. His results were expressed as:

biological value =

$$\frac{\text{food N} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{"endogenous" N}) \times 100}{\text{food N} - (\text{fecal N} - \text{metabolic N})}$$

Values for "metabolic nitrogen" of feces and "endogenous nitrogen" of urine are obtained by means of Kjeldahl analyses of the excreta collected during non-nitrogenous but isocaloric dietary periods. The validity of this procedure has been questioned since Folin's classical distinction between "endogenous" and "exogenous" metabolism seems untenable in the light of evidence that nitrogen metabolism is dynamic and continuous (ref. 39, 40).

2. Nitrogen Balance Index

A practical variant of Mitchell's procedure which yields a "nitrogen balance index" of dietary protein has been described and determined by Allison (ref. 21). This value is obtained from:

$$\text{index} = \frac{B - B_0}{I}$$

where B is the nitrogen balance associated with intake (I), and B₀ is the nitrogen balance found during periods of feeding the protein-free diet. The accuracy of the index demands that B₀, endogenous nitrogen,

should be constant and independent of protein intake. Although the measurements necessary for the calculation of the nitrogen balance index can be readily made in experimental animals, these obviously present serious problems in human studies.

3. Net Protein Utilization

Miller and Bender (ref. 41), as well as Bender and Doell (ref. 42), define their Net Protein Utilization (NPU) by the formula:

$$NPU = \frac{B_F - B_K + I_K}{I_F}$$

where B_F is the carcass nitrogen of protein-fed rats, B_K the carcass nitrogen of rats on a protein-free diet, I_F the total dietary nitrogen, and I_K the nitrogen from the test diet without the test protein. Miller and Payne have developed mathematical formulas containing factors for the influence of protein concentration (ref. 43), caloric restriction (ref. 44), and the use of food composition tables (ref. 45) in problems in the prediction of protein values of diets from net protein utilization data. This approach is similar in many respects to the procedure which measures the per cent nitrogen retained. Since the results obtained by the two methods are practically identical, the need for these newer and more difficult theoretical treatments has been seriously questioned by Frost (ref. 20).

A more recent approach, the concept of "complete protein," is an attempt to combine a somewhat modified chemical score with the per cent protein of a food or diet in a single numerical index (ref. 46).

4. Protein Utilization Index

In order to arrive at a relative utilization value of test protein products which would compensate for weight changes, Albanese and associates (ref. 47) applied the following formulas to their results with infants:

$$P = BW \times N / 1000$$

where P = protein utilization, BW = body weight change in grams per day, and N = nitrogen retention in milligrams per kilogram per day.

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The coefficient of utilization of the test products, P_r , is then expressed as the numerical value of the ratio: P test protein formula/ P evaporated milk formula; or

$$P_r = P_{tp}/P_{em}.$$

Expression of bioassay results in this manner has several advantages. First, it equalizes disparities between body weight changes and nitrogen retention values which often arise in infants from transpositions of body fluid compartments (ref. 26). Second, it relates increments in nitrogenous tissue directly to qualitative amino acid differences of the test nitrogenous moiety of the diets. And last, it provides a simple numerical comparison of the test substance with a standard infant food--e.g., evaporated milk.

An example of such calculations for a male infant, W. M., weighing 3,327 gm at 1.5 months of age, follows: Consumption of a standard evaporated milk formula at the average level of 3.11 gm N/day for 2 weeks resulted in an average body weight change of 23 gm/day and a nitrogen retention of 138 mg/kg/day. Thus, $P_{em} = 23 \times 138/1000$, or 3.17. During the subsequent 2 weeks, this infant consumed an isocaloric bovine plasma digest formula at the average rate of 3.12 gm N/day. This dietary resulted in an average body weight change of 40 gm/day and a nitrogen retention of 180 mg/kg/day. Thus, $P_{tp} = 40 \times 180/1000$, or 7.20. Hence, the coefficient of utilization (P_r) of the bovine plasma digest formula in this single assay is $7.20/3.17$, or 2.3. The average P_r of ten such bioassays with bovine plasma digest in infants 1.5-9.0 months of age was 1.82 (ref. 47).

III. AMINO ACID LEVELS

From determination of biological value and its variants, it has been known for some years that the nutritive value of a dietary protein is in the final analysis determined by its amino acid content. Mitchell (ref. 48) and Oser (ref. 49) have developed scoring methods which relate amino acid profile of protein to biological value. Not all amino acids present in a protein, however, are available to the animal, especially when the protein is either from a vegetable source or has had some of the amino acids destroyed or rendered unavailable by processing.

Methionine balance studies by Melnick and associates (ref. 50) showed that 49 per cent of the methionine present in soybean meal fed to

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rats appeared in the feces and therefore was not available to the animal. Kuiken (ref. 51) reported variations in availability of lysine and methionine in cottonseed meal, depending on the conditions of processing. It would appear that a measure of reduced availability, regardless of cause, could be determined by the extent to which the amino acids appear in the blood stream, a parameter of tissue level.

Richardson and co-workers (ref. 52) reported that amino acids in the plasma of the chick did not parallel the amounts fed in the diet. Some of the amino acids present in low amounts showed an unexpectedly high level in the blood. These same authors, however, as well as Charkey and associates (ref. 53) and Denton and collaborators (ref. 54), presented evidence that the concentration of any one amino acid in the blood is usually in agreement with the relative concentration of that amino acid in the diet, and that the addition of supplemental amino acids to the diet results in an increase in the blood level of the corresponding amino acid. Also, a lowering of the concentration of any one amino acid in the blood has been observed by these authors to be the reflection of a deficiency of this particular amino acid in the diet.

Steele et al. (ref. 55) measured amino acids in the blood and urine of human subjects ingesting 25, 100, and 200 gm of protein daily, contributed by meat, milk, and bread. Microbiological analyses of the urine and blood collected during the test period showed rather poor correlation with changes in protein intake. Subsequently, Charkey and others (ref. 56) found by microbiological procedures that fasting in adult humans led in 48 hours to increased blood levels of leucine and valine, but decreased levels of lysine, threonine, methionine, arginine, and tryptophan. Albanese and Orto (ref. 57) have by paper chromatography noted a correlation between lysine level in the diet and free lysine level in the blood of infants. More recently, Longenecker and Hause (ref. 58) reported that the free amino acid level in the plasma of the dog reflects the composition of the diet. Their procedure, by which amino acid adequacy of a diet can be evaluated by the study of plasma free amino acids, is described. Puchal and co-workers (ref. 59) have also found plasma amino acid levels in young pigs to be correlated closely to the amino acid composition of the dietary.

From the foregoing reports, it has become increasingly clear that measurements of blood, unlike urine, amino acid levels bear considerable promise as a criterion of protein nutrition. Currently, microbiological, chemical, and chromatographic methods are in common

use. Each of these analytical principles has advantages and shortcomings. It is generally agreed that microbiological procedures are the most limited in terms of variety of applications and accuracy (ref. 60-63). These limitations arise primarily from lengthy incubation periods and the sporadic presence in biological fluids of growth-promoting substances other than amino acids. The older literature contains many reports of chemical methods for the determination of amino acids (ref. 64, 65). However, except for certain specific purposes--e.g., food analyses--their use is currently declining because of the greater specificity, rapidity, and ease of operation of chromatographic techniques. Procedures which we have found best suited for metabolic studies in humans will be discussed in some detail.

A. Total Amino Nitrogen

The determination of this constituent of biological fluids and products is still most easily and conveniently determined by chemical procedures. Folin's photometric technique (ref. 66), employing sodium β -naphthoquinone-4-sulfonate has been shown to react with uric acid and ammonia present in the urine or blood. The gasometric-ninhydrin method (ref. 67, 68), generally conceded to be quite specific for the determination of α -amino acid nitrogen, is rather tedious for routine analyses and excessive in sample needs. The gasometric-nitrous acid (ref. 69) and formol (ref. 70) or acetone (ref. 71) titration methods are less specific. The copper method of Pope and Stevens (ref. 72), adapted for urine (ref. 73) and blood (ref. 74) by Albanese and his associates, has found wide usage in a variety of modifications (ref. 75). A number of colorimetric adaptations of the copper method have been reported which have been reviewed by Kekki (ref. 76). A description of these procedures as currently employed in this laboratory in titrimetric and colorimetric forms will be included in this report.

In the course of the last 10 years, Albanese, Orto, and Zavattaro have developed an ultramicro method for the estimation of free plasma amino nitrogen. This procedure is based on the colorimetric determination of amino nitrogen contained in spots of plasma filtrate (5 λ) dispensed on strips of filter paper which are reacted with ninhydrin under rigidly controlled conditions of time and temperature. Details of this technique are given in Section IV.

The employment of plasma amino nitrogen (PAN) levels as an effective criterion of protein metabolism requires that considerable attention be given to the state of the test organism. Stafford (ref. 77) has reported on a very careful study of physiological conditions

influencing PAN levels in rabbits. One parameter believed to be of importance in standardizing test routines is the length of fast necessary to reduce PAN to a basal level. The variations incurred during a 24-hour fast are shown in Fig. 2. The 16-hour fast reduced the PAN of rabbits to a stable level. In humans, Albanese *et al.* found that for practical purposes an overnight fast of 10-12 hours provides a stable level for adults. However, the interval varies somewhat with age and nutritional state of the individual.

In order to evaluate the usefulness of fasting plasma amino nitrogen changes as criteria of the effects of various agents on protein nutrition, it is necessary to make the permissive assumption that the plasma amino nitrogen content of the blood and body fluids constitutes the major effective labile amino acid pool of the body, and that this pool functions metabolically as shown in Fig. 3. The validity of this biochemical schematic and its implications is supported by a considerable body of scientific evidence (ref. 52-55, 58, 59).

1. Nutritional States

The fluctuations of the amino acid pool which have been observed in various nutritional states (% S) are shown in Fig. 4. This biochemical-nutritional relationship is based on published and some unpublished data derived from some four hundred subjects (ref. 78).

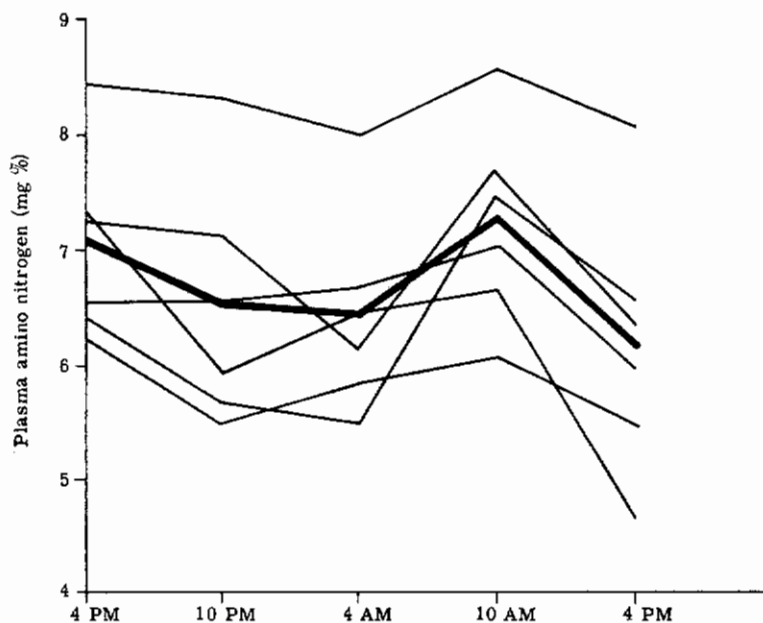


Figure 2. Plasma amino nitrogen of untreated rabbits measured 6 times during a 24-hour period. The fast began 24 hours before the first sample was taken. (From ref. 77)

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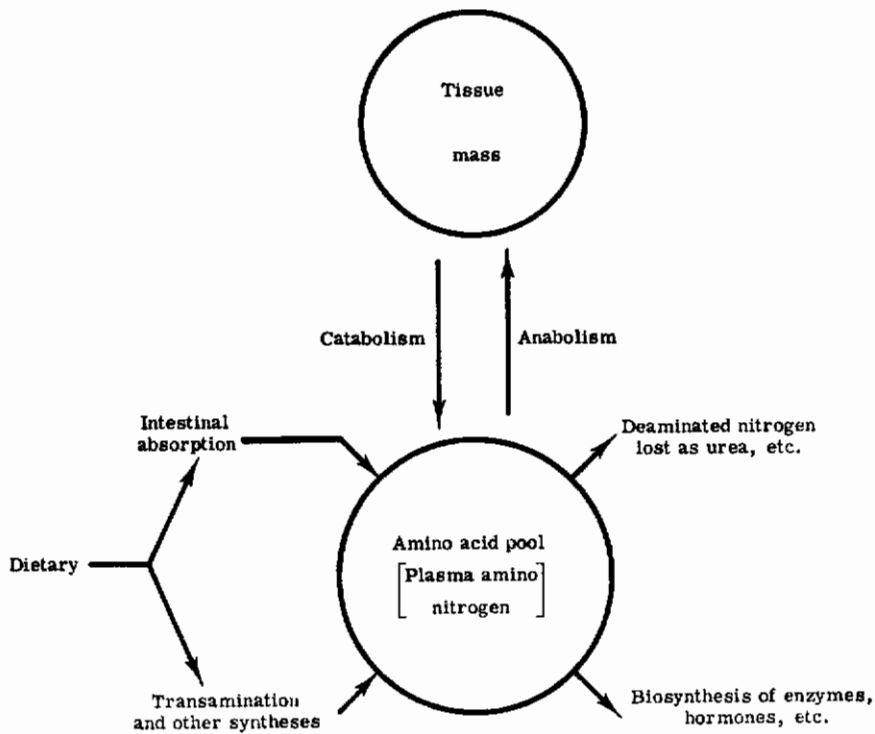


Figure 3. Schematic of body supply and loss of amino acids.

