EFFECT OF AGE AND DIET ON AMINO ACID AVAILABILITY AND FREE AMINO ACIDS OF RAT SKELETAL MUSCLE, LIVER AND SERUM

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Patricia M. Giovannetti

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ABSTRACT

The biological availability of sixteen amino acids from pure wheat to male Wistar rats weighing 80 g or 300 g was determined. These amino acids were 90-98% available with glutamic acid and proline the most available and aspartic acid, alanine or lysine the least available. Among essential amino acids, however, lysine was the least available while histidine and phenylalanine were the most available. The prevention of coprophagy had no effect on the availability of amino acids.

In addition, male weanling rats of Wistar strain were fed diets, that varied in amino acid and protein content, for 28, 56 and 84 days. The protein content of casein diets, C-1, C-2, and C-3 was 6%, 11.5% and 21% while that of the wheat diet (W) and modified wheat diet supplemented with protein (mod. SW), was 11.9% and 19.9% respectively. All tissues analyzed responded to dietary intake but there were less changes in the amino acid concentration of serum than of liver or gastrocnemii. Generally, the essential amino acids were less concentrated in tissue of rats fed either the C-1 or mod. SW diets but were most concentrated in tissues of rats fed the C-3 diet. Non-essential amino acids were higher in tissues of rats fed the C-1, C-2 and W diets, and lowest in rats fed the C-3 and mod. SW diets.

With age, most changes occurred between the start of

the experiment and 28 days later. These changes were exhibited mostly as decreases, the greatest number taking place in the liver; most increases, however, occurred in the free amino acid concentrations of the serum. Though a constancy of concentration was found for most amino acids in tissues of rats fed the C-3 and mod. SW diets from 28 to 84 days, variation was most evident in rats fed the low protein diets, W, C-2 and C-1.

The findings of this study show that intracellular free amino acids are as sensitive as extracellular free amino acids to fluctuations of amino acid intake; in fact, certain intracellular amino acids were more sensitive to excesses and deficiencies of amino acids in the diet than were extracellular amino acids. While the free methionine and taurine contents of liver reflected more accurately than other tissues the variation in the methionine content of the diets, most marked was the response of the gastrocnemii to the lysine content of the diets. Weanling rats fed the W diet, containing only 35% of their requirement of lysine, for 28, 56 and 84 days, had 20, 16 and 19 μmoles lysine/100 ml serum, 139, 98 and 110 μmoles/100 g liver, 83, 27 and 70 μmoles/100 g thigh and leg muscle, respectively; there was, however, no detectable amount of lysine in the gastrocnemii. The need for further investigation into the specific amino acid needs of various intracellular pools is indicated.

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INTRODUCTION

Skeletal muscle is the largest tissue in the body; it makes up 45% of the total body weight. Its metabolism, therefore, is of great importance, especially its protein metabolism during growth and development.

Metabolically, muscle tissue has been considered to be relatively inert compared with liver tissue. Recently, however, the lability of skeletal muscle has been receiving more attention, and, since muscle and its functions are related to the body as a whole, Young (1970) has emphasized the significant qualitative and quantitative interactions of its protein metabolism with other tissues of the body.

Numerous incorporation studies using labeled amino acids have indicated the sensitivity of the protein synthetic and degradative machinery of muscle to dietary amino acid supplies. Though considerable experimentation has sought to establish the relationship of dietary amino acids to the extracellular free amino acids of the blood, only limited attention has been given to how the intracellular free amino acids, especially those of skeletal muscle, relate to amino acid intake and to other tissues of the body. Some of the more comprehensive studies have been reviewed by Rogers and Harper (1968).

However, most of these studies have been conducted on a relatively short term basis, ranging from results

obtained after a twelve-hour fast (Schurr et al., 1950b) to a seven-day fast (Thompson et al., 1950) and after ingestion of one meal, balanced or imbalanced with respect to amino acid composition (Leung et al., 1968b), to consumption of a protein free diet for a period of three weeks (Thompson et al., 1950). The purpose of this study, therefore, was to observe the changes that take place in free amino acid pools of serum, liver and skeletal muscle over a period of time. Since Miller (1969), in his review of protein metabolism during early growth and development stressed the metabolic differences between the neonate and the adult, it was decided that a 12-week period, from weanling to maturity of the rat, would be used to determine adaptive changes in amino acid pools of tissues.

Amino acid metabolism, however, depends not only on the quantity and quality of amino acids supplied in the diet but also on their absorption from the intestinal tract. Generally, the biological availability of amino acids from proteins of animal origin is much higher than that from vegetable origin. Consequently, in this study, attention was given to the quality and quantity of amino acids that were not absorbed from wheat, the chief constituent in two of the diets.

REVIEW OF THE LITERATURE

Protein metabolism is essentially amino acid metabolism so consideration of one must include the other. Nor can growth or development be considered without inclusion of the nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Thus, DNA, RNA, protein and free amino acid metabolism will be considered in this review, in connection with growth and development of the metabolically active organ, the liver, and the less metabolically active skeletal muscle. Since these parameters possibly reflect the nutritive value of the diet, consideration of the availability of amino acids as well as their content and balance of essential amino acids, is justified. Also, certain aspects of free amino acids in serum will be included in this review since the blood is the main carrier of nutrients to tissues of the body.

Availability of Amino Acids

The availability of amino acids from proteins is a measure of their absorption and utilization by a biological system. Different criteria have been used to determine the extent that amino acids are available from intact protein.

Measurement of Availability

Two *in vitro* and three *in vivo* assays have been

developed. *In vitro* methods give 1) a relative measure of the amino N or free amino acid(s) released from various proteins incubated with proteolytic enzymes (Riesen *et al.*, 1947; Melnick and Oser, 1948; Mauron *et al.*, 1955; Sheffner *et al.*, 1956), or 2) a quantitative measure of lysine in the protein reacting with 1-fluoro-2:4 dinitrobenzene, FDNB (Carpenter and Ellinger, 1955; Carpenter, 1960). *In vivo* methods assess 1) the digestibility of a specific amino acid, the unabsorbed amino acid (Kuiken and Lyman, 1948; Kuiken, 1952), 2) the efficiency of utilization as well as digestibility by measuring a) the ability of a protein with known amino acid composition to replace a specific amino acid in supporting growth (Schweigert, 1948; Gupta *et al.*, 1958; Ousterhaut *et al.*, 1959) or repletion of the intact animal (Schweigert and Guthneck, 1953 and 1954) and b) in maintaining N balance in a mature subject (Linkswiler *et al.*, 1958a, 1958b, 1960a, 1960b; Watts *et al.*, 1959a, 1959b).

The *in vivo* methods are of particular importance since the degree of availability of amino acids from protein is influenced by 1) resistance to hydrolysis by digestive enzymes, 2) presence of enzyme inhibitors, 3) competition among amino acids for transport across intestinal mucosa and into cells (Adibi, 1969; Annegers, 1969), 4) absorption rates of individual amino acids (Geiger, 1948; Williams, 1969), and 5) decreased solubility (Mecham and Olcott, 1947)

in some instances. In vitro assays, however, are useful in estimating the effect of processing foods on the rate of release of amino acids by proteolytic enzymes and in quantitating the available lysine content of intact cereal protein by the FDNB method.

Comparison of Methods Measuring Availability

The various methods have been compared to note the sources of error and to estimate the significance of the differences observed.

1. In vitro methods

Szmelcman and Guggenheim (1967) digested processed cereals with proteolytic enzymes and then determined the microbiological availability of six essential amino acids; they found the type of proteolytic enzyme used for in vitro digestion generally determined the extent of availability of 1) a particular amino acid to a specific micro-organism, or 2) lysine by the FDNB method. Dvořák (1968a, 1968b), in efforts to simulate the digestive milieu of the gut, has shown that it was possible to achieve complete hydrolysis of all α-amino peptide bonds with papain, leucine amino peptidase and prolidase so that the microbiologically available amino acids corresponded with theoretical values irrespective of the micro-organisms used.

In all in vitro determinations on the availability of essential amino acids in the proteins, the assumptions

have been that 1) endopeptidases and exopeptidases split all bonds as do the proteolytic enzymes of the digestive tract, and 2) the micro-organisms use only free amino acids of the digest for growth. Melnick and Oser (1948), however, found evidence for the utilization of peptides by micro-organisms, as estimates of enzymatic digestion based on formol titration were lower than that based on microbiological analysis of specific amino acids. Geiger et al. (1952) compared the amino acids liberated during acid and enzymatic hydrolysis of zein using paper chromatographic techniques and showed that certain amino acids liberated during acid hydrolysis were absent in the enzymatic digest. Work by Ford (1965) also showed that in the papain digest of heat-treated proteins, peptides of "larger molecular size" were present and that in these the availability of amino acids was low. Dvořák (1968b) used L-leucinamide, L-leucyl-glycyl-glycine and glycyl-L-leucine substrates for leucine amino peptidase and glycyl-leucine dipeptidase, and found that the availability of leucine was more than 50% after 72 hours incubation, with all the micro-organisms examined. Results by Kuiken et al. (1943) for the availability of leucine from glycyl-L-leucine with Lactobacillus arabinosis agree with these findings and suggest that the micro-organisms used had exopeptidases by which they utilized amino acids of peptides previously released from the proteins by hydrolysis with

endopeptidases. Kinetic studies to measure the uptake of amino acids and dipeptides by micro-organisms (Brock and Wooley, 1964; Yoder et al., 1965; Mayshak et al., 1966; Yoder et al., 1967), however, support the concept of separate transport sites for amino acids and related dipeptides.

