ASSIMILATION OF N^{15}-HISTIDINE IN AMINO ACID IMBALANCE

by

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V. INTRODUCTION

Background

The general concepts of amino acid balance and imbalance will be discussed briefly in this section. A limited number of references have been cited since recent articles (12, 22) have reviewed this area quite thoroughly.

Amino acid balance refers to the relative proportion of amino acids in a protein or diet. The term is most frequently used in reference to the indispensable amino acids; however, the dispensable amino acids must not be overlooked. In recent years, it has been generally accepted that after correcting for digestibility the closer the proportion of indispensable amino acids in the diet corresponds to the amino acid requirements, the greater will be the efficiency with which the protein will be used. The ratio of dispensable to indispensable amino acids in a protein will also effect its efficiency of utilization. Better growth will be attained and higher Protein Efficiency Ratios, Biological Values, Net Protein Utilizations and Chemical Scores obtained when the correct balance of amino acids is absorbed.

The concept of amino acid balance has particular significance since:

> "....first, all of the amino acids needed by higher organisms are obtained together from the proteins of foodstuffs; second, except for minor quantities required for special purposes, all amino acids are

used concommitantly for the synthesis of tissue proteins; third, and most important, there is essentially no storage of amino acids in the body." (22).

Therefore, the body can't compensate for slight amino acid deficiencies even for a short time, and the animal is very sensitive to alterations in the amino acid patterns in the diet.

Harper (22) proposed that: "...the term 'imbalance' be applied to those changes in the amino acid pattern of a diet that cause a growth depression which is completely prevented by a small supplement of the limiting amino acid or acids." There are two methods by which amino acid imbalances can be produced. One involves the addition of a quantity of protein or amino acid mixture lacking in one indispensable amino acid to a diet containing an amount of protein that just satisfies the requirement for maximum growth. Imbalance produced by this method gives more severe and predictable results. The second method involves the addition of a small amount of amino acid or acids to a diet that is low in protein. The amino acid added is usually the one that is second most limiting for growth. This type of imbalance is usually less severe and is influenced more by small changes in diet and environmental conditions. The first method has been used in this investigation.

Although the control and imbalance diets may contain the same amount of the limiting amino acid, the imbalance fed rats grow less rapidly. Harper et al. (12) have postulated that the mixture of amino acids added to the diet to create the imbalance cannot be used for protein synthesis since it is deficient in one indispensable amino acid. This supplement is thought to increase the requirement for the limiting amino acid; and therefore, depress growth below the level observed before the amino acid mixture was added.

Literature Review

In the last decade a number of reviews (10, 11, 12, 23, 25) have dealt with the topic of amino acid imbalance. Most notable are two by Harper's group (12, 22). Since these reviews were quite thorough, only the main points pertaining to this investigation will be discussed in detail.

From casual observation it was noted that food intake depression occurred in 24-48 hours (12). However, Kumta and Harper (20) by measuring food intake at short intervals, noted that the depression occurred in from three to eight hours (20). They also found that the plasma content of the most limiting amino acid in the diet was lowered within one hour after feeding the imbalanced diet (20). The plasma amino acid pattern tended to reflect the indispensable amino acid pattern in the diet, i.e., the plasma concentration of the amino acids in the mixture added, which Harper et al. (12)

hypothesized as not being used extensively for protein synthesis, were high. An amino acid deficient diet has produced similar plasma amino acid alterations (20, 22).

In experiments using imbalanced amino acid mixtures where force-feeding and hormone injection have been used to stimulate food intake, growth depression was not evident (2, 19). It could be said that the depression of growth was caused by the reduced food intake since the imbalanced diet was adequate for growth. Then, what causes the reduction in food intake? Harper (22) discussed the possibility of reduced palatability and refuted it as an unlikely cause of the reduced food intake. Rose (24) has pointed out that "growing animals lose the desire to eat when the food is not suitable for tissue synthesis but regain it when all the components for anabolism are made available." In addition, Mellinkoff (23) has implicated changes in the blood amino acid pattern and concentration in appetite regulation. The time sequence of appetite loss and plasma amino acid pattern changes noted by Kumta and Harper (20) tend to support the idea of Mellinkoff.

Also, it has been shown that rats offered a choice between an imbalanced diet that would support growth and a protein-free diet preferred the protein-free diet over the imbalanced diet in a few days (i.e., when the amino acid pattern of the blood became sufficiently altered), so that in the case of rats fed an imbalanced amino acid mixture there is a reduction or regulation of food intake even though the diet is adequate for growth.

If we assume that the blood amino acid patterns and concentrations, or these factors in a tissue closely associated with the blood, are responsible for the "false" appetite regulation; the problem involves the determination of the cause of the changes in the blood amino acid patterns and concentrations.

Harper et al. (12, 22) have reviewed the possible causes of depressed blood levels of the limiting amino acid (or depressed food intake) in the imbalance phenomenon. Kumta and Harper (19) found no delay in stomach emptying due to amino acid imbalance. A depressed rate of protein digestion due to amino acid imbalance was not a factor since the phenomenon has been demonstrated using diets containing crystalline amino acids as the only source of nitrogen (13).

An increased rate of catabolism of all amino acids was suggested by Salmon (25) as a possible cause of the growth depression (i.e., depressed food intake or blood levels of the limiting amino acid) due to amino acid imbalances. A number of workers (2, 5, 8, 21) have determined the efficiency of utilization of nitrogen (i.e., the efficiency of utilization of the limiting amino acid) in the imbalance situation. In experiments on rats (21) and chicks (8) no evidence for reduced efficiency of utilization of nitrogen was found. Some evidence for reduced efficiency of utilization of nitrogen was found in experiments in rats (5) and dogs (2). Florentino and Pearson (9)

and Wilson et al. (31) studied the metabolism of isotopically-labeled tryptophan in rats fed a diet low in protein and lacking niacin to which threonine had been added to create an imbalance. Florentino and Pearson (9) obtained results supporting Salmon's hypothesis (25) while Wilson et al. found no evidence for increased catabolism of the limiting amino acid.

Since growth depression is primarily due to reduced food intake (12, 19, 22, 32), it is difficult to determine from the above experiments whether the changes in amino acid metabolism (or changes in the efficiency of nitrogen utilization)were due to amino acid imbalance or the reduced food intake. The more direct evidence from isotope experiments (12, 32) from experiments on rats treated with insulin to stimulate their food intake (19), from experiments on rats exposed to a cold environment (16), and from force-feeding experiments (19) does not support the hypothesis of reduced efficiency of utilization of the limiting amino acid (i.e., of reduced efficiency of utilization of nitrogen).

Harper (22) has discussed the possibility of delayed amino acid absorption and transport as a cause of the adverse effects due to amino acid imbalance. He points out specific cases where amino acid imbalances can be caused and then corrected by the addition of one amino acid and then the other which are known not to compete for absorption. Harper (22) also points out that the absorptive capacity of the intestine is more than adequate to cope with the

additional amino acids that cause the imbalance. Although more work is needed in this area, Harper (22) postulated that reduced absorption and transport of the limiting amino acid is probably not the cause of the amino acid imbalance phenomenon.

The above discussion points to the possibility that delayed absorption and transport, and increased excretion and catabolism of the limiting amino acid are not among the causes of the adverse effects evident in amino acid imbalance.

According to Yoshida et al. (32): "The initial metabolic effects of an amino acid imbalance should be detectable before, or at least at the same time as, the food intake of the experimental animals is affected, hence, experiments of relatively short duration appear to be needed to resolve the dilemma posed by these disparate results."

Although Kumta and Harper (19) could not demonstrate the inhibition of tryptophan pyrolase synthesis when an imbalance was induced, there is no question about total protein synthesis being depressed by an amino acid imbalance, because the growth rate is depressed. However, this is probably a secondary effect to lowered food intake and plasma levels of the limiting amino acid. Other possible causes of the imbalance phenomenon (i.e., reduced food intake and blood levels of the limiting amino acid) include increased

incorporation of the limiting amino acid into protein as suggested by Koeppe and Henderson (17) or a decrease in tissue protein breakdown.