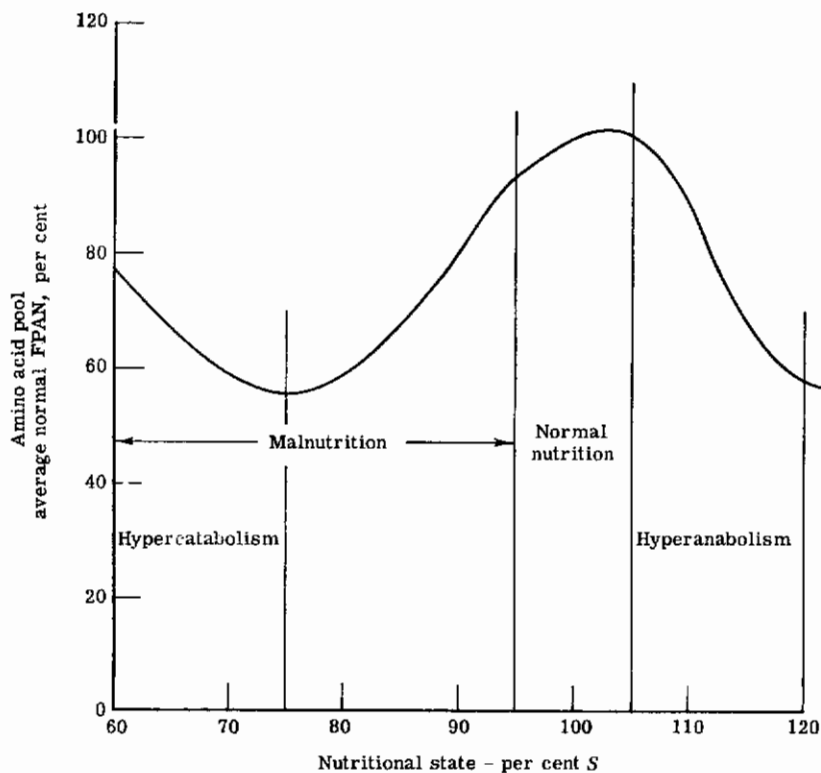


Figure 4. Relation of amino acid pool level to nutritional state as a function of standard body weight (%S). Solid line represents average data on some four hundred subjects (ref. 78).

Contrails

Interpretation of FPAN changes in terms of the metabolic scheme shown in Fig. 4 suggests that, as we pass from the nutritional norms to the first stages of malnutrition, the repletion demands of the tissue mass, owing to limitations of adequate qualitative or quantitative food intake or nutrient absorption, may exceed the amino acid supply in the pool. This results in a reduction of circulating amino acids (FPAN). The degrees of malnutrition comprised in this area (Fig. 4) are generally characterized by low negative nitrogen balances. In hypercatabolic states of malnutrition, destruction of tissue mass floods the pool with amino acid fragments which, because of some metabolic disability, cannot be reutilized by the body for tissue formation. This metabolic defect, which is associated with high negative nitrogen balances, may arise from endogenous deficiencies of hormones or enzymes, or from exogenous deficiencies of essential nutrients, e.g., amino acids, vitamins, or minerals.

In hyperanabolic states, the amino acid pool becomes depleted because the supply of amino acids does not meet the accelerated demands of tissue mass biosynthesis. This metabolic defect may arise from endogenous or exogenous excess of anabolic factors (hormones) prevailing in the face of a relatively inadequate intake of essential or nonessential nitrogen.

Studies by Andrews et al. (ref. 79) on serum amino acid nitrogen in infancy and childhood appear to confirm the presence of elevated serum amino acid nitrogen in certain patients with acute infectious processes, e.g., hypercatabolic states. However, the inconsistency of the findings precludes prognostic usefulness of the measurement in febrile conditions. Malignant protein malnutrition of infancy and cachexia of malignant neoplasias in adults are also often associated with hyperaminoacidemia. Elevated PAN has been reported in hyperthyroidism and rarely in diabetes mellitus. Administration of corticosteroids which induce protein catabolism frequently causes hyperaminoacidemia (ref. 80), which is associated with hyperglycemia (ref. 81). Administration of anabolic agents under conditions of limited nitrogen intake has been noted to cause a significant reduction in plasma amino nitrogen levels (ref. 80) and a lowering of the fasting blood sugar level with impaired tolerance to glucose administered both orally and intravenously (ref. 82). When due consideration is given to these limiting factors, measurements of PAN have proven to be powerful tools in metabolic and nutritional explorations.

2. Nitrogen Utilization and Balance

The foregoing observations suggested that, under conditions of known protein intake, fasting plasma amino nitrogen levels could be correlated with nitrogen balance. To test this possibility, Albanese and Orto (ref. 83) made some 60 determinations of the nitrogen intake, nitrogen balance, and fasting plasma amino nitrogen of 41 male and female adults in the recovery phase of a variety of physically disabling episodes. These subjects were all in a repletion phase of nutrition. Computations from the data so obtained by the principles of nomography (ref. 84) permitted the construction of the nomogram shown in Fig. 5. It will be noted that per cent nitrogen utilization is a logarithmic function of the vectors created by nitrogen intake and FPAN. Since

$$\text{N utilization \%} = \frac{\text{N balance}}{\text{N intake}}$$

and

$$\text{N balance} = \text{N intake} \times \text{N utilization \%}$$

the balance can be readily calculated from nitrogen intake and fasting plasma amino nitrogen. Comparisons of nitrogen balance calculated from these parameters and that obtained by actual measurements are shown in Table II. Although the differences are somewhat greater than might be desirable for certain metabolic investigations, this simplification can be expected to facilitate greatly the undertaking of useful dietary studies and would seem particularly worthwhile for field or population surveys.

It should be mentioned that the nomographic procedure for the estimation of nitrogen balance has not proved completely applicable in the area of negative nitrogen balance nor under conditions of excessive nitrogen intake. Investigations currently in progress give promise of an early resolution of the limitations.

3. Appetite

Mellinkoff and co-workers (ref. 85) reported that in metabolically normal subjects there was a reciprocal relationship between serum amino nitrogen concentration and crude estimates of appetite. A rise in PAN appeared to be accompanied by a waning appetite--and an increase in appetite by a fall in PAN (Table III). A similar relationship between blood sugar concentrations and appetite was found after infusions of glucose and hydrolyzed casein, but the administration of

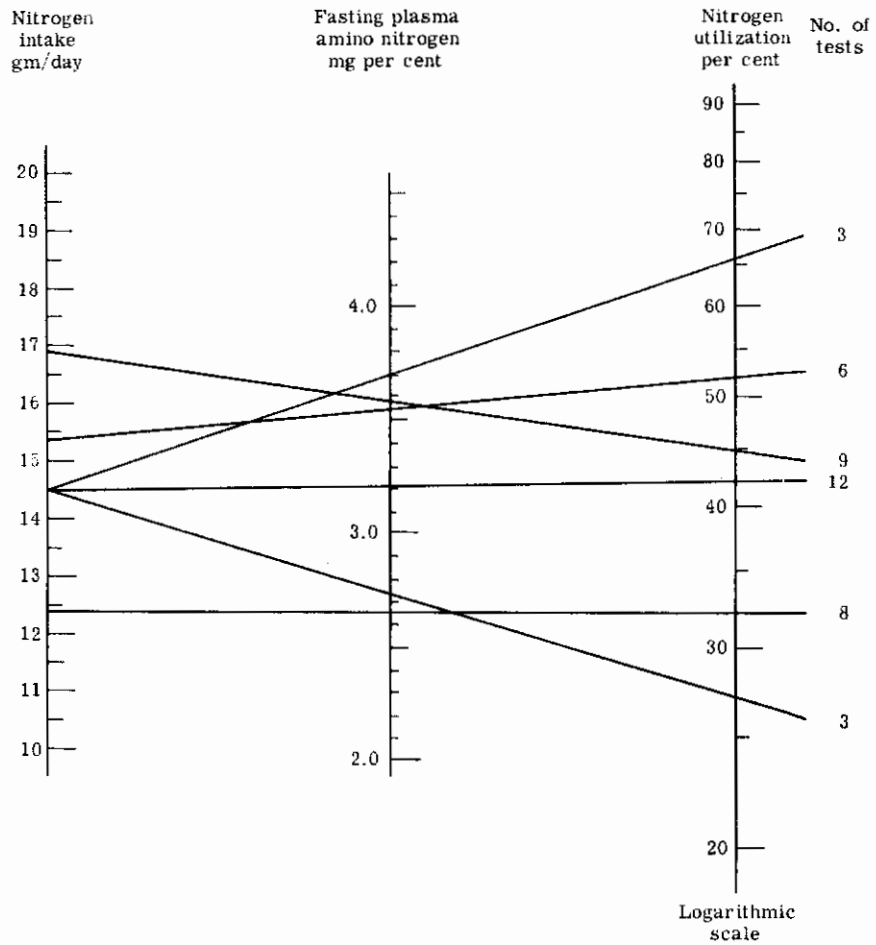


Figure 5. Nomogram for estimating nitrogen utilization from data on nitrogen intake and fasting plasma amino nitrogen.

Table II
Comparison of Nitrogen Balance Values Calculated from Nomogram
and Obtained from Kjeldahl Measurements

| Subject | Nitrogen intake (gm/day) | FPAN (mg%) | Nitrogen ^a utilization (%) | Nitrogen balance | | |
|---------|-----------------------------|---------------|---|---------------------------------------|-----------------------------|--------------------------|
| | | | | Calc. ^b (gm/day) (a) | Observed (gm/day) (b) | Δ (gm/day) (b - a) |
| 1 | 14.3 | 2.7 | 27 | +3.9 | +4.1 | +0.2 |
| 2 | 14.6 | 2.8 | 26 | +3.8 | +3.4 | -0.4 |
| 3 | 14.8 | 3.6 | 56 | +8.3 | +8.9 | +0.6 |
| 4 | 12.7 | 3.2 | 48 | +6.1 | +5.1 | -1.0 |
| 5 | 17.8 | 2.5 | 14 | +2.5 | +1.4 | -1.1 |
| 6 | 17.0 | 3.4 | 37 | +6.3 | +5.9 | -0.4 |
| 7 | 12.0 | 3.0 | 45 | +5.4 | +4.8 | -0.6 |
| 8 | 15.1 | 3.2 | 38 | +5.8 | +6.0 | +0.2 |
| 9 | 12.0 | 2.7 | 34 | +4.1 | +2.7 | -1.4 |

^a Determined from nomogram.

^b Nitrogen utilization (%) = nitrogen balance (gm/day)/nitrogen intake (gm/day) × 100; then: nitrogen balance (gm/day) = N utilization (gm%) × N intake.

Table III
Correlation Between Fluctuations in Appetite and Changes in
Serum Amino Acid and Blood Sugar Concentrations^a

| Exp. | ^b for change in serum amino acid nitrogen conc. and in appetite | | ^b for change in blood sugar conc. and in appetite | |
|------|--|----------|--|----------|
| | <i>r</i> | <i>P</i> | <i>r</i> | <i>P</i> |
| 1 | -0.595 | <0.01 | — | — |
| 2 | -0.405 | <0.01 | -0.318 | <0.05 |
| 3 | -0.509 | <0.01 | +0.232 | <0.05 |
| 4 | -0.526 | <0.01 | +0.297 | <0.05 |

^a From Mellinkoff *et al.* (ref. 85).

^b Correlation coefficient

hydrolyzed casein alone caused the blood sugar concentration and the appetite to diminish simultaneously (Fig. 6).

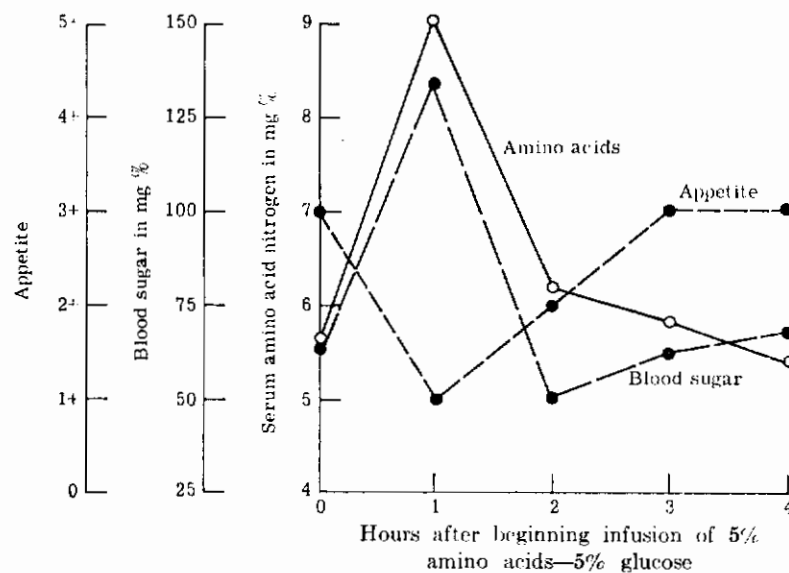


Figure 6. Appetite and blood sugar and serum amino acid concentrations in subject J.K., following infusion of 500 ml of distilled water containing 5% amino acids and 5% glucose in 45 minutes (from ref. 85).