2. In vitro versus in vivo methods

Although in vitro studies are of value in determining the release of amino acids from proteins, the need for an index for measuring the availability of amino acids in the intact animal has also received much attention. Determinations on the availability of lysine (Stott and Smith, 1966) from meat meals to Tetrahymena pyriformis W, the protozoa requiring the same essential amino acids as the growing rat, showed lower values than those obtained by the FDNB method.

Similarily, Ford (1964) and Henry and Ford (1965) found the lysine values from leaf protein were more available by the FDNB method than by microbiological or rat growth assays after enzymatic digestion. Boctor and Harper (1968) showed similar differences between the gain in weight of the rat and the FDNB determination of available lysine in autoclaved egg albumin, beef protein and casein; there was high correlation, however, between the values obtained by both methods for the untreated proteins. These findings further point to the fact that essential amino acid moieties from autoclaved protein preparations were less available to

the rat than they were to micro-organisms.

In connection with this, Ford (1964), who improved the availability of amino acids to micro-organisms by increasing the rate of digestion with grinding and more rigorous pre-digestion, wondered if amino acids locked up in indigestible peptide residues could pass into body fluids and still remain unutilized in the intact animal. Horn et al. (1968) found little of the synthetic fructose-methionine complex available to the rat although microbiologically it exhibited 80% of the growth-stimulating ability of methionine. To study the mechanism by which amino acids are made unavailable in carbohydrate-containing foods, Hagen et al. (1970) chose to complex fructose with glycine, an essential amino acid for Leuconostoc mesenteroides P-60 but a non-essential for the growing rat. When assayed microbiologically, the compound had only 68% of the growth-stimulating ability of glycine, but it produced comparable weight gains when fed to nitrogen deprived rats fed an equivalent amount of glycine. Analyses of sera from rats fed fructose-glycine by ion-exchange chromatography revealed a smaller increase in the glycine peak and a larger increase in the unidentified pre-threonine peak than when glycine was fed. These findings suggested to Hagen et al. (1970) that fructose amino acids may be metabolized in the rat in such a way that nitrogen but little or none of the amino acid itself can be utilized.

Criteria, other than weight gain, have been used to measure animal growth as the index of amino acids available from foodstuffs. Calhoun *et al.* (1966) determined the availability of lysine in wheat, flour, bread, and gluten by rat growth studies using gain in weight, gain in empty carcass weight (gastrointestinal contents removed) and gain in nitrogen content of the carcass over a three week period, and found a high degree of correlation between lysine and growth. This agrees with results obtained by de Muelenaere *et al.* (1967a) and Boctor and Harper (1968). In the assessment of the availability of lysine when purified proteins or proteins from cereal and milk powder were supplements to a wheat gluten diet low in lysine, Gupta *et al.* (1958) obtained evidence that amino acid availability values calculated from weight gain per unit of food consumed for two weeks, were more accurate than values obtained from growth data alone.

3. *In vivo* methods

Studies were conducted by de Muelenaere *et al.* (1967a, 1967b) to determine the availability of lysine, threonine, isoleucine and valine from corn and rice proteins by the growth and fecal analysis methods. Besides considering weight gain and weight of empty carcass as criteria of growth, the content of carcass water, nitrogen and the amino acid being assessed, were also considered with the

availability being calculated in three ways for each criterion. The lysine of rice and corn was found highly available, but threonine, isoleucine and valine were less available in corn products than in rice products. These findings indicated that availability of an amino acid from one particular source of protein may vary greatly depending on the criteria of measurement used. The most reliable and sensitive method of calculation was that based on plotting the total change in criterion against the total amount of amino acid consumed since growth is a function of amino acid intake. An inverse relationship was found between the availability of an amino acid and the protein and/or carbohydrate content of the diet, it being more marked in determinations by the growth method than by fecal analysis method; this was also previously observed (Gupta and Elvehjem, 1957) when apparent tryptophan availability decreased as tryptophan was supplemented in the diet. A discrepancy existed between true protein digestibility values and values for availability of amino acids by the fecal analysis method as found for lysine and threonine from rice and corn, respectively, by de Muelenaere et al. (1967a, 1967b). Ford et al. (1967), however, suggested that factors such as the pattern of available amino acids released during digestion may be responsible for the differences sometimes noted between the availability of amino acids and the digestibility of protein. Although

values obtained by the fecal analysis method are higher than those obtained by the growth method, Ford et al. (1967) found close correlations between results obtained by fecal analysis method, as applied to four whale meat meals and dried skim milk samples having a wide range of nutritive quality, and those predicted from chemical and microbiological tests. Results of Gupta and Elvehjem (1957) from rat bio-assays on the availability of tryptophan in beef, pork, casein, egg albumin, beef fibrin, purified soybean protein and milk powders checked well with those obtained from tryptophan determinations in the feces, with beef being similar to the findings of Kuiken and Lyman (1948) using different experimental techniques. Only lysine from corn was reported by de Muelenaere et al. (1967a) to be lower by the fecal analysis method than by the growth method.

Summary

The term "availability" has been defined by de Muelenaere et al. (1967b) as "that portion of an amino acid present in a protein which is used for growth, development and maintenance of an animal in so far as it is dependent on the digestibility of the protein, presence of enzyme-resistant peptide linkages, enzyme-inhibiting substances and rate of release of the amino acid in the intestinal tract".

In conducting in vivo studies to assess the availability of amino acids, it seems imperative that when using

the growth method consideration be given to possible existence of amino acid deficiencies, imbalances or antagonisms for de Muelenaere et al. (1967b) found isoleucine and valine were more available to rats when the leucine-isoleucine antagonism was eliminated, by omitting leucine and half of the isoleucine or all of the leucine from the zein-simulated diet, in growth studies. Also, the source of carbohydrate, and the levels of protein, calories and cellulose should receive attention for they are influencing factors in both the growth and fecal analysis methods. If processed foods are being assayed, the possibility of amino acid destruction, the formation of unavailable compounds containing amino acids and peptide bonds resistant to enzymatic hydrolysis should not be overlooked. Since the total metabolism of the intact body as well as the method of measurement, determine the outcome of growth assays, availabilities determined by this method are time-consuming, vary by as much as 15-20%, and probably are only semi-quantitative. On the other hand, amino acid availabilities determined by the fecal analysis method are less varied but higher in some instances than those obtained by growth methods, possibly because some amino acids are released into the gut at a slow rate. The former method, however, has been criticized because 1) the digest itself may influence the secretion of protein-rich materials into the intestinal tract to increase the apparent

undigested residue, and 2) the microflora of the lower intestine may alter the concentrations of the different amino acids in the feces. Although no consideration has been given to the effect of coprophagy on biological availability of amino acids, Kuiken (1952) added sulfathiozole to the diet and found that bacterial synthesis and degradation in the intestinal tract had no significant effect on the levels of available amino acids. It would seem, therefore, that if endogenous amino acids could be more accurately measured, fecal analysis may serve to measure the unreleased amino acids.

In vitro assays using proteolytic enzymes to hydrolyse food protein may never precisely simulate the environment of the gut but they may serve as guides to the amount of amino acids released by enzymatic digestion in the intestine. On the other hand, the FDNB method gives at its best only a crude quantitation of lysine as acids used in this procedure can split enzyme-resistant bonds. It may serve the useful purpose of determining if changes in the lysine content occurred during the processing of a food.

Introduction of automated ion-exchange chromatography for the determination of free amino acids may possibly offset discrepancies incurred when peptides as well as available amino acids enhance growth of micro-organisms (as noted above, pages 6 and 7).