At this point a summary can be set forth concerning the events of amino acid imbalance. As stated above, reduced food intake is responsible for the growth depression due to amino acid imbalance. The reduced blood level of the limiting amino acid probably controls the food intake, and ultimately, causes the growth depression. Of the possible causes of the reduced blood level of the limiting amino acid, two (increased excretion and catabolism and delayed absorption and transport of the limiting amino acid) are probably not responsible and two (increased incorporation of the limiting amino acid into protein and decreased tissue protein breakdown) remain open to question.

In view of this evidence, Harper et al. (12) set forth the hypothesis that the limiting amino acid was incorporated into protein at an increased rate during the first stages of the imbalance phenomenon. This increased incorporation of the limiting amino acid into protein caused reduced blood levels of the limiting amino acid which in turn triggered the appetite control center which reduced the animal's food consumption and depressed growth. Evidence pertinent to the first part of Harper's hypothesis concerning the rate of incorporation of the limiting amino acid into tissue protein has been obtained by this laboratory as well as by Harper's (32).

Results from preliminary isotope studies (12) and from recent extensive studies (32) on the fate of the limiting amino acid suggest that the rate of incorporation of the limiting amino acid into protein was increased, and that the rate of absorption and transport, and the rate of excretion and catabolism of the limiting amino acid were not altered in the imbalance situation. In these experiments (12, 32) imbalances were created in rats by adding a mixture of amino acids lacking either threonine or histidine to low casein diets. Threonine-U-C^{14} and histidine-U-C^{14} were used to follow the metabolic fate of the limiting amino acid. Harper et al. (12) and Yoshida et al. (32) both found reduced $C^{14}O_2$ expiration in rats receiving the imbalanced diets, confirming that there was no increased catabolism of the limiting amino acid. Also, liver glycogen and lipids did not exhibit significant differences between the control and imbalance fed rats with respect to radioactivity (32). Data on C^{14} excretion indicated that there was no increase in excretion of the limiting amino acid in rats fed the imbalanced diet (12, 32).

There was increased incorporation of C^{14} into the carcasses of the rats fed the imbalanced diet in 48 hours, indicating an increased rate of protein synthesis in the imbalance situation. Detailed investigations into the amount of C^{14} found in the acid-soluble and protein fractions of various tissues (32) indicated that the rate of incorporation of the limiting amino acid into protein was significantly

increased for liver tissue and not altered in the other tissues tested (kidney, muscle, and gastrointestinal tract). Also, a reduced amount of radioactivity was found in the acid soluble fraction of the blood of rats fed the imbalanced diet, as would be expected.

Data on the absorption of the limiting amino acid were obtained by washing the gastrointestinal tract and determining the amount of radioactivity in its contents. At 3.5 hours and 8.0 hours the rats fed the imbalanced diet absorbed less C^{14} than the rats fed the control diets, however, the differences were not significant.

Although the first part of Harper's hypothesis appears to be correct (the possibility of an increased rate of tissue protein breakdown remains to be tested), more evidence needs to be obtained to determine whether the reduced blood level of the limiting amino acid actually causes the reduced food intake.

Objectives

In the investigation that follows nitrogen-15 was used as a tracer in a short-term experiment to determine whether there were changes in the concentration of L-N^{15}-histidine in the acid soluble and protein fractions of liver, kidney, muscle, gastrointestinal tract, and serum that could be attributed to ingestion of an imbalanced diet. In the same experiment, nitrogen-15 concentrations were followed in the urine, lower gastrointestinal contents and feces, and in the upper gastrointestinal contents for rats fed an imbalanced or corrected diet with reference to histidine.

By testing these tissues and samples for nitrogen-15 content, it was hoped that some insight into the cause of the reduced blood levels of the limiting amino acid might be gained. The nitrogen-15 data proposed above would indicate whether the limiting amino acid was incorporated into protein at an increased rate, whether it was catabolized at an increased rate, whether it was adsorbed and transported at a reduced rate or whether there might be a combination of these factors involved in the imbalanced situation.

VI. EXPERIMENTAL PROCEDURES

N^{15}-Histidine Isolation

Escherichia coli, strain K-12 (American Type Culture Collection No. 10798), was grown in a defined medium using N^{15}-enriched ammonium sulfate as the only source of nitrogen. The medium was prepared according to Ku, et al. (18) using 20 µgm. of ammonia nitrogen per ml. of culture. Ku's data indicated that this amount of nitrogen would support maximum growth with high utilization of the nitrogen source. The culture was maintained on nutrient agar (28) at 37° C. with transfers made once every three months.

N^{15}-enriched medium was prepared in autoclavable five gallon plastic bottles and autoclaved for one hour at 121° C. and 15 lb./in.^{2}. The sterile medium was inoculated with 1 ml. of E. coli from a pre-culture at approximately 0.1 absorbancy units (λ = 615 mµ). The culture was incubated at 37° C. with agitation by a magnetic stirrer.

The culture was stored in the cold room (40° F.) for twenty-four hours after reaching the decelerating growth phase. A Servall centrifuge with four one-liter buckets (2500 x G) was used to harvest the first batch of cells; for the second batch, a Sharples continuous flow centrifuge (62,000 x G) was used. Cell yield was determined on a wet weight basis.

Two batches of cells were grown; for the first batch commercial N^{15}-enriched ammonium salts were used. The second batch was grown on N^{15}-ammonium sulfate recovered from the waste of the first run. In

the first run, total ammonia-nitrogen was determined on the culture supernatant, and ammonia-nitrogen utilization was calculated using the original ammonia-nitrogen content (20 μgm NH_3-N/ml). In the second run, total nitrogen was determined on the medium after autoclaving, on the culture after refrigeration and on the culture supernatant. This permitted the calculation of nitrogen recovery and overall nitrogen utilization.

The kjeldahl procedure was as follows:

The digestion was carried out in 125 ml. round botton flasks with ground glass joints to fit the microkjeldahl distillation apparatus. A sample containing approximately 1 mgm. of nitrogen and 20 ml. of digestion mixture (14) was heated in the flask on a heating mantel until the solution became clear.

After cooling, 40 ml. of distilled water was added to the flask. The flask was then placed on the microkjeldahl distillation apparatus; 20 ml. of 40% sodium hydroxide was added; and the sample was distilled into 10 ml. of distilled water containing 1 ml. of 0.072 N sulfuric acid and 0.5 ml. bromcresol purple (0.05 gm. in 250 ml. ethanol).

The distillate was titrated with 0.005 N sodium hydroxide and the amount of nitrogen in the sample was calculated.

The cells were hydrolyzed for twenty-four hours in refluxing 6 N hydrochloric acid using 100 ml. of 6 N hydrochloric acid per gram (wet weight) of cells. The total volume and nitrogen content of the hydrolysate

was determined. The total hydrolysate was dried several times on a flash evaporator to remove excess hydrochloric acid. The concentrate was adjusted to pH 2.2 and filtered.

The hydrolysate was then placed on a cation exchange column and eluted with pH 5.28 citrate buffer (29) without detergent or mold inhibitor. The column, 58 cm. long and 7.6 cm. in diameter, contained five pounds of Bio-Rad Analytical Grade Cation Exchange Resin AG 50W-X4 (100 - 200 mesh). Fractions of 200 to 250 ml. were collected using a fraction collector. Each fraction was monitored using the ninhydrin test. For the ninhydrin test, 0.5 ml. of sample, 1.5 ml. of distilled water, and 0.5 ml. of ninhydrin reagent (29) were heated in a boiling water bath for 10 minutes. After cooling, 3.5 ml. of isopropanol was added, and absorbancy was determined at λ = 570 mμ in a Spectronic 20 colorimeter. On the second run, a Uviscan unit was also used for monitoring the fractions.