Albanese and associates (ref. 86) made measurements of the midday preprandial, and 1-hour postprandial, blood sugar and amino nitrogen levels of sixteen normal healthy individuals (25-48 years). Subject response to questions regarding "feelings" of hunger in the pre- and post-prandial periods showed much better correlation with amino nitrogen levels than with blood sugar levels. This series included two females who were approximately 50 per cent overweight in terms of the Metropolitan Life Insurance Company tables (ref. 87). Both of these subjects felt severe gastric distress between 3 and 4 P. M. daily, which was relieved by ice cream or other refreshments. Measurements at the height of this gastric distress revealed normal blood sugar levels but amino nitrogen levels approximately 50 per cent below the immediate postprandial levels.

From these and other reports (ref. 88, 89, 90), it would appear that the "glucostatic" theory of food intake regulation proposed by

Mayer (ref. 91) is no longer tenable and that measurements of PAN may provide a more promising elucidation of associated problems, namely idiopathic chronic under- and over-nutrition. The latter, overnutrition or obesity, because it is etiologically suspect in cardiovascular diseases, receives considerable attention in both the lay and the scientific literature.

4. Effect of Carbohydrates

There is as yet insufficient evidence to show whether the rate of protein synthesis is directly influenced by changes in carbohydrate and fat supply or whether the effect occurs primarily through other reactions in protein metabolism. Munro has concluded that the action does not appear to depend primarily on improved synthesis of non-essential amino acids from the carbohydrate, but rather on the temporary increase in the level of available energy shortly after carbohydrate administration with consequent stimulation of protein synthesis (ref.92). A subsequent report by Albanese and co-workers (ref. 93) on the effect of carbohydrates on blood amino nitrogen showed that test doses of fructose induce a higher blood amino nitrogen index than comparable amounts of dextrose in fasting human subjects. This effect was most pronounced in the elderly (ref. 94).

These observations are best explained by the now well established fact (ref. 95) that fructose, because of its relative metabolic independence from insulin, is more rapidly utilized than glucose. Consequently, it would appear that the protein-sparing mechanism may be dependent on the metabolic pathways of the sugars and, contrary to the views of Munro, that the magnitude of the action is probably a function of an improved synthesis of nonessential amino acids as well as an increased level of available energy. The need for additional research in this related aspect of protein and carbohydrates is clearly indicated.

5. Effect of Steroids

Stafford (ref. 77) found that treatment of rabbits with Depo-Nortestonate (DNT) caused a depression of the fasting plasma amino nitrogen (FPAN) to a small but consistent degree. The behavior of the FPAN after administration of exogenous amino acid was found to be variable, but the data indicated that if enough rabbits were tested, a significant effect of DNT on amino acid tolerance could be demonstrated. Further studies showed that two anabolic steroids, testosterone cyclopentylpropionate and nortestosterone cyclopentylpropionate, caused

FPAN depressions of 25 and 27 per cent respectively. The catabolic steroid, cortisone cyclopentylpropionate, caused an increase of 12 per cent. These observations are in good accord with postulates permitted by the data shown in Fig. 4.

B. Individual Blood Amino Acids

As previously noted, it has been known for some years that the nutritive value of a dietary protein is determined mainly by its amino acid content. Not all amino acids present in a protein, however, are available to the animal, especially when the protein is either from a vegetable source or has had some of the amino acids destroyed or rendered unavailable by processing.

Albanese and Orto (ref. 96) reported studies in which the availability of amino acids was determined from differences in blood levels immediately before, and 1 hour after, test meals which supplied proteins and calories in amounts usually consumed by infants and young children. Test feedings with a low-protein infant formula (1.7 gm %) were associated with a decrease in plasma lysine as well as total plasma amino nitrogen. Conversely, feedings with conventional whole or evaporated milk formulas were associated with increases in plasma lysine and total plasma amino nitrogen. Ingestion of milk or egg proteins (0.25-0.50 gm/kg) caused an increase in the lysine content of the blood of young children. Ingestion of cereal or white bread proteins caused a decrease in the lysine concentration of the blood of young children. Supplementation with lysine provided a physiological technique for overcoming the plasma lysine-lowering effect of the foods tested.

Puchal et al. (ref. 59) found that plasma amino acid levels in the young pig are closely related to the amino acid composition of the dietary protein. Different plasma amino acid patterns were observed for the five different protein sources assayed: dried skim milk, soybean meal, fish meal, cottonseed meal, and meat meal. Comparison of the patterns with those of skim milk pointed to some of the amino acids that may be responsible for the poor growth performance of young pigs.

Collectively, the foregoing experiences suggest rather strongly that measurements of free individual blood amino acids provide an effective tool in problems of protein metabolism. Consequently, it is important that the accuracy and operational advantages of the methods employed be carefully scrutinized. Currently, four principal techniques

are in use--namely, isotope and microbiological analyses, and column and paper chromatography.

1. Paper Chromatography

Because of ease of operation and simplicity of equipment, paper chromatography remains by far the most useful and popular of analytical methods for the determination of amino acids in biological fluids and products. The literature contains a wealth of individual reports and a number of books covering every aspect of this subject. We have found "Chromatographic and Electrophoretic Techniques," edited by Ivor Smith (ref. 97), to be the most comprehensive treatise with regard to methodology and data. The many desirable features of paper chromatography are pervaded by difficulties of quantification. However, by choice of solvents which are relatively stable and insensitive to environmental changes, techniques have been developed which bear a degree of accuracy comparable to the aforementioned procedures. Our efforts with this analytical principle are described in Section IV. These methods have been applied in attempts to resolve a variety of problems. Some of the results will now be related in detail.

C. Applications

1. Individuality of Protein and Amino Acid Needs

The assumption that the human population is composed largely of individuals who have about average nutritional requirements has been questioned by some, notably Roger Williams (ref. 98). The assumption is also considered untenable in the light of facts of variability and certain statistical considerations. The findings of Rose (ref. 16), Leverton (ref. 17), Albanese (ref. 99), and others show that there exist large interindividual differences in requirements and metabolism of amino acids. Therefore, the determination of individual needs becomes a "must" in a variety of clinical and general problems of nutrition. For some time we have been concerned with maintenance of nutritional status of men chosen to go on long space or submarine trips. Owing to the logistics of such operations, these personnel will subsist on food limited in quantity, variety, nature, and content. Under these circumstances, large requirements for specific nutrients in the individual, if undetected, may indeed prove disastrous. This is particularly so on trips where men cannot be resupplied. Large individual requirements for specific nutrients pose no great problem as long as these are known.

Careful review of the limitations of the nitrogen balance procedure for these purposes indicated that more facile methods are urgently needed to determine the optimal requirements of man for protein and individual essential amino acids to maintain and augment functional capacity. These problems have been known for some time and led to long-range investigations of other methods of determining essential nitrogen needs of man. Consideration of immediate objectives indicated that new methods shall have the following characteristics:

- (1) Accuracy with less than 10 per cent error. This accuracy is necessary for measuring interindividual differences in requirements;
- (2) Clinical ease, in the sense that it will require little time or effort on the part of the subjects involved;
- (3) Simple techniques, if possible enabling hospital technicians to perform the analyses under suitable supervision;
- (4) Adaptability to circumstances which reflect environmental restrictions.

Also, the method should be capable of yielding the following information: (a) measurement of optimum as well as minimum protein and amino acid requirements; (b) assessment of the range of requirements as a function of biological variation; and (c) determination of requirements, not only when man is adequately fed but also when he is nutritionally compromised.

In attempting to achieve the objectives of this program, the parameters influencing protein utilization were examined (Table IV).

Table IV
Parameters Influencing Protein Utilization

| Exogenous factors | Endogenous factors |
|---|---|
| 1. Amino acid contour of protein | 1. Nutritional individuality |
| 2. Calorie, vitamin, and mineral balance of dietary | 2. Genetic characteristics |
| 3. Artifacts of food processing and preparation | 3. Biochemical individuality |
| 4. Food habits | 4. Hormonal and enzyme balance |
| | 5. Inborn or induced metabolic aberrations for specific amino acids |

In order that the exogenous factors could be minimized, standard test procedures and foods were employed. To explore the endogenous factors associated with nutritional individuality, subjects of various age and stature were tested. The assay protocol is shown in Table V.

Table V
Assay Protocol for Amino Acid Availability Studies

1. Healthy, male or female subjects (21-42 years of age) with normal blood counts and urinalyses were requested to eat, between 7:00 and 8:00 A.M. on test days, a standard breakfast consisting of:

| | Protein | Calories |
|-----------------------------------|---------------|------------|
| Orange juice (4 oz) | 0 | 40 |
| Toast, buttered (2 slices) | 4.3 | 276 |
| Coffee (1 cup) with sugar (1 tsp) | 0 | 20 |
| Total | 4.3 gm | 336 |

2. Two hours after the "standard breakfast," fingerprick blood samples of 0.1 or 0.2 ml were collected for zero-time measurements.
3. Immediately thereafter, the subjects drank the test meal contained in a 200-cc volume.
4. The test meal consisted of varying amounts of a commercially available vitamin- and mineral-fortified milk protein product having a percentile caloric distribution of: protein, 31.0; fat, 9.0; carbohydrate, 48.0.
5. Blood samples were again collected by fingerprick at 1 and 2 hours after ingestion of the test meal.
6. Analyses of samples were begun immediately after collection.

The practical objectives of these researches precluded use of the more cumbersome methods, e.g., microbiological, column chromatography, or radiochemical. The methods employed permit the use of small amounts of sample and the obtaining of rapid results (Table VI).

Table VI
Determination of Amino Acids

| Constituents | Principle | Reagent | Size of sample (λ) | Time (hr) | Accuracy (%) |
|--|--|-----------|--------------------|----------------------------|--------------|
| Plasma amino nitrogen | Multiple spots on paper strips with external standards | Ninhydrin | 5 × 4 | 1.5 | ±5 |
| Lysine | Horizontal, circular paper chromatography with internal standards | Ninhydrin | 30 × 2 | 5 (3-hr wait) | ±7 |
| Methionine, threonine, other amino acids | Ascending paper strip chromatography with external or internal standards | Ninhydrin | 30 × 1 | 21 (overnight, 20-hr wait) | ±10 |
| Sample preparation: | 200λ (0.2 ml) whole blood (fingerprick) + 300λ (0.3 ml) acetone | | | 240λ filtrate | |
| Total sample needs: | | | | - 110λ | |
| Excess for duplicates or other determinations: | | | | 130λ | |

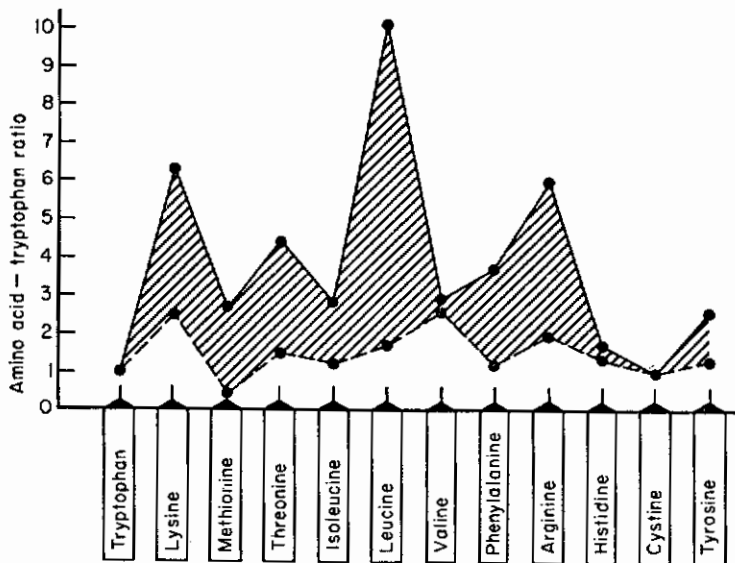


Figure 7. Amino acid pattern differences of muscle proteins and fasting plasma amino acids. The hatched areas represent the specific amino acid deficit. The solid line indicates the mammalian muscle proteins, and the broken line the fasting plasma amino acids (ref. 99a).

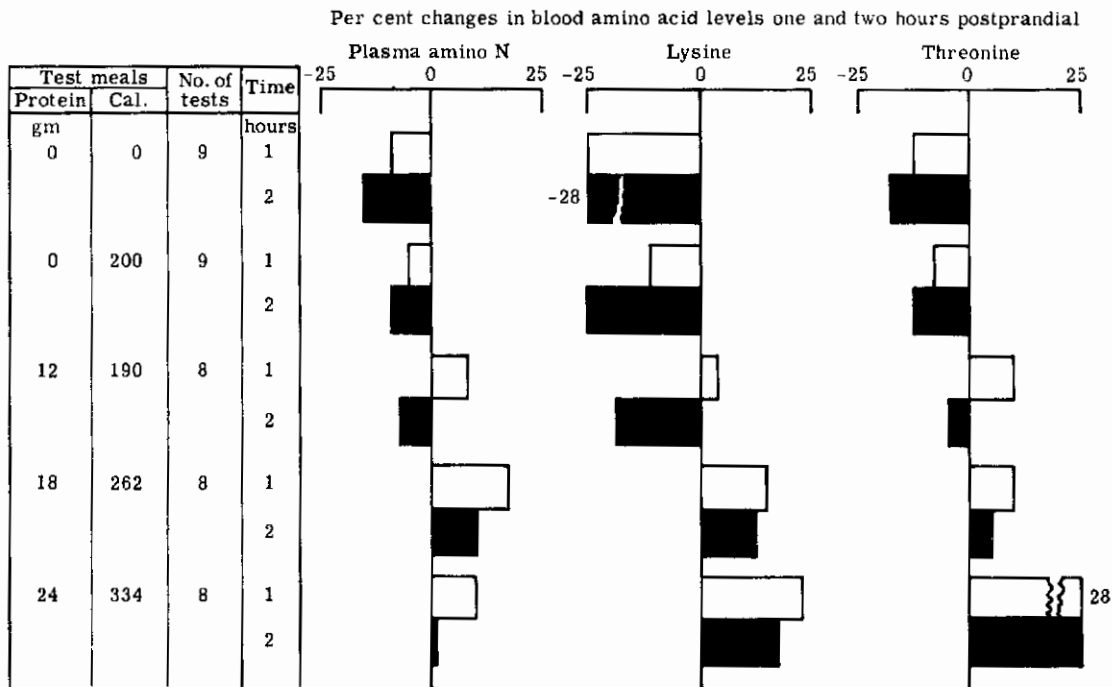


Figure 8. Effect of protein intake on plasma amino nitrogen, lysine, and threonine levels.