Extracellular Free Amino Acids of the Blood

Evidence has been accumulating that extracellular essential amino acid levels in rats (McLaughlan, 1964; Clark et al., 1966; Harker et al., 1968; Anderson et al., 1968), poultry (Richardson et al., 1953; Tonkinson et al., 1959; Hill and Olsen, 1967; Zimmerman and Scott, 1967), pigs (Puchal et al., 1962; Richardson et al., 1965; Braude et al., 1969) and man (McLaughlan et al., 1963; Swendseid et al., 1966; Adibi, 1968; Young et al., 1970) are rapidly affected by dietary changes in amino acid levels. In fact, several investigators (Denton and Elvehjem, 1954; Longnecker and Hause, 1959; McLaughlan et al., 1963; Sanchez, 1969; and Buraczewski et al., 1967) have shown plasma amino acids were proportional to the amount of the amino acids supplied by ingested protein, but Harker et al. (1968) found only the concentration of lysine in plasma related to the dietary intake of amino acids at four different levels. While Almquist (1954), Gray et al. (1960), McLaughlan (1964), and Clark et al. (1966) reported that dietary deficiencies of single essential amino acids may result in depressed plasma levels of that essential amino acid, a low plasma amino acid level need not always indicate a dietary amino acid deficiency; in fact, plasma levels of essential amino acids were not elevated until dietary intake exceeded that found necessary for maximum growth (Morrison et al., 1961; Zimmerman

and Scott, 1967). On the other hand, Peng et al. (1969) have pointed out that accumulation of some essential amino acids in plasma could be a false signal of adequacy if an imbalanced diet had been ingested. Nevertheless, much confidence has been placed in Almquist's suggestion that the amino acid levels of plasma might be considered an indicator of dietary amino acid adequacy in establishing amino acid requirements, for a plasma amino acid ratio was developed by Longnecker and Hause (1961) and a plasma amino acid score by McLaughlan (1964) for the detection of the limiting dietary amino acid. The determination of extracellular free amino acids has been used clinically, also, in the nutritional assessment of human subjects (Swendseid et al., 1963a; Whitehead and Dean, 1964) for usually excesses or deficiencies in the dietary intake are exaggerated in the plasma (Almquist, 1954; Dean and Scott, 1966). In a review of the relationship between protein quality and plasma amino acid levels, McLaughlan (1963) concluded "plasma amino acid levels cannot be used directly to compare the protein quality of one protein with another" but suggests they may be useful in evaluating the availability of amino acids in processed foods.

Extracellular amino acid concentrations, however, are not only influenced by the amino acid pattern of ingested protein, but also by many other factors: 1) the type of protein consumed prior to the test period (Anderson et al.,

1969; Peng et al., 1969), 2) the amount of amino acids consumed during the test (Peraino and Harper, 1962; Alam et al., 1966; Peng et al. 1969), 3) consumption of other nutrients, such as sugars and vitamins (Richardson et al., 1953; Charkey et al., 1954; Swendseid et al., 1967), 4) rate and extent of amino acids released during digestion and their rate of absorption (Nasset and Ju, 1961; Goldberg and Guggenheim, 1962; Snook, 1965; Smith, 1966; Bergen and Purser, 1968), 5) the synthetic and degradative capacity of the animal (Anderson et al., 1968; Anderson et al., 1969; Peng et al., 1969), 6) the amount of amino acids required for protein synthesis, 7) hormones (Leung et al., 1968a; Laudau and Lugibihl, 1967), 8) time of sampling the blood (Longnecker and Hause, 1961; McLaughlan et al., 1963; Clark et al., 1966; Boomgardt and McDonald, 1969); and 9) source of blood, whether arterial or venous (Denton and Elvehjem, 1954; Peraino and Harper, 1962). Peng et al. (1969) have suggested that food intake regulation is an important homeostatic mechanism for the control of plasma concentrations of essential amino acids, low levels of the latter stimulating appetite and high levels depressing appetite.

Growth, Development and Tissue Protein Metabolism

That protein is fundamental to life itself was first

demonstrated when Magendie (1816) showed that dogs fed diets containing only carbohydrate and fat, did not survive. Almost a hundred years later, Van Slyke and Meyer (1913) found that protein entered the blood from the intestine in the form of amino acids which were taken up by the tissues within thirty minutes and retained as protein. In analytical studies, Moulton (1923) compared developmental changes of several different mammalian species with man; he defined chemical maturity as the stage of development at which concentration of water, proteins and salts became comparatively constant, and he related chemical development to the degree of physical development at birth. Decades later, confirmation of this relationship was presented by Widdowson and Dickerson (1964) for eight mammalian species. A recent review (Miller, 1969) focuses on the cellular changes related to protein metabolism during the immediate postnatal period.

Changes in Cellular Constituents during Growth

In an extensive experiment, Enesco and Leblond (1962) weighed each organ and determined the DNA content of rats during different periods from birth to 160 days. They reported that three periods of development exist, as follows: 1) from birth to seventeen days, rapid growth of tissues is due almost exclusively to nuclear mitosis resulting in cell addition, hyperplasia; 2) from seventeen to about

34-48 days growth continues by cytoplasmic enlargement of cells as well as by increase in the number of cells, the latter at a reduced rate; 3) from 48 days on, growth is now mostly due to an increase in the size of cells, hypertrophy. When Winick and Noble (1965) studied growth and development of rats from ten days after conception to maturity (99 days) they also found an increase in DNA content that corresponded with the first stage of growth; in addition, an increase in protein per nucleus as well as a continuing increase in DNA at a slower rate reflected the second period of growth while the third period showed an increase in protein until a constant level was reached and growth ceased. In all organs and tissues studied, levels of RNA increased proportionately with DNA concentration, a high RNA/DNA ratio occurring earlier in development than the protein increase in the same tissues. The data of Enesco and Leblond (1962) and Winick and Noble (1965) also showed that while the sequence of events is similar, the extent of hyperplasia varies from one tissue to another.

1. Liver

Hyperplasia occurs at the cellular level in liver, according to Goss (1966). Evidence for this was supplied by Enesco and Leblond (1962) who found the number of nuclei increased markedly with age; this was reflected also in DNA content of liver which ranged from 1.41-12.18 mg/liver from

7-35 days and then increased more slowly to 25.22 mg/liver by maturity. Histological liver sections showed considerable increase in the size of parenchymal cells between 7-34 days followed by a small increase up to 95 days. The data of Winick and Noble (1965) showed 1) rapid cell division from about 16 days after conception to 44 days post-natally followed by a declining increase up to 99 days, as indicated by DNA content of liver and incorporation of ^{14}C-thymidine during normal growth in the rat, and 2) an increase in protein content from 28.2-121.8 mg/liver from 17 days after conception to 14 days post-partum preceding a rapid increase up to 4240 mg of protein/liver by 99 days. This retardation of liver growth in the neonate was evident also in Enesco and Leblond's work (1962) for the mean weight of rat liver by the seventeenth day after birth was only three times greater than that at 7 days but 37 times greater by maturity. Unlike other tissues, Winick and Noble (1965) found an increase in RNA/DNA ratio in liver between birth and 4 days of age with no increase in the concentration of RNA beyond 14 days, so they assumed that RNA reached its final content per cell during early growth when cells were dividing rapidly. The work of Geschwind and Li (1949) indicated that RNA continued to increase up to 40 days.

2. Skeletal muscle

Goss (1966) indicated that hyperplasia takes place

in skeletal muscle by regeneration or increase in myofibril number but only within the confines of the muscle fiber. A steady increase in weight of the muscle was observed by Enesco and Leblond (1962) when they examined gastrocnemius muscle at 17, 34 and 80-95 days. The increase lessened somewhat, however, after 34 days of age. In fact, as a percent of total body weight, skeletal muscle weight rose from 23% at birth to 45.5% at 140 days, indicating a more rapid increase in weight than that of the body. A steady increase in DNA content was also found from birth onward for the number of nuclei rose steadily throughout and weight per nucleus rose steeply between 17 and 34 days and to a lesser extent between 34 and 95 days. Gordon et al. (1966) also found a linear increase of DNA in quadriceps from 43 to 90 days but no increase during the 50 days that followed. Histological sections of Enesco and Leblond (1962) as well as the increase in protein concentration noted by Gordon et al. (1966) indicated progressive hypertrophy of muscle fibers with age. Winick and Noble (1961) found a high RNA/DNA ratio long before the high protein/DNA ratio was evident so tissues active in protein synthesis also have a high concentration of RNA. Howarth (1969), also, observed that rates of protein synthesis, measured by in vivo and in vitro techniques, were closely associated with rates of RNA and DNA synthesis.

In terms of concentration per 100 g of tissue, Miller (1969) graphically (Fig. 1) indicated that 1) DNA reached its highest peak at birth in both liver and skeletal muscle but declined rapidly in the former compared with the slow levelling off in the latter; 2) liver RNA attained its highest concentration from 7-14 days of age declined at a similar rate up to twenty days before declining at a much slower rate; but, in muscle, RNA was at its maximum at birth and gradually tapered off at a slow rate up to and beyond 25 days; 3) protein synthesis in the liver was at its highest between 15 and 25 days, but in skeletal muscle after a definite neonatal decrease, protein synthesis increased temporarily until the twelfth day when a rapid increase continued until maturity.