Histidine fractions were pooled and concentrated on the flash evaporator; other fractions were also pooled according to the results of the ninhydrin test, concentrated and subjected to kjeldahl (1) digestion (first run) to recover the N^{15} for further use. On the second column, fractions were pooled on the basis of the ultraviolet scanning as well as on the basis of the ninhydrin tests. The amount of nitrogen was determined in each pool of fractions. As a result, we have more complete data on the nitrogen distribution in the fractions

eluted from the second cation exchange column. Histidine determinations using the Beckman-Spinco Automatic Amino Acid Analyzer (29) were obtained to determine recovery of histidine from the column.

The concentrated buffer-histidine solutions from the two columns were combined, adjusted to pH 2.2 with concentrated hydrochloric acid and rerun through the cation exchange column for further purification. The ninhydrin test and ultraviolet absorption were also used to monitor these column fractions. The histidine fractions were pooled and concentrated on the flash evaporator. This concentrated buffer-histidine solution was adjusted to pH <1 with concentrated hydrochloric acid; and ethyl acetate extractions, 80% ethanol precipitations, and an ion retardation column (2 lb. Bio-Rad Analytical Grade Ion Retardation Resin AG 11 A8, 50-100 mesh in a 7.6 cm. x 21 cm. column) were used to remove citric acid and sodium chloride, respectively, according to the following scheme:

1. Three 400 ml. aliquotes were each extracted seven times using 200 ml. of ethyl acetate each time. The water fractions were concentrated several times on a flash evaporator to remove the ethyl acetate.

2. The solution was made 80% (v/v) with ethanol; the supernatant and two 80% (v/v) ethanol washes of the precipitate were concentrated several times on a flash evaporator to remove the ethanol.

3. The solution was made 80% (v/v) with ethanol; the supernatant and two 80% (v/v) ethanol washes of the precipitate were concentrated several times on a flash evaporator to remove the ethanol.

4. 500 ml. of concentrated buffer-histidine solution was extracted 20 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator to remove the ethyl acetate.

5. 450 ml. of solution was extracted 18 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

6. The solution was made 80% (v/v) with ethanol; the supernatant and two 80% (v/v) ethanol washes of the precipitate were concentrated several times on a flash evaporator.

7. 250 ml. of solution was extracted 12 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

8. The solution was made 80% (v/v) with ethanol. Testing of the precipitate indicated that some histidine was present so the precipitate was dissolved in distilled water and combined with the supernatant to be concentrated on a flash evaporator.

9. Another 80% (v/v) ethanol precipitation of the sodium chloride was attempted, but histidine came down again so this procedure was discontinued.

10. 200 ml. of solution was extracted 15 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

11. 200 ml. of solution was extracted 15 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

12. 175 ml. of solution was extracted 15 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

13. 175 ml. of solution was extracted 15 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

14. 175 ml. of solution was extracted 10 times using 200 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

15. 150 ml. of solution was extracted 10 times using 200 ml. ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

16. Three 50 ml. aliquotes were each eluted from the above ion retardation column in 20 ml. fractions with ion exchanged distilled water. The fractions were monitored for histidine with ninhydrin and for the chloride ion with 0.5 M silver nitrate (0.1 ml. sample, 1 drop of silver nitrate and 7 ml. of distilled water with the absorbancy determined at λ = 570 μ. in Spectronic 20). The fractions from each column containing histidine were all pooled and concentrated on a flash evaporator.

17. The above solution was chromatographed on the ion retardation column again using the same procedures as in 16.

18. 125 ml. solution was extracted 20 times using 100 ml. of ethyl acetate each time. The water fraction was concentrated several times on a flash evaporator.

19. The solution was run through the ion retardation column twice using the procedures given in 16.

20. The entire sample (100 ml.) was added to 3000 ml. of cold absolute ethanol and allowed to cool 4 hours in the freezer 0° C. The precipitate was isolated by centrifugation and found to contain half of the total histidine.

21. The supernatant from 20 was concentrated several times on a flash evaporator to remove the excess ethanol. The 60 ml. solution was extracted 20 times with 125 ml. ethyl acetate washes and the water fraction concentrated several times on a flash evaporator.

22. The precipitate from 20 and the solution from 21 were combined, made basic with 40% sodium hydroxide and taken to dryness several times to remove the ammonia. Then the pH was adjusted to 2.2 with concentrated hydrochloric acid.

23. The solution was then chromatographed on the ion retardation column as in 16.

24. Total histidine and total nitrogen were determined to find out if all the nitrogen could be accounted for in histidine.

In the above procedures, all ethyl acetate washes and all 80% ethanol precipitates were saved. All precipitates were tested for

histidine using the ninhydrin test given above after removing ammonia. Recovery data for histidine after extraction and after chromatography on the ion retardation column were calculated from amino acid determinations on the Beckman-Spinco Automatic Amino Acid Analyzer.

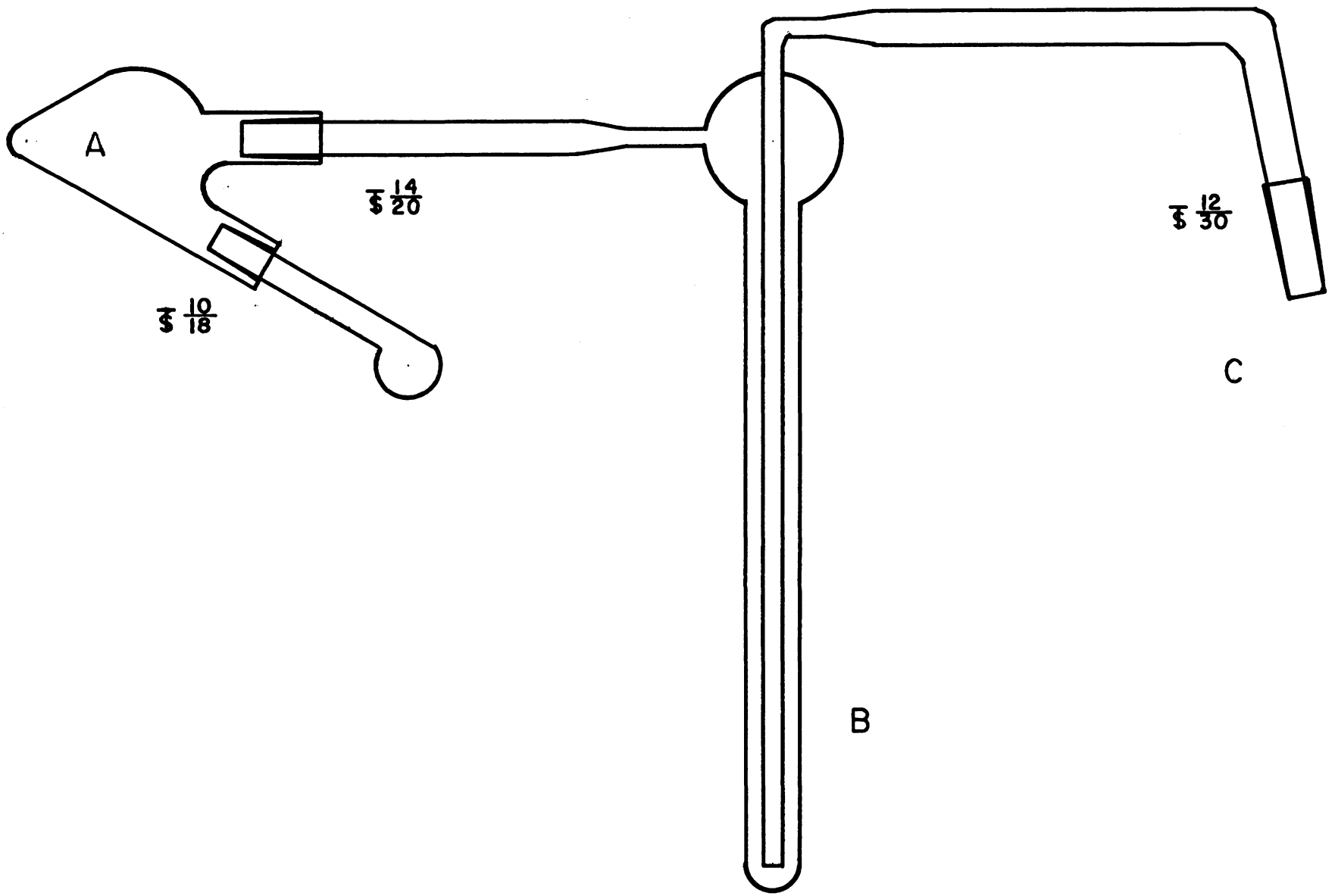
N^{15}-Determination

The nitrogen-containing material to be analyzed was subjected to kjeldahl digestion (1). After titration to determine total nitrogen, the ammonium sulfate solutions were concentrated by boiling on a hot plate in a 250 ml. Erlenmeyer flask. An aliquote of the concentrated solution containing approximately 1 mgm. of nitrogen was dried on a piece of glass filter paper (1 cm. x 4 cm.).