Contrails

The uncertainties of sample storage are avoided; the equipment requirements are relatively inexpensive and simple. The accuracy of these methods in our hands is comparable to that obtained with more elaborate equipment and time-consuming techniques.

Our criterion of individual amino acid needs is based on the capacity of a protein to change the pattern of blood amino acids from the fasting to that prevailing in muscle protein. This relationship is shown in Fig. 7. This pattern, we feel, is a logical one--not based on the untenable assumptions of the FAO pattern (ref. 100). It can be demonstrated that an increase of 20-25 per cent in the fasting level of most amino acids fulfills the requirements of the tissue analysis pattern.

Typical results are shown in Fig. 8. It will be noted that progressive increases in plasma amino nitrogen, at both 1- and 2-hour intervals, occur as the protein intake is increased from 0 to 18 gm in the test meal. Lysine and threonine optima are attained at higher levels of protein intake. In Fig. 9, it will be seen that methionine and the leucines follow this general pattern; cystine does not. Such differences as exist between the total amino nitrogen change and the individual amino acid change provide useful information with regard to limiting amino acid or calorie balance at each level of protein fed. This newly observed phenomenon, the protein overload effect, is under intensive study in our laboratory.

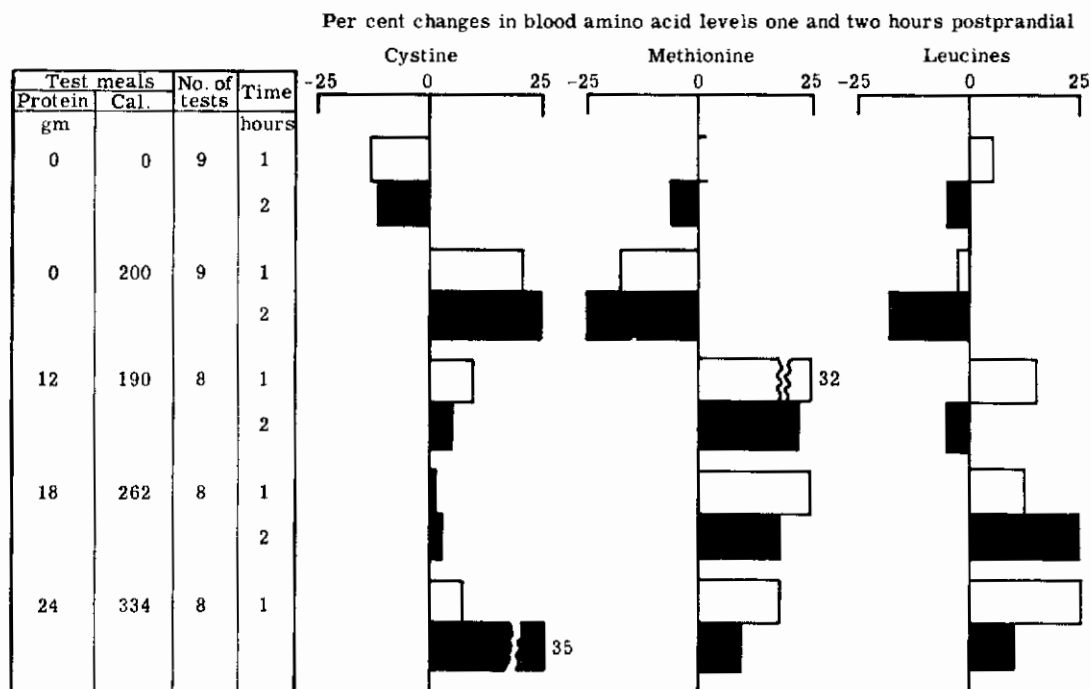


Figure 9. Effect of protein intake on cystine, methionine, and the leucines.

Contrails

An example of these relationships in an individual of normal body weight is shown in Fig. 10a. It will be noted that the zero intercept (a measure of minimal needs) for plasma amino nitrogen was obtained at approximately 0.2 gm of milk protein/kg of body weight. The zero intercept for lysine, however, did not occur until 0.35 gm of milk protein/kg had been fed in the test meal. The individuality of some of our subjects in respect to this characteristic is exemplified by the data contained in Fig. 10b, an overweight individual, and in Fig. 10c, an underweight individual.

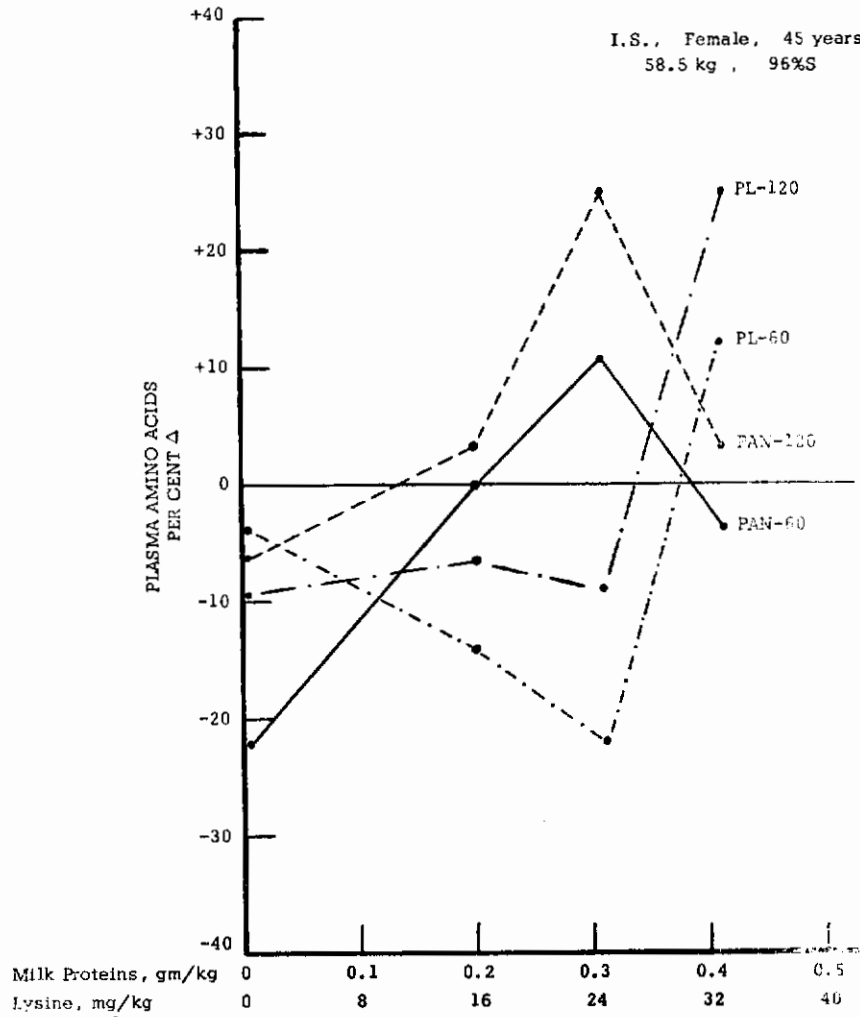


Figure 10a. Effect of body weight on the relationship of dietary protein and lysine to plasma amino nitrogen and plasma lysine levels.

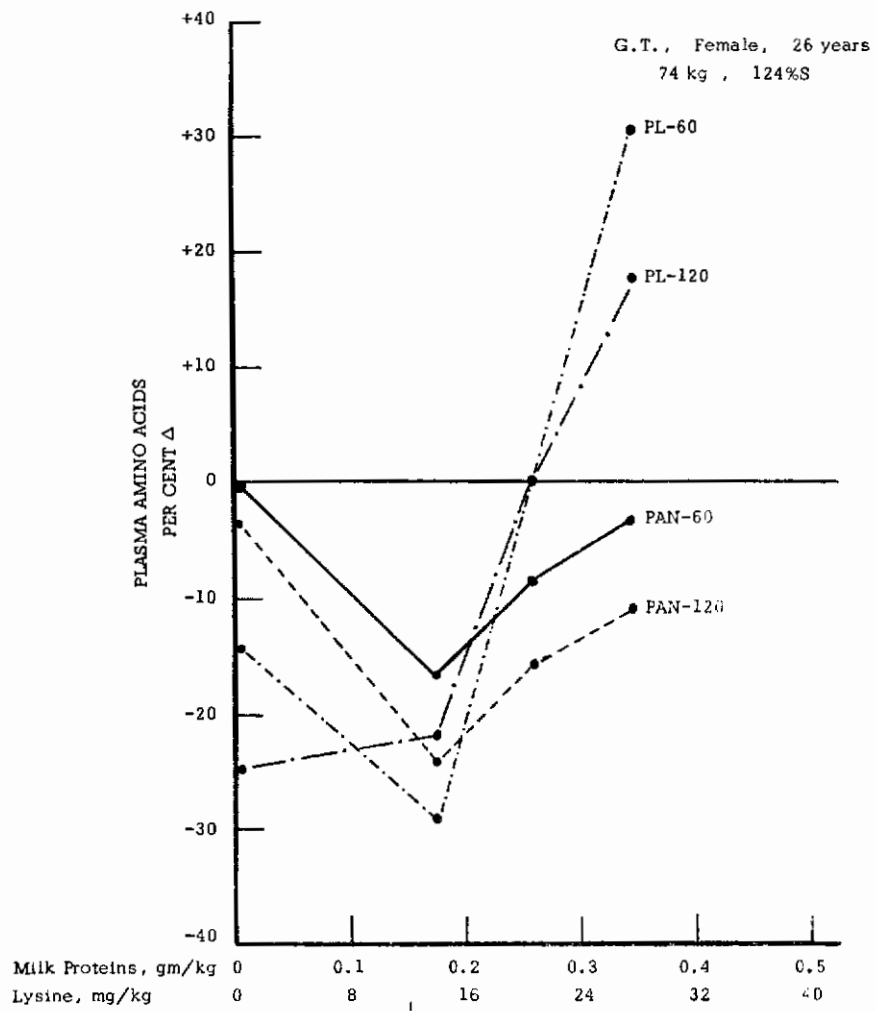


Figure 10b.

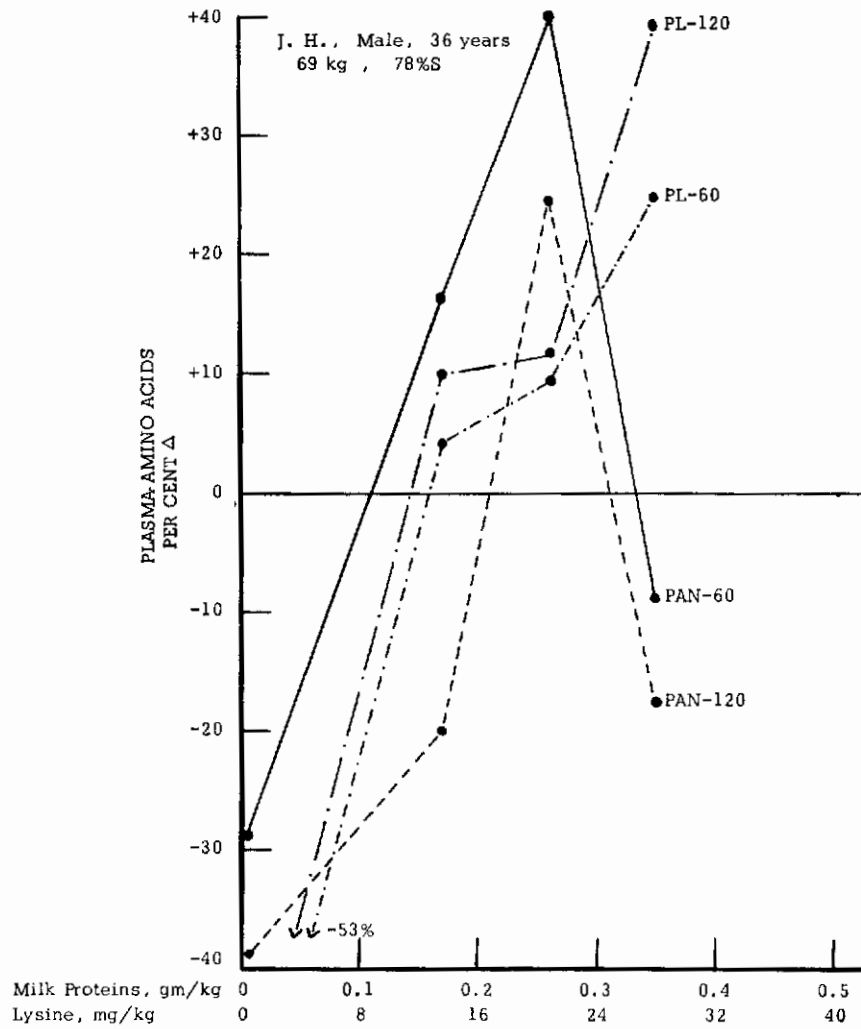


Figure 10c.