Nutritional Implications

For normal growth and development, adequate supplies of nucleic acids and protein are a necessity. De novo biosynthesis of precursors such as nucleotides and nucleosides are easily synthesized from several small molecules. Thus various components of the purine ring can be derived from formate, carbon dioxide, glutamine, aspartic acid and glycine while pyrimidine bases start with carbamyl phosphate and aspartate. Of far more importance, however, are the enzymes required for these synthetic processes. Thus, even without consideration of the protein synthesis necessary for

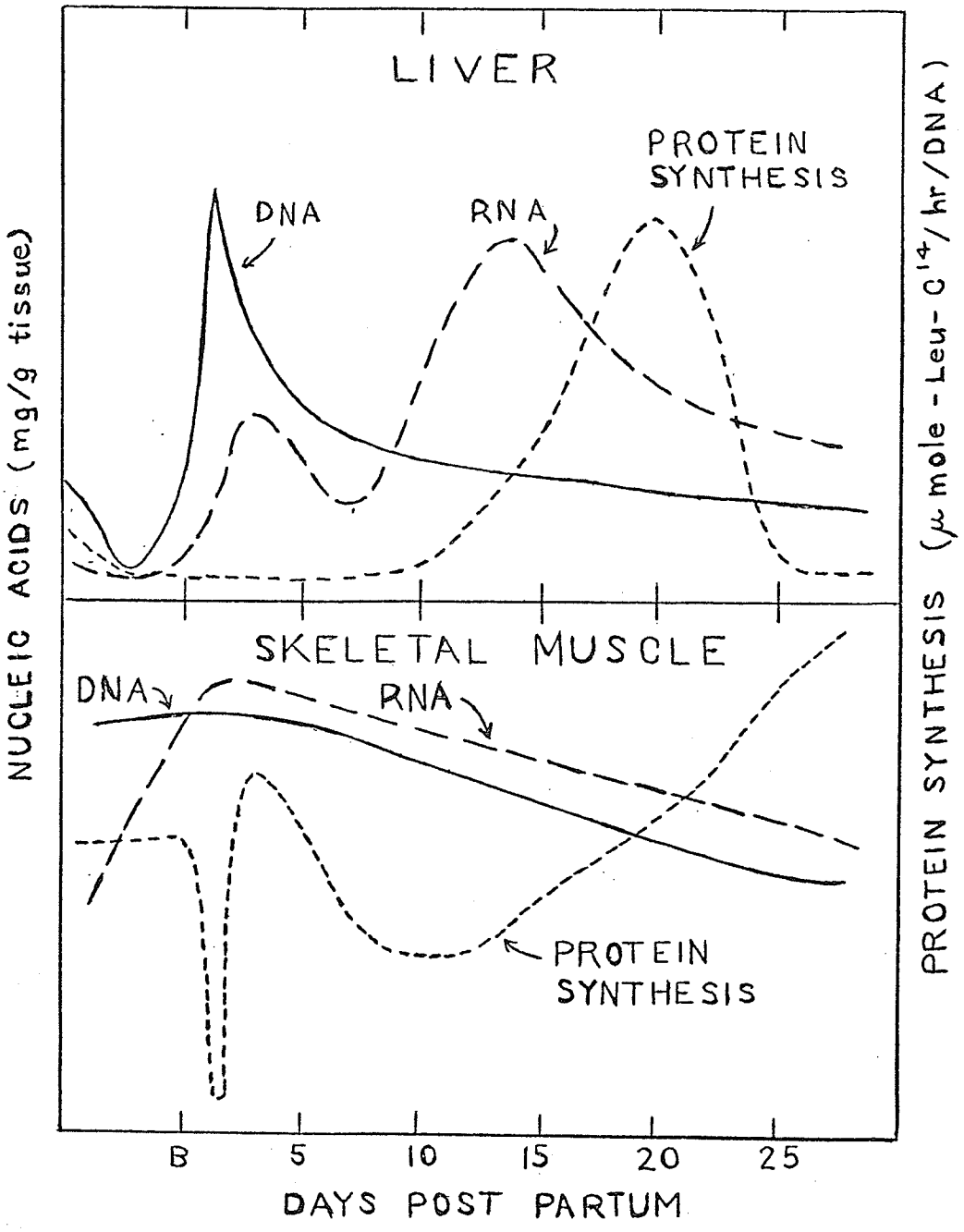

Fig. 1. An integrated picture of immediate pre-natal and post-natal development of protein metabolism in liver and skeletal muscle of the rat (From Miller, 1969)

cytoplasmic enlargement of cells, protein metabolism with its regulation by synthetic and degradative enzymes, hormones and free amino acid pools, is implicated. There is evidence for these involvements in most organs and tissues of the body.

In fact, the literature abounds with studies showing the speed at which liver responded to change in dietary protein. For example, Addis et al. (1936) demonstrated that liver protein was reduced by 20% after two days on a protein free diet and Kosterlitz (1947) showed that there was also a reduction in phospholipids and RNA in the liver when the amino acid supply from the diet was reduced. This quick response of the liver indicated to Munro (1968) that 1) it was related to the immediate amino acid supply to the liver, and 2) there was an integration in metabolic responses since protein, RNA and phospholipid concentrations were affected by intake of protein. In studies with dogs weighing 16.0, 20.4 and 28.3 kg, Elwyn (1970) found 57% of absorbed amino N was converted into urea as it passed through the liver, 6% left the liver as plasma protein, and only 23% entered the general circulation as free amino acids; the remaining 14% possibly was retained as hepatic protein. Although research on skeletal muscle, has not been as extensive as on liver, Allison et al. (1963) found that the adaptive reactions in both liver and muscle play an important role in the

utilization of dietary protein.

1. DNA

The effect of diet on DNA content of the rat is most critical at the post-natal preweaning stage. Work by Winick and Noble (1961) related the state of nutrition during the neonatal period to the rate of cell division and the final number of cells in the organs of the rat. They found rats from small-sized litters had a greater growth rate and a greater number of normal sized cells than less adequately nursed neonatal rats from litters three times the size of the former. Recently, Howarth (1970) found that undernutrition and protein deficiency retarded protein and DNA accumulation in the skeletal muscle of weanling rats and young chicks, the effect on DNA being more severe; repletion of the rats resulted in DNA but not protein being accumulated at a faster rate than was normally found. During calorie restriction (Winick and Noble, 1966) from 1) birth to weaning, 2) weaning to 42 days of age, and 3) 65-85 days of age, there was a corresponding decrease in DNA, RNA, protein and weight of all organs in 1), in 2) except for DNA content of brain and lung, and in 3) except for DNA other than in spleen and thymus. The lung and brain in 2) and all organs in 3) except thymus attained normal size upon refeeding an adequate diet. Thus, recovery depended on the age of onset of the malnutrition, the earlier it occurred the more drastic

the effect and less chance for repletion, hyperplasia being the forerunner of hypertrophy.

The DNA content of diploid nuclei in interphase has often been used as a point of reference in analysis of tissue (Thomson et al., 1953) because of its constancy in "mature" organs. Among the investigators who questioned this constancy of DNA concentration were Mendes and Waterlow (1958), Umana (1965) and Haider and Tarver (1969) who found increased DNA content of isolated cell nuclei and DNA concentration per gram of liver weight when rats were starved or fed diets ranging in protein content from 0-6.4%. However, a 50% dietary restriction (Elliott and Cheek, 1968) resulted in a significant loss of DNA per gram of liver and muscle. Obviously, further research is necessary to determine mechanisms connected with a DNA increase in liver cells, supposedly no longer susceptible to mitosis, in some instances when protein intake is greatly reduced.

2. RNA

The RNA content of normal liver cells varies with the level of ingested protein. The nutritional regulation of protein and RNA metabolism in liver cells has been studied extensively by Munro and co-workers. Changes in RNA content, as noted by Munro and Clark (1960) seemed to be associated with the endoplasmic reticulum in the normal liver cell as no changes were observed in malignant cells

devoid of endoplasmic reticulum. Protein intake found to affect the nucleolar RNA of rat livers (Munro et al., 1965), however, was considered of little importance; but of concern was the 20% loss of liver RNA during protein depletion as possibly it represented ribosomal RNA which makes up 80% of the total RNA. Studies (Clark et al., 1957) to determine whether this was the result of decreased synthesis or increased degradation of RNA suggested that withdrawal of the dietary supply of amino acids accelerated RNA breakdown in liver cells. Accordingly, research at the ribosomal level by Wunner et al. (1966) showed that when rats were fed a tryptophan-deficient mixture, there was a change in the polysome profile. Polysomes disaggregated while the population of monosomes and free ribosomal sub-units increased, the latter event causing activation of latent ribosomal ribonuclease which degraded the RNA that was no longer combined with ribosomes to form polysomes. Indeed, ribonuclease which apparently is resistant to depletion has been shown (Zigman and Allison, 1959; Allison et al., 1962) to have an inverse relationship to both quantity and quality of amino acids supplied in the diet.