The glass papers with the samples dried on them were stored in sealed tubes until the N^{15} determinations were made. A Consolidated Electrodynamics Corporation Model 620-21A Mass Spectrometer with a metal inlet system and a micromanometer was used for all N^{15} determinations.

The apparatus used as a reaction chamber for N^{15} determination is shown in Fig. 1. C was attached to the metal inlet system; B was immersed in an ethanol-dry ice slurry to trap water. The glass paper with the sample on it was placed in A, and 1 ml. of sodium hypobromite (3, 30), cooled in the ethanol-dry ice slurry, was placed in the side arm on A. The reaction was carried out by rotating A so that the side arm moved from bottom to top, pouring its contents into A and onto the sample.

Fig. 1. Apparatus for N^{15} determinations.


A
$\frac{14}{20}$
$\frac{10}{18}$
$\frac{12}{30}$
C
B

The N^{15} determination was carried out according to the standard timing procedure given in Table 1.

From this timing procedure onewill note that peaks for M/e 28 and 29 were monitored on each run, and that background scans were taken before each N^{15} determination. In addition, blanks were run at the beginning of the period when N^{15} was to be determined. The blanks consisted of glass papers with no sample and 1 ml. sodium hypobromite solution (3, 30).

The peaks for M/e 28 and 29 were corrected for blank and background readings. Percent N^{15} was calculated using the following formula (3, 4, 30):

$$\text{atom \% } N^{15} = \frac{100}{2R + 1}$$

where $R = \frac{M/e\ 28}{M/e\ 29}$. M refers to the mass of the particle and e to the charge on the particle.

The atom percent excess N^{15} was determined by subtracting the atom percent N^{15} that occurs naturally from the atom percent N^{15} found for a sample. The naturally occurring atom percent N^{15} was determined at the same time as a group of samples were to be run.

Metabolic Studies

Eighteen male, weanling (40-45 gm.) Sprague-Dawley rats were maintained on a basal diet (Table 2) for eight days. The rats were offered the basal diet twice a day for two-hour intervals. These eight days served as a training period to condition the rats to consume their

Table 1

Timing for N^{15} Determinations

Time (min.)	Manipulation
0	Start pump-out of the reaction chamber
2	Pump-out complete, close off reaction chamber Admit background sample Open valve to molecular leak
2.5	Conduct the reaction
3	Make background scans of M/e* 28 and 29
5	Close valve to the molecular leak Admit sample gas
5.5	Open valve to the molecular leak
6.5	Scan peaks for M/e* 28 and 29
8	Scan again.

*M refers to the mass of the particle and e to the charge on the particle.

Table 2

Diet Composition

Basal diet:

Ingredient	gm./kgm. diet
Amino acid mixture H (27)	102.20
Vitamin mixture (15)*	10.00
Salt mixture (1)	53.90
Choline Chloride (15)	2.00
Corn oil	100.00
α-tocopherol (6)	0.091
Oleum percomorphum (6)**	0.35 (9 drops)
Sucrose	735.759
	1004.3

Imbalanced diet:

Amino acid mixture H (no histidine) (27)	99.69
L-N^{15}-histidine.Hcl.H_2O (97 atom % excess N^{15}	2.77
Essential amino acid mixture***	49.10
Vitamin mixture (15)	10.00
Salt mixture (1)	53.00
Choline chloride (15)	2.00
Corn oil containing α-tocopherol and oleum percomorphum	100.441
Sucrose	686.589
	1004.49

Corrected diet:

Amino acid mixture H (27) (no histidine)	99.69
L-N^{15} histidine.HCl.H_2O (97 atom % excess N^{15})	2.77
Essential amino acid mixture***	49.10
L-histidine.HCl.H_2O	2.74
Sodium bicarbonate	1.10
Vitamin mixture (15)	10.00
Salt mixture (1)	53.90
Choline chloride (15)	2.00
Corn oil containing α-tocopherol and oleum percomorphum	100.441
Sucrose	682.989
	1004.73

*The water soluble vitamins were mixed with sucrose to make up one percent of the diet.

**Mead Johnson Laboratories, three drops contained 3750 I.U. Vitamin A, 540 I.U. Vitamin D.

***The essential amino acid mixture added to create the imbalance consisted of: methionine 4.0 gm., leucine 10.0 gm., arginine.HCl, 4.0 gm., phenylalanine 3.75 gm., threonine 3.75 gm., tryptophan, 2.5 gm., cystine, 1.5 gm., tyrosine, 4.0 gm., valine 7.0 gm., isoleucine, 7.5 gm., and sodium bicarbonate, 1.6 gm. (all L-isomers).

entire day's food supply in two, two-hour intervals. It was also a period of adjustment to the purified diet and a period of protein depletion.

At the end of the sixteen feeding intervals, twelve of the heavier rats (the best eaters) were selected for the experimental diets. These rats were grouped according to weight into six groups, three to be fed the corrected diet and three to be fed the imbalanced diet (Table 2). The amino acid mixtures used had previously been demonstrated to cause and correct an imbalance situation (6). The availability of only 100 mgm. of L-N^{15}-histidine.HCl.H_2O (Schwarz Bio-Research, Inc.) limited the amounts of each diet to 18 gm. Each diet contained equal amounts of label (N^{15}), although the corrected diet contained more histidine. Each rat was offered 2.8 gm. of diet leaving 0.6 gm. for total nitrogen (1) and nitrogen-15 determinations.

A twelve-hour starvation period preceded the feeding of the experimental diets which were offered to the rats according to the schedule shown in Table 3. All rats were housed individually in metabolism cages during the course of the experiment. Table 3 also indicates the order in which the rats were sacrificed.

During the thirty-minute sacrificing period, the rat was anesthetized with chloroform; blood was obtained by heart puncture; the liver was removed, weighed and homogenized in 10% (w/w) trichloroacetic acid (TCA); the gastrointestinal tract was removed; the kidneys were removed, weighed and homogenized in 10% (w/w) TCA; the gastroenemius

Table 3

Schedule for the Feeding and Sacrificing of Rats

Time (hours)	Manipulation
0.0	Feed: I-2*** (1), I-4* (5), I-6* (9)***
0.5	Feed: I-2 (2), I-4 (6), I-6 (10)
1.0	Feed: II-2 (3), II-4 (7), II-6 (11)
1.5	Feed: II-2 (4), II-4 (8), II-6 (12)
2.0	Remove feed: 1, 5, 9 Sacrifice 1
2.5	Remove feed: 2, 6, 10 " 2
3.0	Remove feed: 3, 7, 11 " 3
3.5	Remove feed: 4, 8, 12 " 4
4.0	" 5
4.5	" 6
5.0	" 7
5.5	" 8
6.0	" 9
6.5	" 10
7.0	" 11
7.5	" 12
8.0	

*I Rats fed the imbalanced diet.

*II Rats fed the corrected diet.