The findings with respect to observed extremes in individuality of lysine minima are shown in Table VII. Attention is called to the fact that the estimated lysine minima varied from 246 mg to 507 mg/day. A comparison of these findings with those of other investigators is shown in Table VIII. The lysine minima obtained by our procedures fall well within the range of those obtained by nitrogen balance determinations.

Table VII
Individuality of Lysine Minima of Humans

| Subject | Age | Body weight (%S) | Zero intercept | | Average of minima × body weight in kg (mg) | Equivalent daily minima (load test × 3) (mg) |
|---------|-----|------------------|----------------|-----------------|--|--|
| | | | 60 min (mg/kg) | 120 min (mg/kg) | | |
| J. H. | 36 | 78 | 11.2 | 12.4 | 82 | 246 |
| I. S. | 45 | 96 | 27.2 | 30.0 | 169 | 507 |
| G. T. | 26 | 124 | 21.0 | 21.0 | 147 | 441 |

Table VIII
Lysine Minima of Normal, Healthy Adults

| Investigators | Methods | | Subjects | | Lysine minima (mg/day) |
|----------------------------------|---------------------------------|-------------------------|----------|-------|------------------------|
| | Test substances | Criteria | No. | Sex | |
| Rose <i>et al.</i> ^a | Mixture of 8 amino acids | Nitrogen balance | 6 | M | 400-800 |
| Clark <i>et al.</i> ^b | Cereal proteins and amino acids | Nitrogen balance | 10 | M & F | 300-700 |
| Albanese and Orto ^c | Milk proteins | Blood amino acid levels | 22 | M & F | 300-650 |

^a (ref. 100a).

^b (ref. 100b).

^c Interpretation: Present studies indicate that N balance determinations of minimum requirements represent only a measure of needs for maintenance of fasting or 2-hour postprandial blood levels of the essential amino acids.

Contrails

It is abundantly clear that the validity and practical significance of the foregoing observations would be greatly enhanced by a resolution of the factors causing the "protein overload effect." As a first step in this direction, the influence of a calorie supplement (50 gm of sucrose) to the standard test product at the 18- and 24-gm protein levels was studied (Table V). Total calories and per cent of protein calories of the test meals, and results of the assays, are shown in Table IX. It will be noted that, when the contribution of protein calories was reduced from 31 to 16 per cent (test meals A and B), the plasma amino nitrogen index of protein utilization for the 60-minute interval was significantly increased in all three subjects. This effect persisted, but to a lesser degree, at the 120-minute test interval. In general, the measurements with test meals C and D were in good accord with those obtained with test meals A and B. However, the "protein overload effect" of meal C appears to be far greater than that of meal A, and the results indicate the need for a larger calorie supplement than was provided in meal D.

Although the available data are as yet quite inadequate, it seems in order here to speculate briefly on the possible application of these results to estimations of nitrogen balance. If the not altogether unreasonable assumption that per cent plasma amino nitrogen changes (% Δ PAN) provide a fair measure of protein nitrogen utilization be allowed, then, since

$$\text{protein N utilization, \%} = \frac{\text{N balance}}{\text{N intake}}$$

it follows that

$$\% \Delta \text{PAN} = \frac{\text{N balance}}{\text{N intake}}$$

or

$$\text{N balance} = \text{N intake} \times \% \Delta \text{PAN}$$

Obviously, the PAN changes observed at either the 60- or 120-minute interval could be employed in these calculations with equal validity. However, only those results obtained at the 120-minute interval--a possibly steadier metabolic state--are recorded in Table IX. Considered in this light, it is readily apparent that the carbohydrate supplement exerts a significant protein-sparing effect or reduction in the "protein overload effect." Attention is also called to the magnitude of interindividual differences of estimated nitrogen balance obtained when similar amounts of protein (gm/kg) are fed in the test meals.

Table IX
 Influence of Calorie and Protein Supplements on Plasma Amino Nitrogen
 and Estimated Nitrogen Balance

| Subjects | Test meals | | Plasma amino N index of | | Protein in test meal (gm/kg) | Calc. nitrogen intake ^a (gm/day) | Estimated N balance ^b (gm/day) |
|-----------------|------------|-------|-------------------------|----------------|------------------------------------|---|---|
| | Code | Total | protein utilization | | | | |
| | | | Calories | Protein (%) | | | |
| J. H., M, 69 kg | O | 0 | 0 | -13 | 0.0 | 0 | 0 |
| | A | 262 | 31 | +11 | 0.26 | 8.7 | +1.5 |
| | B | 462 | 16 | +25 | 0.26 | 8.7 | +1.7 |
| | C | 334 | 31 | -5 | 0.35 | 11.5 | +0.8 |
| | D | 534 | 18 | +19 | 0.35 | 11.5 | +2.2 |
| I. S., F, 58 kg | O | 0 | 0 | -7 | 0.0 | 0 | 0 |
| | A | 262 | 31 | +25 | 0.31 | 8.7 | +0.7 |
| | B | 462 | 16 | +47 | 0.31 | 8.7 | +0.9 |
| | C | 334 | 31 | +4 | 0.40 | 11.5 | +0.6 |
| | D | 534 | 18 | +19 | 0.40 | 11.5 | +1.5 |
| G. T., F, 70 kg | O | 0 | 0 | -6 | 0.0 | 0 | 0 |
| | A | 262 | 31 | +22 | 0.26 | 8.7 | +0.5 |
| | B | 462 | 16 | +52 | 0.26 | 8.7 | +1.7 |
| | C | 334 | 31 | +2 | 0.35 | 11.5 | -0.6 |
| | D | 534 | 18 | +5 | 0.35 | 11.5 | +0.3 |

^a Daily N intake = (gm protein in test meal X 3)/6.25.

^b Estimated N balance (gm/day) = $PAN\Delta_{120}\%$ X calculated nitrogen intake (gm/day).

Conclusions

In an effort to overcome some of the shortcomings of the foregoing, the following approach was assayed. The nitrogen intake of adult subjects was determined from diet records, and nitrogen balance determined from 24-hour urine collections. Subjects were then given a protein load containing one-third of the previously observed nitrogen intake. Levels of plasma amino nitrogen (PAN), as well as other amino acids, were determined on samples collected before, and one and two hours after, ingestion of the test meal. Calculations disclosed that the PAN change following the test load is related to the nitrogen balance by the equation:

$$Y = A - 16X$$

where:

Y = nitrogen balance, gm/day;
A = intercept of y axis; and
X = $\Delta\text{PAN}_{60} + 120/2$.

Nitrogen balance so calculated showed a better than ± 5 per cent agreement with that observed for 8 subjects studied. Available data show that A may vary with the amount of protein in the test meal, which is a function of the nutritional individuality or status of the test subject.

In this connection, attention is called to the very interesting report of Kraut and Zimmermann-Telschow on the alterations in free amino acids of blood and urine in relation to diet (ref. 101). In a 5-month dietary trial in a healthy 19-year-old male, pure whole egg protein was given for a period of 3 weeks and a lesser amount of whole egg protein together with other nitrogen sources for two periods each of 3 weeks. The amount of nitrogen in the diet corresponded roughly to the minimum required for balance. Analyses of free amino acids in fasting serum and urine chromatographically showed a significant increase in essential amino acids in the serum during the pure whole egg diet over the levels during normal diet. In the two periods with rather more than half the amount of whole egg plus diammonium citrate and glycine, the levels of all essential amino acids except lysine were significantly lower than in the whole egg protein period. The hypothesis is put forward that variations in the valine:lysine ratio in the serum between the different diet periods enable one to determine whether anabolic or catabolic conditions predominated at the time the blood sample was taken.

Urinary amino acids remained substantially unaltered throughout the whole dietary study; only glycine elimination is influenced by the composition of the diet.

IV. METHODS

It is clear from the literature that the challenge of problems of protein nutrition and metabolism have resulted in a continuous flow of new techniques and concepts. Consequently, there is a constant need for appraisal of the old and the new. Too often, the novelty of methods, especially those requiring elegant and costly instrumentation, obscures their lack of basic improvement over the old. The methods described here have proved to be convenient and adequate for the study of problems of human nutrition and biochemistry in the areas of our cognizance.

A. Nitrogen Determination--Kjeldahl Method

1. Principle

The sample is boiled with concentrated sulfuric acid and a "digestion mixture" to convert all forms of nitrogen to ammonium sulfate. Subsequent addition of an excess amount of alkali in a closed system neutralizes the acid and releases ammonia which is distilled into boric acid solution. On completion, the bound boric acid is back-titrated with hydrochloric acid of known strength.

2. Reagents Required

- a. Sodium Hydroxide. A 40% solution.
- b. Hydrochloric Acid. A 0.1 and 0.01 N solution.
- c. Ammonium Sulfate. A 0.1 N primary standard is prepared and a 0.01 N standard is made by a dilution of the primary standard.
- d. Methyl Red Solution. Dissolve 200 mg of methyl red in 50 ml of 95% ethanol and make to 100 ml with water.
- e. Hydrogen Peroxide. 30% (Superoxol, Reagent Grade).
- f. Boric Acid. A 4% solution. Dissolve with the aid of heat 40 gm of boric acid in 1 liter of water and add 5 ml of methyl red solution/liter.
- g. Digestion Mixture. Dissolve with the aid of heat 300 gm of K_2SO_4 in 1 liter of water; dissolve with the aid of heat 100 gm of $CuSO_4$ in 1 liter of water; and dissolve with the aid of heat 40 gm of $HgCl_2$ in 1 liter of water. The solutions are cooled, combined, and mixed thoroughly. This mixture serves to raise the boiling point and catalyze the oxidation of the reduced nitrogen forms present in the sample.

h. Mixture for Steam Generator of Kjeldahl Apparatus. For each liter of water add 5 ml of methyl red and 2 ml of concentrated H_2SO_4 . Water is added as needed and with usage the color will fade and additional methyl red and H_2SO_4 must be added from time to time.

3. Procedure for Urine Samples

a. Digestion. (1) To a 100-ml Kjeldahl flask add 1 ml of urine, 2 ml of concentrated H_2SO_4 , 2 ml of digestion mixture, and 2 glass beads.

(2) Digest over a moderate but constant flame under a hood for 1 hour.

(3) Allow the flasks to cool, add 8-10 drops of superoxol, and digest for an additional 20 minutes.

(4) When the flasks have cooled, add 10 ml of water.

b. Distillation. (1) Steam out Kjeldahl apparatus (Fig. 11) for 15-20 minutes.

(2) Place a 125-ml Erlenmeyer flask containing 5 ml of boric acid in a tipped position so that the stem tip is below the level of the acid.

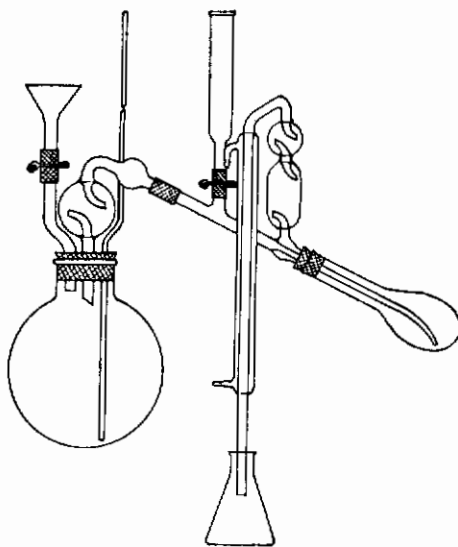


Figure 11. Kjeldahl distilling apparatus for micro and semimicro determination of nitrogen. Available with ground joints from Eck & Krebs, Inc., New York, Catalog No. 1280.

Contrails

- (3) Place the cooled Kjeldahl flask containing the digested sample in position so that the stem tip is below the liquid surface.
- (4) Add slowly 10 ml of NaOH (40%) to the Kjeldahl flask.
- (5) Distill the ammonia into the boric acid; within a few minutes the pink-colored boric acid solution turns yellow. The distillation is continued until about 10 ml of distillate are collected.
- (6) Lower the Erlenmeyer flask to a vertical position and allow the stem to drain thoroughly.
- (7) First remove the Erlenmeyer and then the Kjeldahl flask; rinse both tips carefully with distilled water.
- (8) Titrate the distillate in the Erlenmeyer flask with 0.1 N HCl.

4. Calculation

gm urinary nitrogen/24 hours =

$$\frac{\text{ml of 0.1 N HCl used in titration} \times 0.1 \text{ N HCl factor} \times \text{urine volume (ml)}}{1000}$$

The 0.1 N HCl solution is factored prior to determination of the samples. The purpose of the factor is not to obtain normality but to obtain the N equivalent for the acid used. Therefore, the acid is equated to a solution of standard ammonium sulfate:

Place 5 ml of the 0.1 N standard plus 5 ml of water into a Kjeldahl flask and distill (digestion is not necessary) as described above. Then, titrate the distillate with 0.1 N HCl. The ammonium sulfate standard contains 1.400 mg N/ml.

Hence,

$$1 \text{ ml of exactly } 0.1 \text{ N HCl} = 1.400 \text{ mg nitrogen.}$$

However, if the titration requires more or less than 5 ml of 0.1 N HCl, then the factor is obtained by:

$$\frac{5.00}{\text{titer}} \times 1.400 = \text{factor}$$

B. Determination of Amino Nitrogen of Blood Filtrates by the Copper Method (Ref. 74).

1. Normal Values

5.8 - 8.9 mg%.