Some of the more pertinent facts on protein synthesis in skeletal muscle as related to nucleic acids and brought out in a review by Young (1970) may be summarized as follows: 1) While a large proportion of the DNA of muscle

is present in the non-contractile cells, RNA is thought to derive mainly from contractile cells. 2) There are more free ribosomes in skeletal muscle than in liver but bound ribosomes are more plentiful in the liver. 3) During protein synthesis in muscle cells, polysomes containing as many as 50 ribosomes aggregate on messenger RNA. 4) Proteins, such as actin and tropomyosin are not synthesized simultaneously.

3. Protein

In light of the full array of essential amino acids required for polysomal formation, adequate supply of RNA, and eventual protein synthesis, it is perhaps redundant to consider the effect of diet on the protein content of tissues and organs since it has been reviewed by Waterlow (1969). Nevertheless, there is a great deal of interest in the synthesis (reviewed by Korner, 1964) and degradation (reviewed by Neuberger and Richards, 1964) of protein in estimating the turnover rate of protein in the various tissues. Such attempts, whether by the method of 1) San Pietro and Rittenberg (1953), 2) cumulative excretion of ^{15}N-labeled compounds (Olesen et al., 1954; Wu and Bishop, 1959), 3) rate of disappearance of labeled free amino acid from the blood after single injection (Grümer et al., 1961) and 4) constant infusion (Waterlow and Stephen, 1967; Waterlow, 1967; Gan and Jeffay, 1967; Waterlow and Stephen, 1968) are dependent

on the acceptance of certain assumptions and approximations. Such experimentation, however, does serve a purpose, especially if used as the basis of comparison.

Work by Widdowson and McCance (1956) indicated that there may be a specific species response to dietary intake. Recognizing the possibility that each organ or tissue of a particular species may have a characteristic way of adapting to a situation, researchers studied the effect of dietary protein concentration on the various organs. Mendes and Waterlow (1958) found weanling rats maintained on a 6.4% protein 'Jamaican diet' for 28 days did not grow; the liver, muscle and body weights remained constant even though protein per gram of tissue decreased slightly in muscle. A similar effect was noted by Allison et al. (1963) for DL-methionine-^{35}S concentrated more in the liver than in the muscle after rats had been fed a protein-free diet or starved. On the other hand, Waterlow and Stephen (1966) found that while liver contributed 7.8% to the total nitrogen losses of rats fed a protein-free diet for three days muscle showed no loss of nitrogen. The liver of rats fed a 6% casein diet from 5-8 weeks or from 5-8 weeks followed by a protein-free diet for three days, however, did not contribute as much to the total N deficit as it did in acute depletion, for liver losses were 4% of the total N lost in each instance; muscle over this 5-8 week period of depletion

lost considerably more N than the liver for 26 and 30% of total body nitrogen losses, were contributed by muscle of rats fed the low protein and protein-free diets, respectively, muscle N depletion being fairly proportional to total body N. It is evident, therefore, that the amount of protein lost as a result of malnutrition varies in different tissues and over a period of time. Numerous other studies have shown that the protein content of the liver changed rapidly in proportion to the protein content of the diet, while muscle response was much slower. Using various techniques, many investigators have made an attempt to determine the adaptive reactions of a biological system and/or its component parts to variation of protein intake. While the results are overlapping in scope, they might best be categorized under the following subheadings:

a) Synthesis

Evidence indicates that the distribution of a labeled free amino acid is dependent on the dietary state of the biological system, the needs of the most crucial metabolic processes being satisfied first. This was observed by Waterlow (1959) for three days after he injected DL-methionine-^{35}S into adequately fed rats and protein-depleted rats, he found 75% of the label in the carcass and 25% in the visera of the former, but 50% in the internal organs of the latter. Also, repletion of the rats depleted on the 'Jamaican diet'

(Mendes and Waterlow, 1968) was rapid in the liver but not so in the muscle. The rate of protein synthesis 4 hours after injection of DL-lysine-1-^{14}C in rats fed a high, normal or low protein diet was similar but it was slightly higher for rats on a protein-free diet (Haider and Tarver, 1969). Waterlow and Stephen (1966) again found depleted rats retained a higher ratio of activity in the internal organs and less in the carcass than normal rats. At the ribosomal level, Cooper et al. (1968) observed no difference between uptake in the liver and muscle, possibly because of the method used to prepare the ribosomes. Although Short (1969) found labeled methionine incorporation into protein was more rapid in red muscle containing mostly stromal protein, than in white muscle containing mostly sarcoplasmic protein, Waterlow and Stephen (1966) noted no significant difference in muscular uptake of labeled lysine between the sarcoplasmic and fibrillar fractions of the muscle even though there was a difference in the well-nourished and protein-deprived rats. Consequently, Waterlow's group (1966, 1967, 1968) concluded that low protein diets resulted in a real decrease in the rate of amino acid uptake into muscle protein generally, and that rats adapted to dietary protein intake by change in the synthetic pattern.

Other factors influencing the uptake of labeled amino acid into cellular protein were: 1) age, for Waterlow

and Stephen (1966) and Srivastava and Chaudhary (1969) found less incorporation of L-lysine-^{14}C into the various fractions of muscle with increasing age of the rat, paralleling a decrease noted in muscle RNA by Waterlow and Stephen and reduction in polysomal content found by Srivastava and Chaudhary; 2) hormones, for both growth hormone and insulin are needed not only to transport amino acids into cells but possibly to stimulate more than one step in the activation of amino acids into cellular proteins (Snipes, 1968; Waterlow and Stephen, 1968; Short, 1969); 3) membrane permeability for Henriques et al. (1955) postulated that there is a barrier to the entry of amino acids into muscle cells and Waterlow and Stephen (1967) found some evidence to indicate lysine penetrated muscle tissues more slowly than other amino acids.

Consideration of the mechanism(s) that may control protein synthesis has implicated factors at the subcellular level, also, for incorporation of a labeled amino acid was 32-54% less into microsomes prepared from perfused and non-perfused livers of fasted rats (Rothschild et al., 1968), and about 50% less into ribosomes of rats on a low protein diet six days (Young and Alexis, 1968). Young and Huang (1969) also found reduced amino acid incorporation by ribosomes of right thigh muscles of rats with a 3-day old fracture in left femur and fed a 25% casein diet for 14 days; incorporation

in the liver was similar for injured and uninjured rats. The attachment of mRNA to ribosomes can be inhibited by antibiotics such as chloramphenicol (Murthy, 1966). Enzymes of protein metabolism have been implicated also for Mariani et al. (1963), Gaetani et al. (1964) noted an increase in the activity of the amino-acid activating enzymes in the liver of protein-depleted rats while Stephen (1968) found a similar increase in the liver of twenty-two malnourished infants. Mariani et al. (1963) and Gaetani et al. (1964) also found activity of the amino acid activating enzymes was less than normal in the gastrocnemicus of protein-deprived rats, thus suggesting that the amount of protein synthesis is regulated through modifications in the level of amino acid activating enzymes. On the other hand, Schimke (1962a, 1962b) observed a decrease in the urea cycle enzymes under similar conditions but there was an increase in activity upon refeeding.

b) Catabolism

Most of our knowledge on protein catabolism is based on what has been known for years. Once proteins are synthesized in mammalian cells they are degraded at their own characteristic rates (Sprinson and Rittenberg, 1950; Velick, 1956). Well-known is the fact that when protein intake is decreased a fall in urinary nitrogenous products follows. Stephen (1968) found there was a reduction in urinary

excretion of N products as great as 33-50%. And, since there was no change in catabolism between adequately-fed and nutritionally-deprived rats, Waterlow (1968) suggested that a large proportion of the amino acids liberated by catabolism are re-synthesized into protein within the same cell. Gan and Jeffay (1967) found re-utilization of endogenous amino acids varied with nutritional status, as much as 50% in the liver and 30% in the muscle for normally-fed rats, with 90% in the liver early in a fast and 65% in muscle after a period of fasting. Sidransky and Verney (1970), found skeletal muscle protein breakdown contributed amino acids to the blood from which they were incorporated into hepatic protein when animals were force-fed a threonine-deficient diet for only one day. Unlike protein synthesis, a missing amino acid did not influence degradation of a protein for when Fashakin and Hegsted (1970) varied lysine in the diet of rats and then infused L-valine-^{14}C or vice versa, the loss of activity observed was not affected by the degree of an amino acid deficiency.