**2, 4, 6 Hours after the onset of feeding that the rats were sacrificed.

***( ) Rat number.

Hence, I-2 (1) represents the first rat fed the imbalanced diet and sacrificed two hours after feeding, and II-6 (12) represents the twelfth rat fed the balanced diet and sacrificed 6 hours after feeding.

muscle was removed from both hind legs, weighed and homogenized in 10% (w/w) TCA; and the gastrointestinal tract was washed of its contents with physiological saline, weighed and homogenized in 10% (w/w) TCA in that order, for each rat. The contents washed from the cecum and large intestine were placed with the feces while the contents of the stomach and small intestine were saved separately. Equal volumes of 10% (w/w) TCA were used for each homogenization (10 ml.) and for rinsing the equipment (15 ml.).

Later the liver, kidney, muscle, and gastrointestinal tract homogenates were centrifuged, the TCA fractions saved and the pellets homogenized for five minutes in 10 ml. of 10% (w/w) TCA. The pellets were then suspended in 15 ml. 10% (w/w) TCA and heated in a water bath (80-85°C.) for 30 minutes. The suspension was centrifuged and the TCA fraction decanted. The three TCA fractions were pooled and made up to 80 ml. (extra TCA came from rinsing homogenizer parts) with 10% (w/w) TCA and divided into two, 40 ml. fractions for kjeldahl digestion (1). The pellets were washed twice with 15 ml. of acetone to facilitate drying. After drying, the pellets were weighed and stored in a freezer until kjeldahl digestions (1) could be run.

The entire urine sample from each rat was subjected to kjeldahl digestion (1). The upper gastrointestinal tract contents, and the lower gastrointestinal tract contents and feces were homogenized for five minutes, diluted to 100 ml. with distilled water, and divided

into two, 50 ml. fractions for kjeldahl digestion (1). Ten milliliters of 10% (w/w) TCA was added to 0.4 ml. of each sample of rat serum, mixed and centrifuged. The supernatants were saved, and the pellets were washed once with 10 ml. of 10% (w/w) TCA. The entire TCA fraction and the entire pellet from each sample were subjected to kjeldahl digestion (1). The carcass, left over blood, and the acetone fractions from each rat were pooled and 200 ml of 6 N HCl was added. These mixtures were autoclaved for 8 hours (121° C, 15 lb./in.^{2}) to effect a solubilization so that aliquotes could be taken for kjeldahl digestion (1). After the total nitrogen in the samples had been determined by the procedures above, the samples were boiled down to about one milliliter and stored in sealed tubes at 0° F. until nitrogen-15 determinations could be run.

Total food intake was determined for each rat, and total nitrogen and nitrogen-15 determinations were run on the diet. With the above data, nitrogen-15 (i.e., histidine) absorption and incorporation into protein in the various tissues were compared for the two diet groups as well as for the three times of sacrificing.

Before running the above experiment with N^{15}-histidine, a complete dummy experiment without labeled histidine was carried out. This enabled me to perfect my procedures and to determine whether the amount of nitrogen present in the various samples would be sufficient for N^{15} determinations.

VII. RESULTS AND DISCUSSION

Results of N^{15}-histidine Isolation

Not all parameters of the recovery of nitrogen and histidine were determined on every run of each phase of the procedure; however, a given determination obtained from several runs should be indicative of the rest.

In the first batches of E. coli that were cultured, one percent of the ammonia nitrogen was left in the culture supernatant. In the second group of cultures, it was found that no detectable nitrogen was gained or lost during growth and storage of the cultures. Also, it was found that 6.2% of the total initial nitrogen was left in the culture supernatant. These data were of particular significance since I was trying to attain the greatest N^{15}-enrichment with a minimum loss of N^{15}.

In the first batch of cultures the actual cell yield was approximately 33 gm. of cells (wet weight) per gram of nitrogen in the medium. For the second batch it averaged 54 gm. of cells (wet weight) per gram of nitrogen. This increase in cell yield per gram of nitrogen was probably due to the decreased amount of nitrogen (10 µgm./ml. culture medium) in the second batch of medium.

For the second batch of cultures 88.7% of the total initial nitrogen was recovered in the cell hydrolysate. During the concentration of the hydrolysate and preparation for the cation exchange column another 14.5% of the total initial nitrogen was lost. This left 74.2%

of the total initial nitrogen to be chromatographed on the cation exchange column; the recovery from this column was 90.3% with respect to nitrogen and 77.5% with respect to histidine. The distribution of nitrogen in the various fractions is shown in Table 4.

Seven extractions with ethyl acetate using one-half the volume of the aqueous buffer-histidine solution from the above column (concentrated and adjusted to a pH < 1) did not remove any detectable histidine. No detectable histidine was discarded with the salt precipitated with 80% (v/v) ethanol.

From the results given above, it is obvious that the culture conditions and the Bio-Rad AG SOW-X4 column worked quite well. However, much difficulty was encountered in removing the sodium citrate from the histidine-buffer solution eluted from the column. Even though no detectable histidine was lost when a limited number of ethyl acetate extractions were conducted, the repeated use of this procedure in conjunction with 80% (v/v) ethanol precipitations resulted in the loss of approximately 20% of the histidine through step 15 in "Experimental Procedures, N^{15}-histidine Isolation." After this step, I had 73 mgm of histidine left from a total of 170 mgm obtained before rechromatography on the Bio Rad AG SOW-X4 column. From this step (15) on a considerable amount of histidine was lost because of the ion retardation column where recovery was found to be approximately 50% on two passes through the column.

Unfortunately, only nine milligrams of N^{15}-histidine remained after the purification procedure had been completed, and even then, approximately six percent of the total nitrogen could not be accounted

Table 4

Distribution of Nitrogen in Fractions from the Bio Rad AG-50W-X4 Column

Fractions	Comments	Nitrogen (mgm)
1-4 *		0
5-14	Ninhydrin Peak I (Acetic and Neutral A.A.)	166.7
15-23		15.3
24-28	Ninhydrin Peak II (Acetic and Neutral A.A.)	18.6
29-38		7.5
39-51		9.0
52-61	Ninhydrin Peak III (Lysine)	37.2
62-68		27.8
69-79	Ninhydrin Peak IV (Histidine)	8.7
80-89		9.1
90-98		2.2
99-107	NaOH Wash	16.5
		318.6

*Each fraction contained 200-250 ml. of effluent.

for in histidine. This system for the isolation of nitrogen-15 enriched histidine would be considerably improved if the Bio Rad AG 50W-X4 column effluent contained a volatile or an otherwise easily removable acid or buffering compound.

Comments on the N^{15} Determinations

A number of problems were encountered during the process of perfecting the N^{15} determination. Besides being a delicate instrument subject to many breakdowns, the mass spectrometer required that certain regimens be followed when conducting the reaction mentioned earlier.

In the early stages of setting up the N^{15} determination, extreme inaccuracy and bad precision were encountered. Atom percent N^{15} calculated using M/e 28 and M/e 29 was as little as 50% of the estimated values. Also atom percent N^{15} calculated using M/e 29 and M/e 30 was ridiculously high. A typical set of values were: Calculated, 59.0% N^{15}; determined using M/e 30 and M/e 29; 70.8% N^{15}; determined using M/e 28 and M/e 29, 32.3% N^{15}.

A large portion of this error was found to be due to the spotting technique used in drying the samples on the filter paper. Originally I had spotted the N^{15} enriched ammonium sulfate solution on the paper first, dried it, then spotted some N^{14} ammonium sulfate solution on the same area of the paper to make the desired concentration of N^{15}. This technique was apparently producing areas on the paper that held concentrated amounts of N^{14} or N^{15}, thus yielding unusually high amounts

of N^{15} N^{15} and N^{14} N^{14} when the sodium hypobromite solution released the nitrogen.

Mixing of the two solutions before spotting on the filter paper greatly improved the results; atom percent N^{15} Calculated using M/e 28 and M/e 29 were within 2% of those calculated by using M/e 29 and M/e 30 when the atom percent N^{15} was near 25. However, when atom percent N^{15} approached the naturally occurring level (0.36%) increasing errors were encountered. This error was found to be attributable to the filter papers on which the samples were dried. Evidently the paper gives off a material that produces contamination of the M/e 28 peak only when ammonium sulfate was present. Substitution of glass filter paper for the paper yielded excellent results as shown in Table 5.