2. Principle

Amino acids form complexes with many of the heavy metals. In this procedure, the amino nitrogen reacts with copper to form a chelated compound, and the copper is determined iodometrically with sodium thiosulfate (ref. 72).

3. Reagents Required

- a. Cupric Chloride • 2 H₂O. Dissolve 27.3 gm in 1 liter of water.
- b. Trisodium Phosphate. Dissolve 64.5 gm in 1 liter of water.
- c. Borate Buffer. To 57.21 gm of sodium borate in 1.5 liters of water add 100 ml of N HCl and make to 2 liters with water.
- d. Copper Phosphate Suspension. Mix 1 volume of cupric chloride solution with 2 volumes of trisodium phosphate solution and 2 volumes of borate buffer (pH 7-9).
- e. Thymolphthalein Indicator. Dissolve 0.25 gm of thymolphthalein in 50 ml of 95% ethanol and make to 100 ml with water.
- f. Sodium Thiosulfate Stock (0.1 N). Dissolve 49.6 gm in 200 ml of water; add 20 ml of amyl alcohol which serves as a stabilizing agent, and make to 2 liters with water. The 0.01 N working solution is prepared by diluting the stock solution.
- g. Potassium Iodate Standard (0.01 N). Dry potassium iodate in an oven at 110° C for 1 hour and dissolve 0.3567 gm in 1 liter of water; use to standardize the thiosulfate solution.
- h. Starch Indicator. Dissolve 1 gm of Lintner soluble starch in 100 ml of saturated NaCl by heating over a steam bath; cool overnight and decant the supernatant for use.
- i. Potassium iodide. Make daily in quantities needed, 1 gm of KI/ml of water.
- j. Glacial Acetic Acid.
- k. Sodium Hydroxide. A 1N solution.

4. Procedure

a. To 3 ml of 10% trichloroacetic acid in 15-ml conical centrifuge tubes add 1 ml of unhemolyzed plasma or serum.

b. Mix the tubes thoroughly, allow them to stand for 10 minutes, and centrifuge the samples for 10 minutes at 3000 rpm.

c. Decant the supernatant solutions through 2.5-cm funnels containing Whatman No. 5 filter papers into 100 X 13 mm test tubes; remove 2-ml aliquots to graduated 15-ml conical centrifuge tubes.

d. To each sample filtrate add, in succession, 1 drop of thymolphthalein indicator, N NaOH until the appearance of the blue color, 5 ml of copper phosphate suspension, and distilled water to the 10-ml mark.

e. Shake tubes vigorously, allow the reaction mixture to stand for 5 minutes, and centrifuge the tubes for 10 minutes at 3000 rpm.

f. Transfer 5-ml aliquots of the supernatant from each tube to 125-ml Erlenmeyer flasks and determine the copper content iodometrically as follows:

- (1) Acidify each sample with 0.5 ml of glacial acetic acid.
- (2) Add 1.0 ml of freshly prepared KI solution followed by 4-5 drops of starch solution.
- (3) Titrate the samples with standardized 0.001 N sodium thiosulfate from a 10-ml microburette.

5. Calculation

Since each cubic centimeter of 0.001 N thiosulfate is equivalent to 0.028 mg of amino N, the milligrams of amino N per 100 ml of plasma or serum are found from the following formula:

ml of 0.001 N thiosulfate required for 5 ml of final filtrate X 0.028 X 400.

C. Ultramicro Technique for Plasma Amino Nitrogen

1. Normal Values

2.5-5.0 mg%

2. Principle

The proteins of the blood are precipitated with acetone and the free amino acids subjected to the ninhydrin reaction. The resulting color is read with a recording densitometer and compared with that of the standard.

3. Reagents Required

- a. Acetone. Cleared with norite and sodium sulfate and re-distilled for optimum purity.
- b. Ninhydrin. A 5% solution of ninhydrin dissolved in 95% ethyl alcohol. This solution must be clear and prepared daily.
- c. Benzoic Acid. A saturated solution stored in refrigerator.
- d. Alanine Stock Standard. Dissolve 100 mg of DL-alanine (15.7% N) in 100 ml of the saturated benzoic acid solution.
- e. Diluting Fluid. Mix 4 volumes of acetone with 3 volumes of distilled water.

4. Materials and Apparatus

- a. Schleicher and Schuell No. 507 filter paper cut to strip size of 1.8 X 29 cm by supplier.
- b. A supply of 1-ml centrifuge tubes and No. 00 corks.
- c. A supply of 0.1- and 0.2-ml blood pipettes.
- d. Microcentrifuge.
- e. Ultramicroburette (Standard Industries Ultra-Buret, Model 200) or a supply of 5 λ Kirk ultramicropipettes.
- f. A 45° C oven.
- g. A densitometer (Chromaphor I) coupled to a recording galvanometer (Bristol Dynamaster, Model 560).

5. Procedure

- a. Collection and Preparation of sample. (1) Collect exactly 0.1 ml of blood by fingerprick and add to a 1-ml centrifuge tube containing 0.15 ml of redistilled acetone. If other measurements are needed (see Table VI), double quantities of filtrate must be prepared.

(2) Mix the sample thoroughly with the aid of a copper or platinum wire (No. 20) stirrer, and store the tightly corked sample for 1 hour in the freezing unit of a refrigerator.

(3) Centrifuge the sample for 15 minutes; repeat if the supernatant is not clear.

b. Preparation of Working Standards. (1) Dilute the stock alanine standard 1 ml to 10 ml with diluting fluid. This first standard (A) contains 1.57 mg% of alanine amino nitrogen.

(2) Dilute the above working standard 1:1 with diluting fluid. This second standard (B) contains 0.78 mg% of alanine amino nitrogen.

c. Determination of Plasma Amino Nitrogen. (1) Load the rack (see Fig. 12) with three lengths of 29 cm S & S No. 507 paper.

(2) Fill the microburette with alanine working standard "B". At half-inch intervals from one end of a paper strip, apply 4 "spots" of 5λ each.

(3) Wash the burette with water, dry with alcohol and acetone, and refill with alanine working standard "A". At the opposite end of the paper strip, apply 4 "spots" of 5λ each.

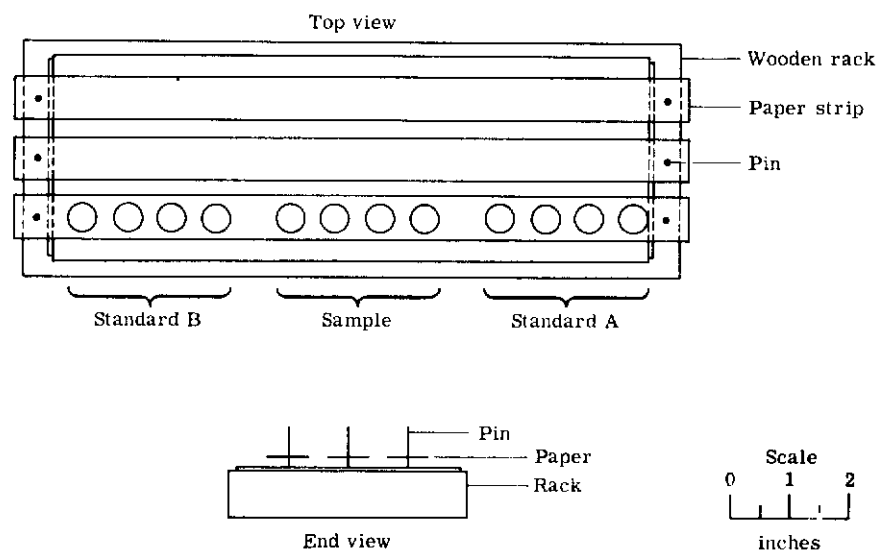


Figure 12. Drawing of racks for holding paper strips in spot amino acid analysis.

Contrails

(4) Repeat the washing procedure before filling the burette with the sample. Draw up enough of the sample supernatant so that 4 "spots" of 5λ each can be applied to the central area of the paper strip.

(5) Place the strip-laden racks in a 37° C drying oven for 15 minutes.

(6) Remove the racks from the oven and apply the ninhydrin solution by brush (3/4-inch camel hair) to each strip. The strips should be thoroughly and evenly saturated with the solution.

(7) Place the racks in an oven controlled at 45° C and $30 \pm 5\%$ humidity for 30 minutes to fully develop the color reaction.

(8) Within 10 minutes of this time, the linear projection of the relative optical density of the "spots" is traced by the densitometer coupled to the recording galvanometer.

(9) The standard and sample "spots" can be applied to the paper strips with Kirk ultramicropipettes with an accuracy comparable to that obtained with the ultramicroburette.

6. Calculation

a. Draw the baseline for all "spots" on the recorded strip.

b. Measure the heights from peak to baseline for all spots.

An average of the 4 "spots" of each set of standards and of the sample is taken.

c. Then, by the method of intercepts:

$$\text{mg\% NH}_2\text{N} = \frac{\text{RU} - \text{I}}{\text{RS} - \text{I}} \times \text{conc. of standard (1.57 or 0.78)} \times \text{dilution (2.5)}$$

where:

RU = height of sample in mm,

RS = height of standard in mm, and

I = intercept.

The intercept is obtained by the following calculations:

$$\begin{aligned} &(\text{ht. in mm of alanine standard "A"}) - \\ &(\text{ht. in mm of alanine standard "B"}) = K, \text{ a constant.} \end{aligned}$$

Then

$$(\text{ht. in mm of alanine standard "B"}) - K = \text{I.}$$

Under circumstances when maximum precision is not required (e.g., only relative values are necessary) results can be obtained by the calibration curve method:

mg% $\text{NH}_2\text{N} =$

$$\frac{\text{reading of unknown}}{\text{reading of standard}} \times \text{concentration of standard} \times \text{dilution.}$$

In this method, the intercept is not subtracted from sample and standard values, but rather the sample and standard values are taken as a ratio of one to the other (Fig. 13).

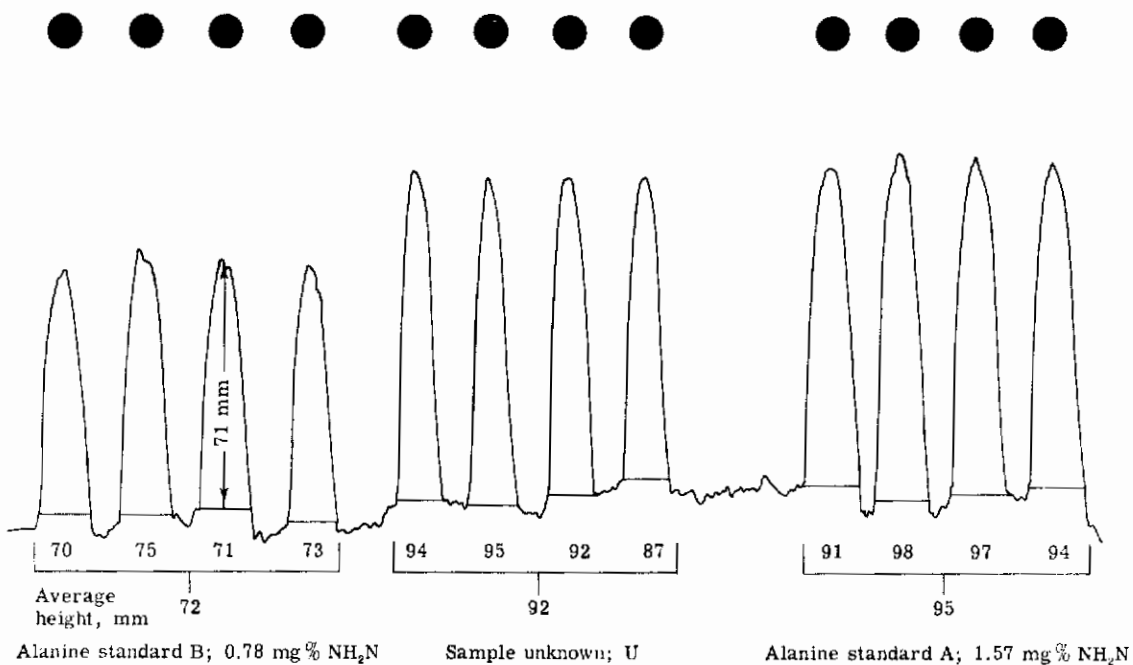


Figure 13. Calculation of plasma amino nitrogen determined by the "spot" method. Example: $K = \text{standard A} - \text{standard B} = (95 - 72) = 23$; $I = \text{standard B} - K = (72 - 23) = 49$; $\text{PAN mg\%} = (\text{RU} - I) / (\text{RS} - I) \times \text{standard A} \times \text{dilution} = (92 - 49) / (95 - 49) \times 1.57 \times 2.5 = 3.7$.

D. Paper Chromatographic Analysis of Amino Acids in Body Fluids

The procedure to be described here is a single-dimension technique based on the ascending principle which in our experience affords maximum ease of operation. We have found that for most purposes this method provides a rapid means of obtaining satisfactory results. However, when the identification of unusual components is required, a two-dimensional system is recommended.

1. Reagents Required

a. Butanol-Acetic Acid-Water Solvent. To 120 ml of n-butyl alcohol in a mixing cylinder, add 30 ml of glacial acetic acid and 50 ml of distilled water. After thorough mixing, allow the solvent to stand for 24-48 hours before use. Multiples of this solvent should be made fresh weekly or as required within each week.

b. Ninhydrin. A 5% solution dissolved in 95% ethyl alcohol. This solution must be clear and prepared daily.