Goldberg (1969a, 1969b) conducted studies to determine rates of protein catabolism in non-growing skeletal muscle of hypophysectomized rats. He found protein catabolism more decreased in sarcoplasmic than in myofibrillar muscle, as well as an increase in synthesis of new protein when hypertrophy was induced by sectioning the tendon of the

gastrocnemius muscle two days after injection of ^{3}H-leucine. Under similar circumstances, two hormones were injected separately and the response to each was noted as follows: ten days after the administration of growth hormone, there was an increase in protein synthesis in the muscle but no change in the catabolic rate; cortisol acetate, on the other hand, increased protein degradation of sarcoplasmic and myofibrillar muscle, similarily, but decreased protein synthesis.

c) Turnover

From isotope studies on muscle proteins, Velick (1956) concluded that individual muscle proteins turnover at different rates. However, turnover of the total proteins making up a tissue or organ does not decrease during adaptation of a rat to lower protein intakes (Waterlow and Stephen, 1968) as the fractional liver turnover rate was 0.80/day for rats starved two days, 0.86/day for rats fed a protein-free diet 3 days, 0.96/day for rats fed a low protein diet for 10 days and 0.61/day for rats fed a low protein diet for 5 weeks, all within the statistically non-significant range of 0.76/day for the control rats. In other turnover studies by Waterlow and Stephen, lysine flux 1) decreased significantly with advancing age (Waterlow and Stephen, 1967) in both liver and muscle protein (Waterlow and Stephen, 1968), 2) was lower for female rats than male rats (Waterlow and Stephen, 1967 and 1968) and for alloxan-induced diabetic

rats compared with normal rats (Hay and Waterlow, 1957).

4. Intracellular free amino acids

Retention of intracellular free amino acids requires a balance between the production and utilization of amino acids. Amino acids are supplied by a release of amino acids during protein catabolism in the tissues, synthesis of non-essential amino acids from suitable precursors, and acceptance of essential as well as non-essential amino acids from the circulatory system. On the other hand, the free amino acids of intracellular spaces are utilized in the synthesis of protein and non-nitrogenous compounds, released to extracellular spaces, or degraded for energy purposes.

That intracellular free amino acid pools are receiving a constant supply of free amino acids from breakdown of tissue protein has been demonstrated by Gan and Jeffay (1967). When labeled lysine was infused into normally fed rats, the intracellular specific activity in the liver reached a plateau within 60 minutes but never attained the high levels of the extracellular specific activity. This indicated an intracellular dilution, which in the case of lysine or other essential amino acids could not result from synthesis, if the assumption is that free amino acid pools are homogenous. If pools are not homogenous, as indeed Waterlow and Stephen (1968) have indicated they are not, then consideration must be given to the presence of non-essential amino acids as

contributing to the dilution of the specific activity in the intracellular pool. In fact, when labeled lysine was infused at a constant rate into rats fed normally for periods up to 20 hours (Gan and Jeffay, 1967), the ratio, liver lysine/plasma lysine specific activities rose to a plateau at a value of about 0.4-0.45 after 45 minutes and remained essentially constant for at least 10 hours before gradually increasing to 0.6 by 20 hours, this last increase indicating that there was an increase in the labeled intracellular pool as a result of degradation of labeled proteins in the cell. Uptake of amino acids by thigh muscle cells was much slower for it took at least ten hours to plateau at a muscle lysine/plasma lysine specific activity ratio of 0.65-0.75. A similar study with the non-essential amino acid, tyrosine, indicated that during the three hour period of the study, results were similar to those found for lysine. Thus, it was concluded that 40-45% of liver lysine and 70% of muscle lysine came directly from the plasma. In addition, a reverse type of experiment showed that protein catabolism continued even in the presence of a more than adequate supply of plasma amino acids. Twenty hours after intraperitoneal injection of labeled lysine when Gan and Jeffay (1967) infused unlabeled lysine for periods up to two hours, the specific activities of both intracellular liver lysine and muscle lysine were reduced 28% and 43%, respectively, by

the end of the infusion period.

Gan and Jeffay (1967) also considered the influence of the size of intake on the amount of intracellular free amino acids contributed by cellular protein catabolism. When large amounts of labeled lysine were infused for one hour, there was an increase in lysine concentration, above perfusion levels, of 66% in plasma, 179% in liver and 100% in muscle. Since the infusion rate was greater than the utilization rate, the size of the intracellular pool was enlarged with the contribution from the plasma to the liver being increased to 90%. When rats, fasted for varying periods of time, were infused with trace quantities of labeled lysine for ten hours, liver lysine pools that remained essentially constant throughout a seven-day fast received about 90% of their lysine from breakdown of protein by the second day; degradation then gradually decreased to about 50% until a normal uptake of free amino acids was attained from the plasma; the ratio of muscle lysine: plasma lysine, on the other hand, gradually decreased from 60% to 40% over the period of the seven day fast. These findings suggest that amino acid intracellular levels were maintained by breakdown of the more labile protein of liver and other viseral organs during the first days of a fast and by degradation of the less labile protein of muscle in the later stages of the fast (Munro, 1964).

Elucidation of the precise mechanisms involving amino acid metabolism will require, however, more than *in vivo* and *in vitro* incorporation studies using labeled amino acids. Indeed, Scharff and Wool (1964) have questioned the reliability of what they called these "black box" investigations which may or may not accurately reflect the concentration ratio of unlabeled amino acids; they argued that chemical analyses are needed to determine the exact amount of free amino acids in tissues. Considerable extracellular and intracellular determinations were made previously, however, by Elvehjem's group using microbiological methods. Schurr *et al.* (1950a, 1950b) and Thompson *et al.* (1950) compared free amino acid levels in the tissues of the mature rat fasted from 1/2-7 days and then fed a nitrogen-free diet from 1-21 days or an 18% casein diet for 15 days (Table 1).

The short term study of Schurr *et al.* (1950b) indicated that regardless of the dietary regimen followed 1) leucine, phenylalanine, tryptophan, valine and isoleucine were 2-4 times higher in liver than in muscle, 2) histidine, lysine and tyrosine were slightly higher in liver than in muscle, while 3) proline, methionine, threonine and arginine were similar in both tissues. Comparison of treatments for each tissue showed 1) no consistent trend in liver except that the total values for the fasting rats, regardless of size, were higher than for fed rats, 2) the total amino acid

Table 1. Summary of studies determining intracellular concentration of amino acids of muscle and liver as influenced by diet

Author	Weight of rats (g)	Test diet (ad libitum)	Time on diet
Schurr et al. (1950)	300-350 (5)[x]	18% casein[1]	12 hours
	350-400 (9)[x]	None[1]	12 hours
	200-250 (9)[y]	None[1]	12 hours
Thompson et al. (1950)	250-300 (9)[x]	18% casein[1]	1, 3, 5 and 7 days
	250-300 (9)[x]	None[1]	1, 3, 5 and 7 days
	250-300 (9)[x]	Nitrogen-free[1]	1, 3, 5, 7, 14 and 21 days
Ryan and Carver (1963)	90-120 (5)[x]	None[2]	12 hours
Allison et al. (1963)	Weanling (7)[z]	18% casein[2]	3 days
	Weanling (7)[z]	Nitrogen-free[2]	3 days
	Weanling (7)[z]	None[2]	3 days
Scharff and Wool (1964)	400 (3)[x]		

No. in bracket indicates number of rats on treatment

[1]Pretest diet of 18% casein for 2 weeks

[2]Presumed rats fed lab chow previous to experiment

[x]Sprague-Dawley rats; [y]Holtzman rats; [z]Wistar rats

values for muscle of fed rats was slightly higher than that of 200-250 g fasted rats, 3) all values were larger for muscle of the 350-400 g fasted rats than either of the other two groups. Relative concentrations of free amino acids in tissues of fed rats were highest 1) in plasma for tryptophan, lysine, tyrosine and methionine, 2) in liver for leucine, phenylalanine, valine, histidine and isoleucine, and 3) in muscle for proline, threonine and arginine.

The data of Thompson et al. (1950) indicated similar trends in the concentration of free amino acids in the liver and muscle of rats, regardless of the diet or the length of time on the diet, except for histidine, lysine and arginine which were higher in muscle than in liver. When rats were fasted seven days, 1) the concentration of most amino acids in liver decreased within 24 hours and approached or exceeded normal levels of control rats by the end of the fast, 2) in muscle, there was an increase in the concentration of most amino acids during the first twenty-four hours, with approximately normal levels being reached by the seventh day of fasting. Changes in the free amino acids in the tissues of rats fed a nitrogen-free diet were different than those of fasted rats for 1) the concentration of most amino acids of liver and muscle increased the first day and then most amino acids other than arginine, histidine and threonine tended to decrease, 2) the amino acid concentration in both

muscle and liver reached a minimum value by the end of the 2nd week and then remained more or less constant. In all cases, proline was significantly decreased by the dietary conditions imposed. The sum of the concentrations of the twelve amino acids for the fasted rats was less than that for the rats on the nitrogen-free diet, possibly because protein catabolism was less rapid in the latter instance. Since the weight loss of the rats on the nitrogen-free diet averaged 17% as compared with 25% for fasted rats over the 7-day period, less degradation of protein in rats fed the nitrogen-free diet is a definite possibility.