In these determinations (Table 5) day to day fluctuations did not exceed 3.0% and repeated determinations on the same sample were within 1.2%. Also, these results show that differences in atom percent N^{15} of at least 6% can be determined reliably.

Metabolic Studies

The Appendices contain data on rat and rat tissue weights as well as raw data on total nitrogen and atom percent excess N^{15} determinations. The nitrogen and N^{15} content of the diets are given in Appendix iii. Duplicate nitrogen and N^{15} determinations were only conducted on the diets. In the data that follows N^{15} has been used as a tracer for histidine. The underlying assumption that was the basis on which all the data has been interpreted, was that there was little or no catabolic degradation of the histidine.

Table 5

Results of N^{15} Determinations on Standard Solutions of N^{15}-Ammonium Sulfate

	Atom % N^{15}		
	Day 1	Day 2	Average
Sample 1	1.33	1.37	1.35
Sample 2	1.28	1.26	1.27
Repeat on Sample 2	1.29	1.28	1.285

Each sample contained approximately 500 µgm. of nitrogen.

Average values (two rats) for N^{15} consumption and absorption are shown in Table 6. Where the N^{15} consumption was similar (two and four hours after feeding) for the rats fed the imbalanced or corrected diets, N^{15} absorbed as percent of the N^{15} consumed was also similar. However, the two groups of rats sacrificed at six hours after feeding consumed quite different amounts of N^{15}. Although the absolute amount of N^{15} absorbed was greater for the imbalanced group than for the corrected group (as was the food consumption), the percent of the consumed that was absorbed was considerably less for the imbalanced group. This left a higher percentage of N^{15} remaining in the gastro-intestinal tract and feces of the imbalanced group (6 hours) as is evident in Table 7. This effect (the lower efficiency of absorption for the imbalanced group) in the groups sacrificed at six hours after feeding may be attributable to the higher intake of N^{15} rather than to the imbalance phenomenon.

Tables 8, 9, 10, 11, and 13 show the amount of N^{15} retained in the protein and the non-protein fractions of the various tissues that were analyzed. The acid-soluble and protein fractions of liver show increasingly wider margins of N^{15} content (Table 8) for the rats fed the imbalanced and corrected diets from two hours to six hours. For the imbalanced groups, more N^{15} was incorporated into protein and less N^{15} was found in the acid soluble fractions at each time of sacrificing.

Table 6

Nitrogen-15 Consumption and Absorption

Time (hours) after onset of eating	Diet	Average μM N^{15} (excess) Consumed	N^{15} Absorbed as % of N^{15} (excess) Consumed
2	Imbalanced	90.66	45.57
	Corrected	86.88	41.45
4	Imbalanced	76.72	60.09
	Corrected	73.58	59.94
6	Imbalanced	92.41	75.78
	Corrected	64.72	87.88

Table 7

Nitrogen-15 Remaining in the Gastrointestinal Tract

Time (hours) after the onset of eating	Diet	% of N^{15} (excess) Consumed	
		Upper Gastrointestinal Contents	Lower Gastrointestinal Contents + Feces
2	Imbalanced	54.28	0.14
	Corrected	58.39	0.17
4	Imbalanced	39.69	0.22
	Corrected	39.86	0.19
6	Imbalanced	23.68	0.53
	Corrected	11.40	0.22

Table 8

Nitrogen-15 Retained in the Liver Fractions*

Time (hours) after the onset of eating	Diet	μM N^{15}(excess)/gm.Fresh Tissue		μM N^{15}(excess)/gm.Protein		μM Histidine from the diet/gm.Fresh Tissue		Total
		Acid Soluble	Protein	Acid Soluble	Protein	Acid Soluble	Protein	
2	Imbalanced	0.63	0.97	4.61	6.88	1.75	3.00	4.75
	Corrected	0.75	0.64	5.14	4.41	4.64	3.96	8.60
4	Imbalanced	0.70	1.46	4.60	9.66	2.16	4.52	6.68
	Corrected	0.94	1.14	5.83	7.05	5.81	7.05	12.86
6	Imbalanced	0.51	2.53	3.78	18.81	1.58	7.82	9.40
	Corrected	1.14	1.60	8.34	11.72	7.05	9.90	16.95

*For these data the μM of N^{15} found in acid-soluble fractions were divided by the amount of protein precipitated with 10% (w/w) Trichloroacetic acid.

Table 9

Nitrogen-15 Retained in Kidney Fractions

Time (hours) after onset of eating	Diet	$\mu M\ N^{15}$ (excess)/gm. fresh tissue		$\mu M\ N^{15}$ (excess)/gm. protein	
		Acid Soluble	Protein	Acid Soluble	Protein
2	Imbalanced	0.12	0.62	0.80	4.00
	Corrected	0.24	0.58	1.44	3.43
4	Imbalanced	0.28	1.18	1.75	7.31
	Corrected	0.29	1.05	1.82	6.45
6	Imbalanced	0.26	2.17	1.59	13.14
	Corrected	0.41	1.48	2.28	8.22

Table 10

Nitrogen-15 Retained in Muscle Fractions

Time (hours) after onset of eating	Diet	$\mu M\ N^{15}$ (excess)/gm. Fresh Tissue		$\mu M\ N^{15}$ (excess)/gm. Protein	
		Acid Soluble	Protein	Acid Soluble	Protein
2	Imbalanced	0.14	0.18	0.75	0.95
	Corrected	0.26	0.19	1.50	1.06
4	Imbalanced	0.26	0.27	1.45	1.31
	Corrected	0.30	0.26	1.62	1.34
6	Imbalanced	0.19	0.57	0.98	3.06
	Corrected	0.43	0.55	2.32	2.89

Table 11

Nitrogen-15 Retained in Gastrointestinal Tract Fractions

Time (hours) after the onset of eating	Diet	$\mu M\ N^{15}$ (excess)/gm.Fresh Tissue		$\mu M\ N^{15}$ (excess)/gm.Protein	
		Acid Soluble	Protein	Acid Soluble	Protein
2	Imbalanced	0.66	0.92	4.91	6.77
	Corrected	0.50	0.56	3.59	3.97
4	Imbalanced	0.46	1.48	3.31	10.65
	Corrected	0.47	1.30	3.99	10.14
6	Imbalanced	0.50	3.01	3.69	21.90
	Corrected	0.41	1.71	2.85	11.63

Table 12

Nitrogen-15 Retained in the Serum Fractions

Time (hours) after the onset of eating	Diet	$\mu M\ N^{15}$ (excess)/ml. Serum	
		Acid Soluble	Protein
2	Imbalanced	0.77	0.39
	Corrected	0.12	0.30
4	Imbalanced	0.26	0.76
	Corrected	0.38	0.79
6	Imbalanced	0.31	1.71
	Corrected	0.50	1.10