2. Materials and Apparatus

a. Schleicher and Schuell No. 507 filter paper cut to strip size of 1.8 X 29 cm by supplier.

b. A supply of Sahli-type hemoglobinometer pipettes to contain 30 λ of sample.

c. Several rectangular No. 19 museum jars (31 X 29 X 11 cm) with lids (Standard Scientific Supply Corp. No. 70600).

d. Parafilm M for sealing the museum jars.

e. A cabinet or chamber to house the museum jars with constant conditions of temperature, 80° F, and humidity, 70%.

f. Frames for holding the paper strips. These are constructed of glass rod 8 mm in diameter (Fig. 14).

g. Teflon clamps supplied by Kensington Scientific Corp., Berkeley, California.

h. Drying pans.

3. Procedure

a. The freshly prepared solvent (600 ml) is poured into the museum jar. The solvent level should be 2 cm. This is covered with parafilm and a lid and placed in the chromatography chamber overnight before use.

b. Collection and preparation of sample. The sample is treated exactly as described in the ultramicro procedure for the determination of plasma amino nitrogen (Method C).

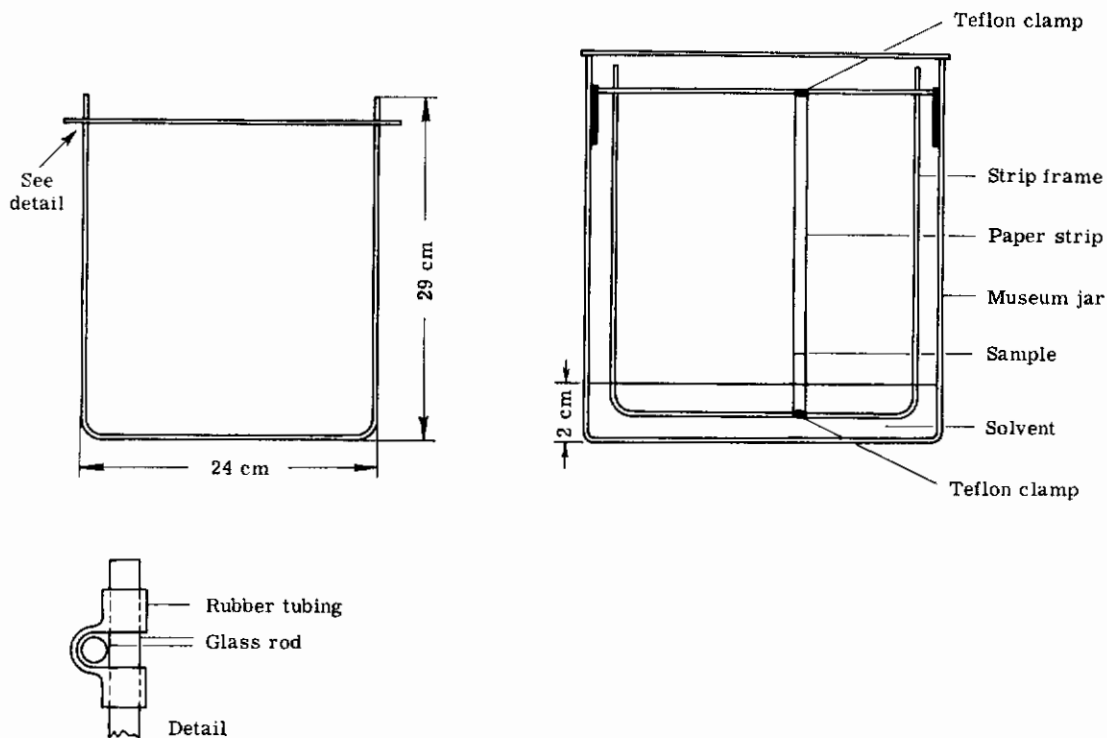


Figure 14. Drawing of glass frames and chromatography vessel.

c. Each pipette is filled with 30λ of supernatant fluid.

d. The paper strips are held in place on the glass frame by means of teflon clamps. Identifying notations are made within 2 cm of the top of the paper strip, and a pencil line, $1\frac{1}{2}$ inches from the bottom of the strip, is drawn lightly as a guide. Each frame will accommodate a maximum of six paper strips.

e. The tip of the pipette is drawn across the pencil guide line, and ten to twelve applications usually suffice to empty the pipette completely. To avoid diffuse bands, the sample should be applied in such a manner that the width of the streak is no more than 3-4 mm.

f. The sample portions should be allowed to dry between applications. A student's gooseneck lamp (60-watt bulb) placed 6 inches above the line of application serves to hasten the drying.

g. The strip-laden glass frames are now set into the partitioning chamber (museum jar) so that the sample line is at least 1 cm above the solvent. The jar is covered with parafilm and the lid set in place to produce a tight seal.

h. With suitable samples and stated temperature-humidity conditions, the solvent will rise to about 20 cm above the fluid level in 18-20 hours (overnight), and a good separation of amino acids is accomplished.

i. At this time, the strip-laden frames are removed from the partitioning chamber and hung in an exhaust hood.

j. The height of the solvent level is marked with pencil, and excess solvent from the bottoms of the chromatograms is dried by careful blotting with paper toweling.

k. The strips are allowed to dry in a moderate flow of air for 1 hour.

l. After drying, the chromatograms are treated with ninhydrin solution by painting with a 3/4-inch camel's-hair brush.

m. The color is developed in a 37°C oven for 1 hour. The relative optical density of the bands is traced by the densitometer coupled to the recording galvanometer.

4. Results

The position of a substance on a chromatogram is specified by its " R_f " value which can be defined as follows:

$$R_f = \frac{\text{distance in cm solvent travels from the origin}}{\text{distance in cm solvent front travels from the origin}}$$

The R_f value is reproducible when chromatographic procedures, conditions, and apparatus are carefully standardized. However, it is quite common for published R_f values to vary. These variations are due to a number of causes, a few of which are: temperature, humidity, grade of paper, existence of polyionic forms, substances with the same R values, "salt" effect, choice and application of location reagent, and length of the solvent flow.

After many trials, we selected the BuA solvent because it has the singular property of "compacting" the bands so that little or no tailing occurs during chromatography. Also, "clean" chromatograms are obtained with this solvent because it is relatively insensitive to

environmental changes. In our experience we have found that phenolic solvents leave residues and have a deleterious effect on some amino acids, especially tryptophan. Ethanol-ammonia and methanol-pyridine are quite volatile, while tert-butanol-methyl ethyl ketone requires special treatment if ninhydrin is to be used after this solvent. Lutidine is not recommended for general use.

In practice, it will also be found that the R_f values vary with concentration of total N content of the sample and distribution of amino acids. These variations always involve a shift in the R_f spectrum upward or downward as a whole group, but seldom a change in spatial sequence for any individual amino acid. After some experience, the correction for these shifts presents no serious problems.

To secure reference guides for nutritional studies, 2 γ quantities of individual L-amino acids were added to 30 λ of pooled plasma filtrate. These samples were chromatographed as described.

The R_f values we have obtained using the BuA solvent are well in accord with those reported by Smith (ref. 97). A graphic representation of this data is shown in Fig. 15.

Certain amino acids, notably lysine and cystine, are detected and easily identified in this one-dimensional system. Others, however, have proximate R_f 's and the need for further separation, e.g., two-dimensional chromatography, is indicated. Accordingly, we have designated the area which includes glycine, aspartic and glutamic acids, hydroxyproline, threonine, and alanine as Zone I; the area comprised of tryptophan, methionine, and valine as Zone II; and the area which includes phenylalanine and the leucines as Zone III. The resolution of each of these areas is under intensive investigation in our laboratory. (Fig. 16, 17, 18, 19).

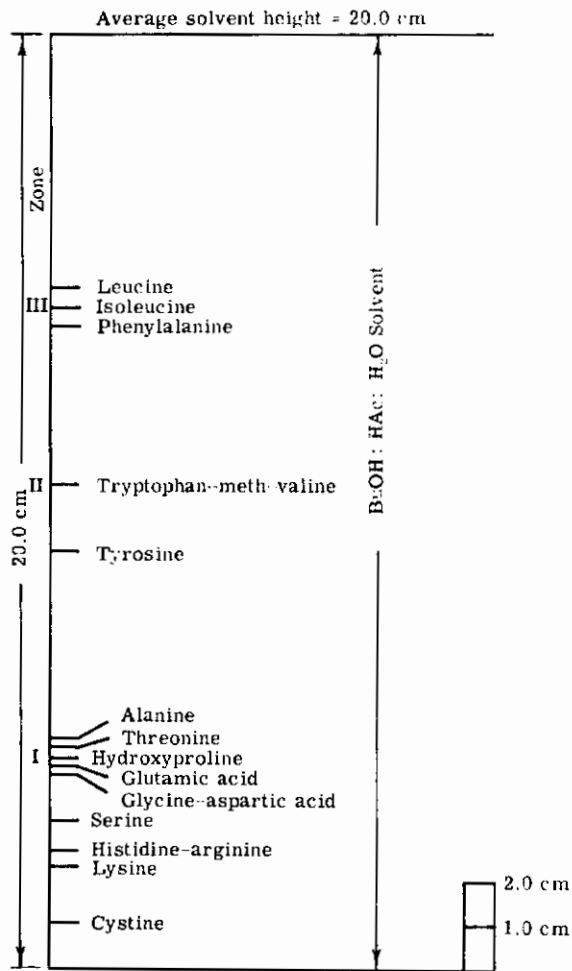


Figure 15. Spatial relationship of amino acids for whole blood filtrates.

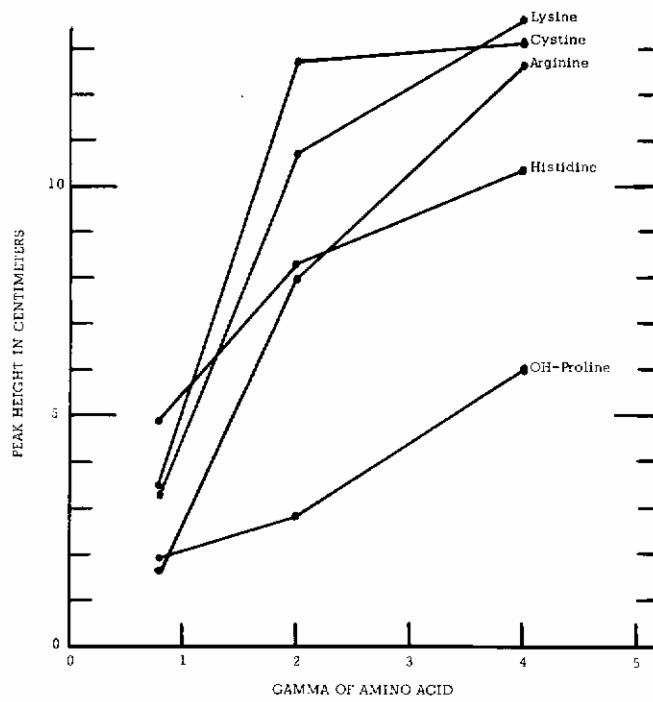


Figure 16. Calibration curves of some amino acids for the ninhydrin reaction.

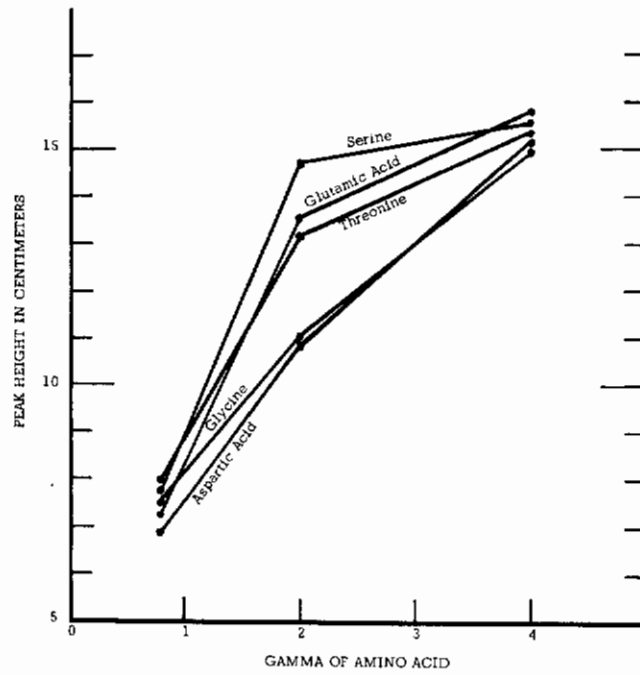


Figure 17. Calibration curves of some amino acids for the ninhydrin reaction. (Continued)

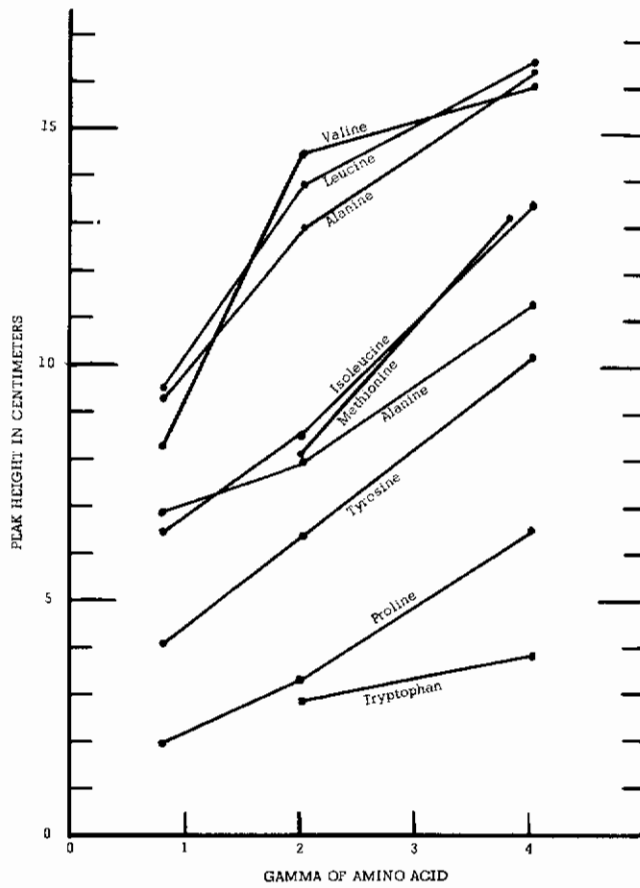


Figure 18. Calibration curves of some amino acids for the ninhydrin reaction. (Continued)