More recently, amino acid analyses of tissues have been accomplished by the use of automated ion-exchange chromatography. Scharff and Wool (1964) studied the plasma and muscle (diaphragm and heart) free amino acids of rats (Table 1). A comparison of essential amino acids from plasma, the only tissue analysis common to the studies of Schurr et al. (1950), Ryan and Carver (1963) and Scharff and Wool (1964) has been made (Table 2). The findings of Schurr et al. (1950) and Scharff and Wool (1964) were similar in spite of age differences of the rats and the rations consumed; determinations made microbiologically were higher by 4.9-48% than those obtained by ion-exchange chromatography, possibly because small peptides that may have been in the tissue extract, were utilized by micro-organisms. Values

Table 2. Comparison of essential amino acid concentrations of plasma as determined microbiologically (Schurr *et al.*, 1950) and by ion-exchange chromatography (Ryan and Carver, 1963; Scharff and Wool, 1964)

Essential amino acids	Plasma (μmoles/100 ml)		
	Schurr *et al.* (1950)	Ryan and Carver (1963)	Scharff and Wool (1964)
Arginine	18.4	7.5	17.5
Histidine	6.2	4.5	7.3
Isoleucine	10.1	6.7	8.8
Leucine	20.3	9.6	15.2
Lysine	39.7	26.6	34.6
Methionine	6.4	3.7	4.5
Phenylalanine	8.3	4.9	7.6
Threonine	37.3	16.7	25.2
Tryptophan	8.2	0.4	6.9
Valine	22.8	12.3	18.8

found by Ryan and Carver (1963) are much lower (21.6-133.3%) than those of Scharff and Wool (1964), no doubt because the younger rats were in a fasting state in the Ryan and Carver (1963) study. Scharff and Wool (1964) also found their values for alanine, aspartic acid, glutamic acid, glycine and serine from muscle were similar to those of Kaplan and Shimizu (1963) as well as those of Ryan and Carver (1963) for they made up about 75-79% of the amino acids of the muscles which had most of its ninhydrin-reacting substances in the form of ammonia and taurine.

When Allison et al. (1963, Table 1) compared intracellular free amino acid concentrations from liver and skeletal muscle as related to DNA, they found 1) the five non-essential amino acids mentioned above, were also the most highly concentrated in liver and skeletal muscle of rats fed an 18% casein diet, with the exception of aspartic acid in muscle, 2) alanine was most highly concentrated in the liver and muscle followed by glycine in liver, and taurine and glycine in muscle, 3) lysine of liver and muscle as well as threonine, leucine and valine of liver were the most highly concentrated among the essential amino acids. Rats fed a protein free diet for three days had lower concentrations of both essential and non-essential amino acids in liver and muscle except for tyrosine, phenylalanine, glycine and taurine in liver. With the exception of lysine, rats

fasted three days contained even less free amino acids in the liver and muscle but more lysine and taurine. More recently, Noda et al. (1969) showed that intracellular levels of free amino acids in liver and muscle initially decreased after feeding a 15% balanced amino acid mixture to rats for one hour after a 23 hour fast; then, they returned to original levels.

Pawlak and Pion (1967, 1968a, 1968b) have looked at the effect that an inadequate intake of one amino acid has on the tissues of the body. In a preliminary experiment (Pawlak and Pion, 1967), when 100 g Wistar rats were fed herring meal supplemented with methionine and wheat flour supplemented with tryptophan for 18 and 35 days, the lysine level in blood and muscle of rats fed the herring meal was about four and fifteen times higher, respectively, than that of rats fed the wheat flour. Also noted were the threonine levels: 1) for blood, 3.9 and 3.6 mg/100 ml in rats fed the herring meal 18 and 35 days compared with 1.9 and 1.7 for those fed the wheat flour for a similar period, and 2) for muscle, 12.9 and 12.0 mg/100 g wet tissue for the herring-meal fed rats compared with 7.2 and 6.9 for the wheat flour-fed rats, the latter suggesting that the lack of lysine in the wheat flour limited the utilization of threonine for protein synthesis in muscle. In later experiments (Pawlak and Pion, 1968a, 1968b), rats were fed wheat diets

supplemented with graded levels of lysine or threonine for two weeks only. The concentration of amino acids other than lysine and threonine were similar whether the limiting amino acid was lysine or threonine. The threonine levels of blood and muscle increased more slowly in rats fed the lower levels of threonine supplementing a wheat diet but began to accumulate in these tissues when 4.2 g of threonine/kg dry diet was consumed. Unlike lysine, however, the percent of increase of threonine in blood and muscle was augmented three and seven times, respectively, while the threonine content of both blood and muscle rose 10 times higher than that of unsupplemented wheat. Figure 2 shows that free lysine became more concentrated in tissues, particularly in muscle, for upon consumption of approximately 8.5 g and 12.5 g of lysine/kg dry matter, there was a large increase of free lysine in muscle with a constant concentration when animals were fed approximately 9.5 to 11.5 g of lysine/kg dry matter.

The effect of imbalanced diets on concentration of amino acids has also been studied. When adult rats (Sanchez, 1969; Sanchez and Swendseid, 1969) were force-fed either a complete amino acid mixture, a mixture devoid of methionine and cystine or a mixture containing 4% methionine, an inverse relationship was found between the amount of methionine in the diet and the concentration of serine and

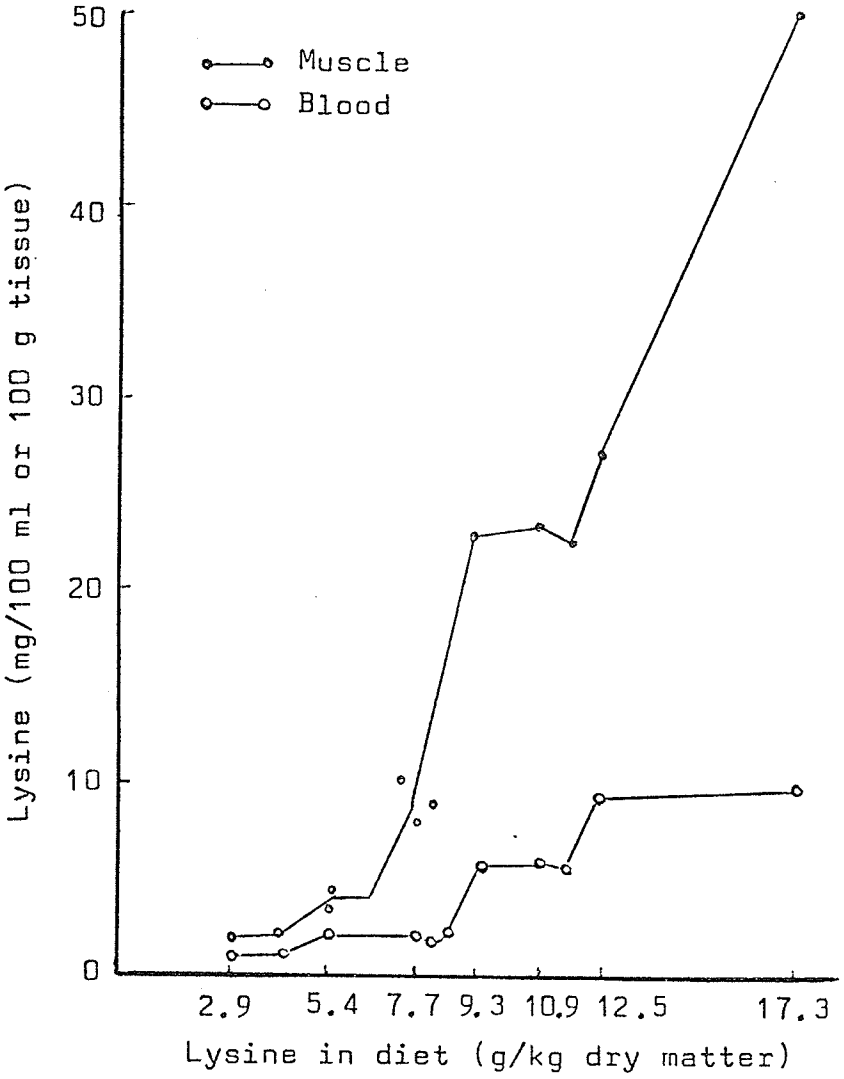

Fig. 2. Levels of free lysine in the blood and muscle of rats fed a wheat diet supplemented with graded levels of lysine (From Pawlak and Pion, 1968b).

glycine in plasma and liver for the activity of threonine dehydrase in the liver was influenced by the amount of sulphur-containing amino acids ingested. The effect of these diets on plasma and liver free amino acids was similar whether the study was 2-8 hours after rats ingested a single meal or eight hours after the last meal, following 3 days on the diets.