Table 13

Nitrogen-15 Retained in the Samples as % of N^{15} (excess) Absorbed

	Time (hours) after the onset of eating					
	Two		Four		Six	
Sample	Imbalanced	Corrected	Imbalanced	Corrected	Imbalanced	Corrected
Urine	0.54 ± 0.13	1.27 ± 0.03	0.28 ± 0.07	0.39 ± 0.26	0.69 ± 0.16	0.83 ± 0.59
Liver: protein	6.12 ± 1.54	4.55 ± 0.76	8.64 ± 0.76	6.74 ± 1.02	11.20 ± 0.33	8.11 ± 0.42
acid soluble	4.09 ± 0.62	5.29 ± 0.97	4.18 ± 0.52	5.58 ± 1.58	2.21 ± 0.79	5.76 ± 0.44
Kidney: protein	0.75 ± 0.02	0.82 ± 0.04	1.40 ± 0.07	1.18 ± 0.04	1.55 ± 0.01	1.30 ± 0.01
acid soluble	0.15 ± 0.05	0.34 ± 0.05	0.34 ± 0.04	0.34 ± 0.11	0.19 ± 0.03	0.36 ± 0.03
Gastrointestinal: tract: protein	7.78 ± 0.10	5.31 ± 0.39	11.36 ± 0.11	10.93 ± 0.81	14.69 ± 3.36	9.33 ± 0.24
acid soluble	5.65 ± 0.28	4.80 ± 0.16	3.55 ± 0.22	4.28 ± 0.74	2.49 ± 0.72	2.28 ± 0.16
Muscle:* protein	10.86 ± 2.71	13.28 ± 0.96	15.09 ± 1.33	13.82 ± 1.84	20.46 ± 1.53	23.67 ± 8.74
acid soluble	8.93 ± 1.95	14.14 ± 1.43	15.29 ± 2.73	17.00 ± 5.25	6.65 ± 3.81	18.44 ± 2.11
Serum:** protein	1.95 ± 0.13	1.69 ± 0.17	3.44 ± 0.82	3.42 ± 0.13	4.89 ± 0.06	3.73 ± 0.33
acid soluble	3.93 ± 0.50	0.67	1.12	1.73	0.89	1.70 ± 0.33
Recovery from the above tissues only	50.75 ± 8.03	52.16 ± 4.96	64.69 ± 6.67	65.41 ± 11.78	65.91 ±10.80	75.51 ± 13.40
Carcass	53.44	76.29	86.89	73.32	74.32	83.76
Total Recovery***	80.50	100.54	118.76	104.90	109.62	114.97

*Total muscle mass was taken as 50% of body weight.

**Total serum volume was taken as 4% of body weight.

***This tabulation does not include the muscle and serum data as it is listed above; only the data for muscle and serum actually analyzed was used and the rest was represented in the carcass.

Data on the histidine content of the two fractions of liver tissue is also shown in Table 8. It is evident that more histidine has been retained in the acid soluble and protein fractions of the liver from the rats fed the corrected diet than from the imbalanced group. However, the histidine was used much more efficiently by the rats fed the imbalanced diet. Histidine data are not included in the other tables (9, 10, 11 and 12) since they are not demonstrative of the effects that took place. One must remember, although a higher percentage of the absorbed histidine was retained in the protein of liver for the imbalanced groups, a larger amount of histidine was retained by the liver protein of the corrected groups. The ratio of the total histidine retained in the liver tissue of the imbalanced group to the total histidine retained in the liver tissue of the corrected group corresponded closely to the ratio of the histidine found for the respective diets. This is a direct indication that the absorption and transport of the limiting amino acid was not affected in the imbalance situation.

The tissue fractions of the kidneys (Table 9) exhibited effects similar to those of liver but not as pronounced. The values for rats sacrificed six hours after feeding show a wide margin between the imbalanced and the corrected groups in both tissue fractions.

The N^{15} content of the muscle protein (Table 10) of the imbalanced group did not differ from that of the corrected group. However, the N^{15} content of the acid-soluble fraction appears to be lower for the imbalanced group at six hours. This is in marked contrast to the clear-cut differences observed in the metabolically more active tissues--liver and kidney.

For the gastrointestinal tract acid-soluble fractions (Table 11) there was no apparent difference in N^{15} content between the two groups at any time. However, there was quite a large difference in the amount of N^{15} retained in the protein fractions of the two groups at six hours after feeding; considerably more N^{15} was retained in the gastrointestinal tract protein of the rats fed the imbalanced diet. The gastrointestinal tissue was more responsive than the muscle, but the clear-cut differences observed for liver and kidney were not evident here.

Nitrogen-15 content of the serum fractions is shown in Table 12. The N^{15} content of the acid-soluble fractions is somewhat questionable since there was very little nitrogen on which to conduct N^{15} determinations; all data that were one order of magnitude off were discarded. However, there appears to be less N^{15} in the acid-soluble portion of the serum from rats fed the imbalanced diets at four and six hours after sacrificing. Nitrogen-15 content of the serum protein seems to be higher for the imbalanced group at six hours.

In Table 13 percent of the absorbed N^{15} found in the tissue fractions and urine is shown. No urinary effect due to the imbalance was apparent confirming Harper's data (12, 22).

The percent of the absorbed N^{15} retained in the tissue fractions reflects the trends observed for the "specific activity" data shown in Tables 8, 9, 10, 11 and 12. The trend of higher incorporation of N^{15} into liver protein and lower percentages of N^{15} in the acid-soluble fractions for the imbalanced group can be seen at each time of sacrificing. However, significant differences were noted only at the four and six hour times of sacrificing for the protein and at six hours for the acid-soluble fraction.

The percentage of the absorbed N^{15} retained for kidney fractions behaves similarly with significant increases in N^{15} incorporation into protein at four and six hours and a significant decrease in the acid-soluble fraction at six hours.

Increased incorporation of N^{15} into gastrointestinal tract protein of the imbalanced group was noted at six hours after feeding only. No decrease in the percentage of N^{15} retained in the acid-soluble fraction of the gastrointestinal tract was evident at any time for the imbalanced group. The failure to see any effect in the gastrointestinal tract acid-soluble fraction may have been due to the readily available supply of N^{15} in lumen and to the rapid recycling of nitrogen due to enzyme secretion.

No significant changes in the percentage of N^{15} retained in muscle protein were noted for the imbalanced group at any time. However, the acid-soluble fraction of the muscle for the imbalanced group had a lower percentage of N^{15} retained than did the corrected group at six hours after feeding. As noted in Table 13, the data for the muscle fractions were made to reflect the percentage of the absorbed N^{15} retained in total muscle mass.

Similarly, the data for the serum fractions were adjusted to show the percent of the absorbed N^{15} retained in the total serum volume. As noted above, the data for the acid-soluble fractions of serum (Table 13) is not very reliable; however, there appears to be a reduction in the percentage of the absorbed N^{15} that is retained in this fraction for the imbalanced group at four and six hours after feeding. Serum protein fractions for the rats fed the imbalanced diet contained a lower percentage of N^{15} than did those for the rats fed the corrected diet at six hours after feeding.

Nitrogen-15 recovery data is shown in Table 13. The percent recovery from the experimental tissues (using estimates of total muscle mass and total serum volume) agree quite well for the corrected and imbalanced groups, however, somewhat wider variation is seen for the different hours of sacrificing. There appears to be no plausable explanation for this other than the possibility of increased incorporation of N^{15} into the tissues analyzed with time since the recovery increased from two to six hours. Total recovery as percent

of the N^{15} absorbed is also shown in Table 13. The total recovery was quite high in most cases and varied considerably between the balanced and imbalanced groups. The author has reason to believe that the carcass data are not completely reliable because of only partial solution of clotted blood in preparing carcass samples for kjeldahl and N^{15} analyses.

VIII. SUMMARY AND CONCLUSIONS

The metabolism of the limiting amino acid (histidine-U-N^{15}) in the imbalanced diet was followed in rats fed an imbalanced or a corrected diet. The gastrointestinal contents and urine as well as the acid-soluble and protein fractions of selected tissues (liver, kidney, muscle, gastrointestinal tract, and serum) were examined for changes in the concentration of N^{15}.

Liver, kidney, gastrointestinal tract, and serum protein from the imbalanced group had retained more N^{15} at six hours after feeding than had the protein from the rats fed the corrected diet. The acid-soluble fractions of liver, kidney, muscle, and serum from the imbalanced rats had retained less N^{15} than the respective control tissues at six hours after feeding. The metabolically more active tissues (liver and kidney) exhibited these effects at earlier times as well.

The N^{15} excreted in the urine showed no significant difference between the balanced and imbalanced groups. A higher percentage of the consumed N^{15} was absorbed at six hours after feeding by the rats fed the corrected diets; however, the close correspondence of the ratio of histidine in the corrected and imbalanced diets to the ratio of dietary histidine retained in the respective livers indicates that the above absorption data was an anomaly due to the heavy consumption of the rats fed the imbalanced diet.