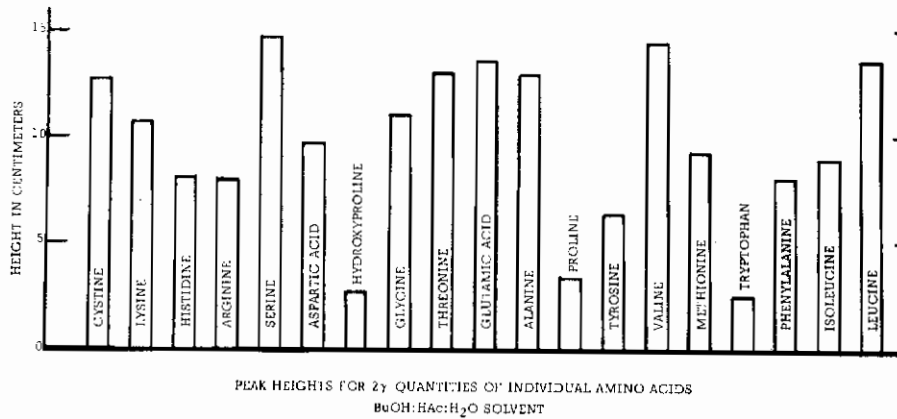


Figure 19. A comparison of the color index of various amino acids when reacted with ninhydrin in 95% ethanol.

E. Quantitative Chromatographic Determination of Lysine and Other Amino Acids in Biological Products.

1. Principle

The amino acids are separated rapidly by stationary circular chromatography. The position and quantity of individual amino acids are determined by means of internal standards.

2. Reagents Required

- a. Lysine Stock Standard, 1 mg/ml. Dissolve 125 mg of 95% L-lysine as the monohydrochloride in 100 ml of distilled water.
- b. Working Standard. The stock solution is diluted 1:5 with distilled water; this solution is further diluted 2:1 with distilled water to obtain a working standard of 0.6 γ /4 λ .
- c. Butanol-Acetic Acid-Water Solvent and Ninhydrin Solution are prepared as previously described (Method D).

3. Materials and Apparatus

- a. Whatman Filter Paper No. 4, diameter 24.0 cm.
- b. Aluminum pie plate with transparent plastic clamp-on domed cover. This plate is manufactured for household use as a pie carrier (No. 2349C) by The Enterprise Aluminum Co., Massillon, Ohio.
- c. Reservoir for solvent. A Pyrex glass Conway diffusion dish (Standard Scientific Supply Corp., No. 52470) is used as a solvent reservoir.
- d. A supply of 4 λ Kirk ultramicropipettes.
- e. A supply of 30 λ Sahli-type hemoglobinometer pipettes.
- f. Oven thermostated at 37°C.
- g. Densitometer coupled to a recording galvanometer as noted previously (Method C).

4. Procedure

- a. Collection and Preparation of Sample. Exactly 0.2 ml of blood is collected by fingerprick and added to a 1-ml centrifuge tube containing 0.30 ml of redistilled acetone. The sample is then treated exactly as has already been cited for the ultramicro determination of plasma amino nitrogen (Method C).

Contrails

b. Preparation of Filter Papers. (1) The circular paper is marked off lightly with pencil into quadrants. The paper is slit along the pencil lines, cutting from the center to within 6 cm of the edge.

(2) The point of application of the sample is marked lightly with pencil at the midpoint and 1 inch from the apex of each quadrant. Necessary notations are made along the quadrant lines.

c. Application of Sample and Internal Standard. (1) Four 30 λ aliquots of supernatant are drawn into the Sahli pipettes. Each of these aliquots is dispensed sequentially in drops onto its respective quadrant. The drops are allowed to dry between applications so that the final diameter of the spot will not exceed 1 cm.

(2) To the spots of a pair on opposite quadrants, 4 λ of the working standard are applied from the Kirk pipette as 1 drop. Therefore, the spots of two quadrants will contain 30 λ of sample supernatant, and the spots of the other two quadrants will contain 30 λ of sample supernatant plus 4 λ of the lysine standard.

d. Resolution Process. (1) The solvent reservoir is filled with 15 ml of the BuA mixture and placed in the center of the plate.

(2) The four apices of the disc are curled under. The disc is then laid over the plate, and the four apices drop into the trough, so that contact is made with the solvent.

(3) The cover is clamped on and the resolution is accomplished in 2 hours at room temperature.

(4) After 2 hours, the paper discs are removed from the chamber, suspended on drying racks, and air-dried in a hood for 20-30 minutes.

(5) The disc is painted with ninhydrin, using a 3/4-inch camel's-hair brush, and dried for 1 hour in a 37°C oven.

(6) The outside edges of each quadrant are cut away so as to leave a center strip 1 inch wide. These strips are read in a recording densitometer.

5. Calculation

plasma lysine, mg% =

$$\frac{B}{C} \times 0.6 (\gamma \text{ lysine in internal standard}) \times 2.5 (\text{dilution})$$

where

A = peak height (cm) of plasma filtrate (30 λ) + 0.6 γ of lysine,

B = peak height (cm) of plasma filtrate (30 λ), and

C = A - B.

6. Results and Comments

Numerous measurements of plasma free lysine have been made by this procedure. Typical results are shown in Fig. 20, and indicate a linear relationship between lysine added ($0.2-0.8\gamma$) and peak heights.

Recovery of added lysine by circular paper chromatography yielded highly reproducible results for all amounts of lysine added to a given plasma sample. Preliminary tests indicate that this procedure will yield satisfactory results with threonine, methionine, tryptophan, valine, phenylalanine, isoleucine, and leucine. (Fig. 21, 22, 23)

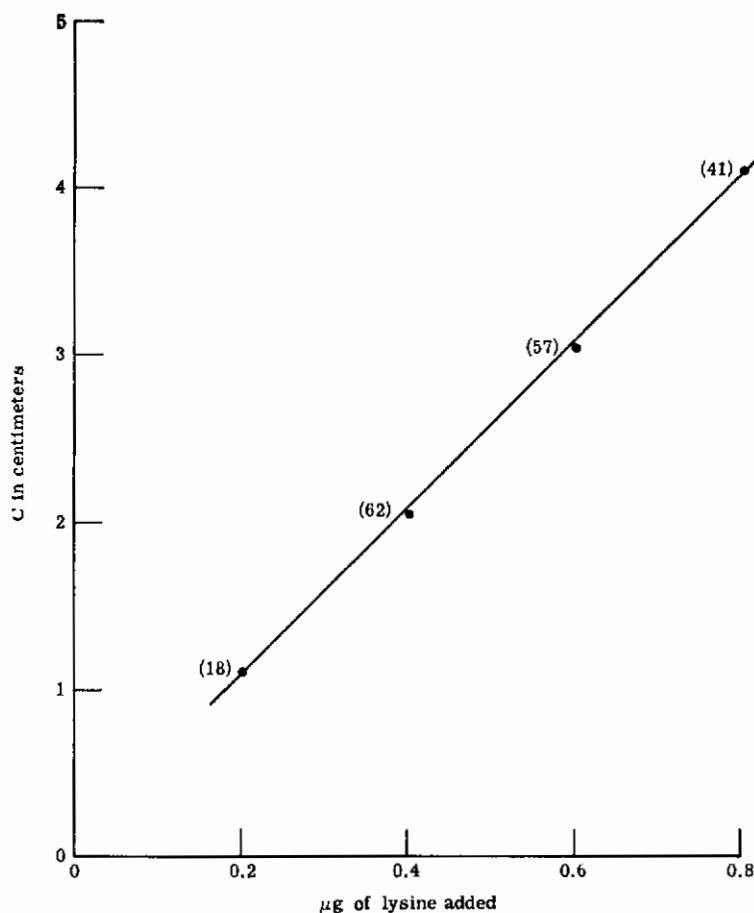


Figure 20. Recovery of lysine added to plasma filtrates. C in centimeters equals peak height of filtrate (30λ) with added lysine minus peak height of filtrate (30λ). Figures in parentheses represent number of tests done.

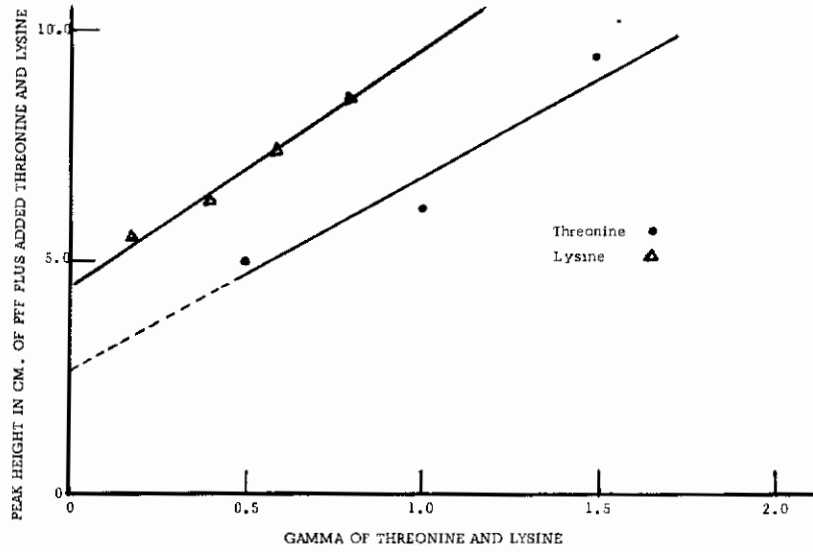


Figure 21. Calibration curve for threonine and lysine added to 30 cubic mm of protein free filtrate (PFF).

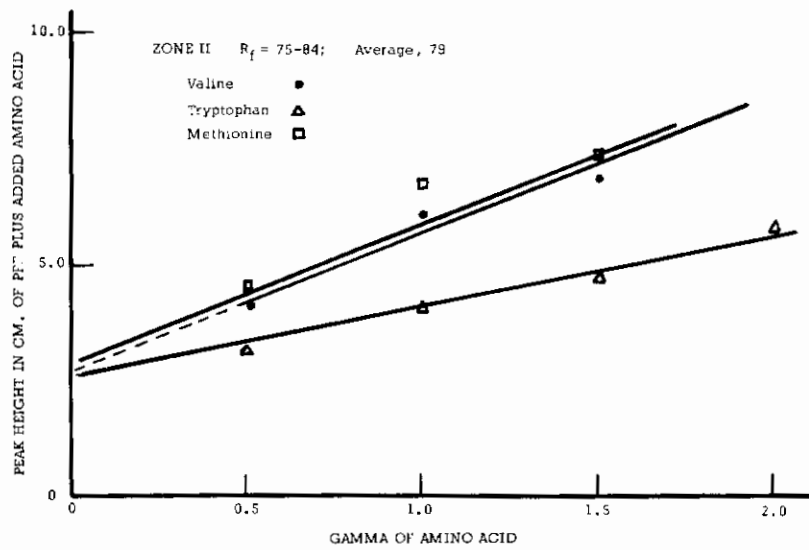


Figure 22. Calibration curve for valine, tryptophan, and methionine (Zone II) added to 30 cubic mm of protein free filtrate (PFF).

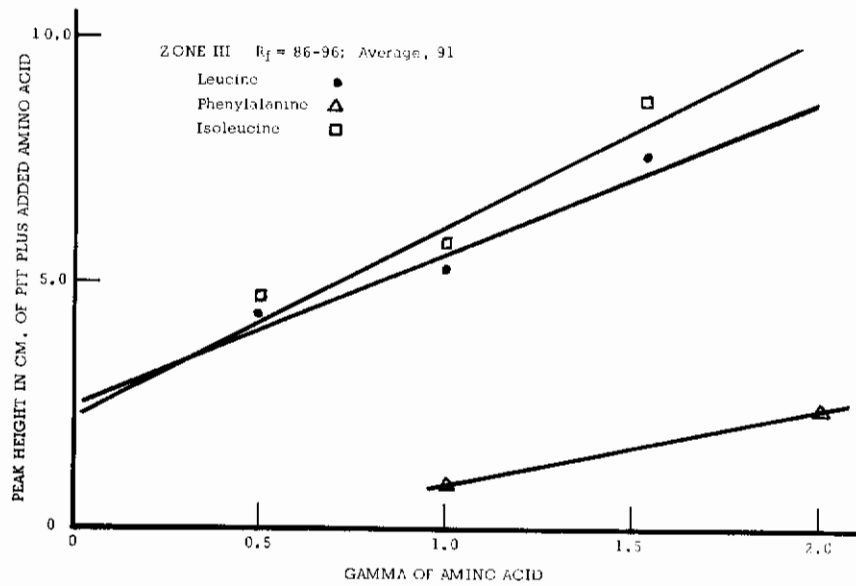


Figure 23. Calibration curve of leucine, phenylalanine, and isoleucine (Zone III) added to 30 cubic mm of protein free filtrate (PFF).

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