The above data confirm the earlier evidence that indicated no increase in the catabolism of the limiting amino acid (8, 12, 16, 19, 21, 31, 32) and no delay in the absorption and transport of the limiting amino acid (32) in the imbalance situation. The first part of Harper's hypothesis (12, 32) stating that there was increased incorporation of the limiting amino acid into tissue proteins of rats fed an imbalanced diet is supported by this investigation. However, differences in the control diets of this investigation and that of Yoshida et al. (32) permit us to note that the limiting amino acid was incorporated into certain tissue proteins more efficiently for the rats fed the imbalanced diet even though less was actually incorporated.

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APPENDICES

Appendix I

Experimental Rat and Rat Tissue Weights (gm.)

Rat No.*	Rat	Liver	Kidneys	Muscle	Gastrointestinal tract (empty)
1	52.0	2.6	0.5	1.2	3.4
2	52.0	2.7	0.5	1.1	3.6
3	50.0	2.7	0.5	1.1	3.4
4	49.0	2.3	0.5	1.2	3.4
5	48.5	2.5	0.6	1.2	3.3
6	55.5	2.9	0.5	1.2	3.7
7	48.5	2.75	0.5	1.1	3.4
8	47.5	2.45	0.5	1.15	---
9	50.0	3.2	0.5	1.3	3.7
10	50.5	3.0	0.5	1.2	3.1
11	49.0	2.8	0.5	1.1	3.1
12	47.5	3.0	0.5	1.3	3.1

*Refer to Table 3 in the text to find the diets and hours of sacrificing which correspond to these numbers.

Appendix II

Isotope Composition of Diets

	μM N/gm. diet	μM N^{15} (excess)/gm.diet
Imbalanced	1.33	34.87
Corrected	1.40	35.46

Appendix III

Total Nitrogen Content (mM) and Atom % Excess N^{15} of the Samples Indicated

Rat No.	Diet Consumed	Upper Gastrointestinal Contents	Lower Gastrointestinal Contents + Feces		Urine	
1	3.28	2.30 (2.264)*	0.36	(0.053)	0.11	(0.238)
2	3.28	2.16 (2.146)	0.36	(0.020)	0.07	(0.289)
3	3.75	2.72 (2.306)	0.30	(0.055)	0.14	(0.320)
4	2.81	1.86 (2.146)	0.32	(0.037)	0.11	(0.407)
5	2.27	1.20 (1.950)	0.38	(0.033)	0.13	(0.073)
6	3.28	1.82 (2.097)	0.36	(0.057)	0.18	(0.097)
7	2.75	1.64 (1.820)	0.30	(0.032)	0.12	(0.200)
8	2.81	1.46 (1.972)	0.42	(0.042)	0.16	(0.056)
9	3.28	1.20 (1.729)	0.32	(0.114)	0.21	(0.266)
10	3.40	1.40 (1.646)	0.34	(0.189)	0.21	(0.192)
11	2.61	0.82 (1.058)	0.24	(0.044)	0.18	(0.141)
12	2.27	0.72 (0.941)	0.34	(0.049)	0.18	(0.366)

*Numbers in parenthesis represent atom percent excess N^{15}.

Appendix IV

Total Nitrogen Content (mM) and Atom % Excess N^{15} of the Tissue Fractions

Rat No.	Liver				Kidney				Muscle			
	Acid Soluble		Protein		Acid Soluble		Protein		Acid Soluble		Protein	
1	0.64	(0.267)	2.68	(0.071)	0.08	(0.053)	0.62	(0.046)	0.40***	(0.044)	1.88***	(0.008)
2	0.68	(0.240	3.89	(0.083)	0.18	(0.045)	0.67	(0.049)	0.46	(0.030)	1.58	(0.015)
3	0.76	(0.281)	3.20	(0.057)	0.21	(0.064)	0.73	(0.041)	0.52	(0.060)	1.69	(0.013)
4	0.62	(0.260)	2.74	(0.051)	0.18	(0.060)	0.69	(0.040)	0.58	(0.051)	1.95	(0.011)
5	0.74	(0.242)	3.05	(0.105)	0.22	(0.067)	0.80	(0.072)	0.54	(0.062)	1.87	(0.015)
6	0.82	(0.241)	3.38	(0.141)	0.22	(0.072)	0.77	(0.091)	0.52	(0.058)	1.58	(0.019)
7	0.76	(0.375)	3.18	(0.085)	0.17	(0.103)	0.69	(0.075)	0.48	(0.082)	1.70	(0.014)
8	0.70	(0.290)	3.08	(0.089)	0.18	(0.066)	0.73	(0.072)	0.56	(0.051)	2.05	(0.016)
9	0.78	(0.147)	3.25	(0.245)	0.21	(0.056)	0.72	(0.150)	0.58	(0.024)	2.18	(0.036)
10	0.78	(0.250)	3.16	(0.244)	0.20	(0.072)	0.72	(0.152)	0.52	(0.060)	1.96	(0.033)
11	0.74	(0.494)	3.13	(0.162)	0.19	(0.121)	0.68	(0.115)	0.48	(0.112)	1.80	(0.026)
12	0.76	(0.381)	3.20	(0.130)	9.19	(0.095)	0.76	(0.092)	0.64	(0.078)	2.30	(0.038)

*mM Nitrogen per ml of serum.
**The numbers in parentheses represent atom % excess N^{15}.
***mM nitrogen in total sample taken.

- Continued -

Appendix IV (continued)

Rat No.	Gastrointestinal Tract				Serum			
	Acid Soluble		Protein		Acid Soluble		Protein	
1	1.00	(0.221)	3.54	(0.084)	0.10*	(0.776)	0.60*	(0.061)
2	1.00	(0.244)	3.97	(0.087)	0.13	(0.592)	0.50	(0.079)
3	1.28	(0.131)	3.44	(0.058)	0.04	(0.296)	0.53	(0.052)
4	1.34	(0.128)	3.60	(0.049)	0.08	(0.347)	0.58	(0.055)
5	1.28	(0.114)	3.53	(0.126)	0.10	(0.035)	0.40	(0.146)
6	1.32	(0.133)	3.69	(0.161)	0.13	(0.201)	0.63	(0.149)
7	1.24	(0.129)	3.40	(0.130)	0.07	(0.541)	0.60	(0.122)
8	1.36	(0.161)	3.77	(0.139)	0.08	(0.664)	0.67	(0.126)
9	1.40	(0.049)	3.81	(0.312)	0.13	(---)	0.61	(0.338)
10	1.08	(0.129)	3.21	(0.070)	0.06	(0.517)	0.54	(0.313)
11	1.18	(0.122)	3.29	(0.174)	0.09	(0.650)	0.48	(0.255)
12	1.16	(0.099)	3.41	(0.143)	0.10	(0.407)	0.45	(0.218)

*mM nitrogen per ml. of serum.
**The numbers in parentheses represent atom % excess N^{15}.
***mM nitrogen in total sample taken.

Assimilation of N^{15}-Histidine in Amino Acid Imbalance

by David R. Hartman

ABSTRACT

An attempt was made to isolate L-N^{15}-histidine from Escherichia coli cultured in a defined medium containing nitrogen-15 enriched ammonium salts as the only source of nitrogen.

Commercially prepared L-N^{15}-histidine.HCl.H_2O was used to follow the limiting amino acid in the imbalanced diet in rats fed a chemically-defined imbalanced or corrected diet. Total nitrogen content and atom percent N^{15} were determined on the urine, gastrointestinal contents, diet, and the acid-soluble and protein fractions of the liver, kidneys, muscle, gastrointestinal tract, and serum at two, four, and six hours after the onset of feeding.

The data were examined for changes in the concentration of N^{15} which might cause the imbalance phenomenon (reduced blood levels of the limiting amino acid). It was found that the limiting amino acid was not catabolized at an increased rate and that it was not absorbed or transported at a reduced rate due to the ingestion of an imbalance amino acid mixture. The increased efficiency with which the histidine was found to be incorporated into metabolically active proteins appears to be a primary cause of the reduced blood levels of the limiting amino acid and, therefore, of the imbalance phenomenon.

Experimental verification was, therefore, obtained for the first unverified aspect of Harper's (12) thinking about the cause of the reduced appetite that is characteristic of the imbalance phenomenon.