In a study by Leung et al. (1968b), 100-120 g rats trained to eat a single meal for 1 hour daily, were fed a basal diet consisting of a 6% casein diet supplemented with 0.3% DL-methionine or an imbalanced diet that contained the basal diet plus 5.4% of an amino acid mixture devoid of threonine. Rats were sacrificed at 1.5, 3, 5, 8, 13, 19 and 24 hours after feeding. Analysis of tissues revealed 1) the concentrations of all the essential and non-essential amino acids were higher at 3 and 5 hours in the livers of rats fed the imbalanced diet than in those of the rats on the basal diet, 2) most of the free amino acid concentrations in the muscle of rats on the imbalanced diet were either similar or lower than rats on the basal diet except for higher levels of lysine at 8, 13, 19 and 24 hours and 2 to 3 times the concentration of valine at 1 1/2, 3 and 5 hours in the muscular tissue of rats fed the imbalanced diet. Leung et al. (1968) reported the pattern of amino acids in the muscle more closely reflected those of the systemic plasma than did

the liver.

Young (1970), in his current summary of findings on free amino acids, reports that while there is little difference in concentration of most amino acids found in the plasma and in the muscle, threonine, histidine and all non-essential amino acids other than tyrosine are more concentrated in the muscle than in the plasma and that taurine has been noted by many (Awapara, 1956; Stern and Stim, 1959; Ryan and Carver, 1963; Boquet and Fromageot, 1965; a review by Jacobsen and Smith, 1968) to be exceedingly high in skeletal muscle, its slow turnover in muscle no doubt contributing to its concentration (Boquet and Fromageot, 1965). The high concentration of taurine in muscle probably is related also to inadequate essential amino acids for protein synthesis for Allison et al. (1953) found high levels of taurine in the muscle of rats fed a protein free diet or fasted, for three days. Further evidence of taurine synthesis has been reported by Fallon et al. (1968) for a 2% cystine or methionine supplement to a 2% casein diet resulted in increased levels of taurine in the liver.

Summary

Many investigators have shown that man and rat can adjust to a low protein intake by altering and diversifying metabolic pathways. There is no reduction in over-all nitrogen turnover but a smaller proportion of the available

nitrogen is excreted and a larger amount is recycled for protein synthesis. To a great extent, the latter is accomplished by a decrease in the synthetic rate of muscle protein as evidenced by a reduced concentration of muscle RNA and decreased synthetic activity of ribosomes isolated from muscle of rats. No data are available to indicate the role of free amino acids in skeletal muscle during these periods of adaptation to prolonged restriction of protein intake.

Restatement of the Problem

Studies to date indicate that adaptation to a change in quantity and quality of dietary protein is accomplished by a change in the protein synthesis. Most attention has centered on adaptation 1) during fasting and 2) diets that contain either no protein or low protein. In the latter case, the rate of synthesis in the liver remains normal or increases above normal but the synthetic rate in the skeletal muscle is greatly reduced, possibly making more amino acids available for synthesis in other tissues. How intracellular amino acids react and adapt to changes in the quantitative and qualitative intake of protein during development, needs further attention.

The objectives of this study, therefore, were 1) to determine, if and how, intracellular free amino acid concentration of skeletal muscle vary with ingestion of protein

differing in quantity and quality; 2) to find out a) the relationship between possible changes in free amino acid concentrations of skeletal muscle and those of liver and plasma, b) if the gastrocnemius is truly representative of most skeletal muscle; 3) to investigate the long term effect of a particular diet on a) the extracellular free amino acid levels of the blood and intracellular free amino acids of other tissues, b) the DNA, RNA levels of liver and skeletal muscle, and the extent of adaptation to the diets.

However, since the effect of protein intake is dependent on actual release and absorption of amino acids within a certain period, the biological availability of wheat was determined as it made up the greater part of two diets. The questionable influence of the microflora on the amino acids of the gastrointestinal tract suggested investigation to see if coprophagy prevention had an effect on amino acid availability.

MATERIALS AND METHODS

Male weanling rats of Wistar strain were housed individually in suspended wire bottom cages, in an environmentally controlled room at a temperature of 23°C. Diets used were made into pellets of 0.8 cm in diameter. The diet and water were fed ad libitum up to the time of sacrifice. During the adjustment period, twenty-three day old rats were fed wheat supplemented (SW) with soybean meal and fishmeal (Table 3). This diet met or exceeded the National Research Council (1964) recommendations in all required nutrients.

Availability Study

Twenty-four rats, weighing 30-42 g at the start of the adjustment period, were used in a study to determine the biological availability of amino acids from pure wheat. Four days later, the rats were divided at random into three groups of eight rats each, two groups being fitted with tail cups (similar to those described by Barnes et al., 1963) as follows: 1) to cover the anus and prevent coprophagy, 2) to act as a sham tail cup further down the tail but allowing coprophagy, while the controls wore no tail cups. The test period was begun four days after placement of tail cups when rats weighed approximately 80 g.

Rats were fasted the first day of the test period to

Table 3. Formulation and composition of diets for availability of amino acid study

Diets	Supplemented wheat (SW)	Unsupplemented wheat (W)	Nitrogen-free
Ingredients (%):			
Wheat	69.0	91.3	
Soybean meal	18.0		
Fishmeal	5.0		
Alfalfa	2.0	2.0	
Dextrose			75.0
Alphacel			20.0
Vegetable oil	3.0	3.0	5.0
Defluorinated rock phosphate	1.5	2.2	
Vitamin premix[x]	1.0	1.0	
Mineral premix[y]	0.5	0.5	
Chemical analysis (%):			
Nitrogen	4.21	2.45	

[x]Contains per kg diet: vitamin A (7150 IU), vitamin D_3 (818 ICU), vitamin E (5.5 IU), vitamin B_{12} (11 µg), vitamin B_{58} premix (5.5 mg riboflavin, 11.0 mg pantothenic acid, 16.5 mg nicotinic acid, 275 mg choline chloride), DL-methionine (0.5 g), menadione (1.1 mg), Santoquin (1.1 mg) and penicillin - streptomycin (26.4 mg)

[y]Contains per kg diet: manganese oxide (81.4 mg Mn), zinc oxide (11 mg Zn), ferrous sulfate (35.2 mg Fe), copper sulfate (6.6 mg Cu) and iodized salt (4.719 g)

induce normal consumption of the nitrogen-free diet (Table 3) on the second day. The test diet, pure wheat, was fed on the third day. Fecal collection was facilitated by including a marker, ferric oxide, in the wheat diets (unsupplemented and supplemented with protein, that is, W and SW diets, respectively, as listed in Table 3), each of which was fed to twelve rats on the fourth day. Rats were fed these diets throughout the remainder of the experiment. A similar availability study was conducted when rats weighed approximately 300 g. Rats wearing tail cups did so throughout the entire experimental period. The feeding schedule, the amount of diet consumed and feces produced are indicated in Table 4.

Fecal samples from any two rats within a specific group were pooled on the basis of similar consumption of food. Feces and diets were ground in a Glencreston micro-mill before drying of a two-gram sample in a vacuum oven at 90^{o}C for three hours. The moisture content of both diets and fecal samples was considered when calculating the dry matter consumption of diet and the production of feces.

While N content of diets and feces was determined by the macro-Kjeldahl method (Horowitz, 1965), samples for amino acid analyses were hydrolyzed according to the method of Bragg et al. (1966). Modifications included a hydrolysis period of 15 hours and reconstitution to a volume of 100

Table 4. Feeding procedures, amount of diet consumed, and feces produced by 80 g rats in the amino acid availability study

Sequence of diets[1]	Average consumption of diet (g)[2]		Average production of feces (g)[2]	
	Younger rats (80 g)	Older rats (300 g)	Younger rats (80 g)	Older rats (300 g)
None				
Nitrogen-free	9.65	16.54	1.55	3.14
Pure wheat	13.35	24.43	1.36	2.68

[1]Rats were fasted and fed each diet 24 hours

[2]Air-dried

milliliters (ml) with a sodium citrate buffer at pH 2.2. One half ml of each pooled sample was analyzed on a model 116 Beckman amino acid analyzer.

Tissue Study

Environmental conditions were similar for the tissue studies as those noted above. In addition, controlled lighting of 12 hours light and 12 hours darkness was used, for daily rhythm in some metabolic activities has been observed (Halberg and Barnum, 1961; Potter et al., 1966a, 1966b, 1967; Baril and Potter, 1968; Elwyn, 1970; Wurtman, 1970). In the following experiments, after a 2-day adjustment period on laboratory chow, base-line controls were sacrificed and 12 of the 25-day old rats were randomly assigned to each diet (Tables 5 and 6): 1) experiment I, rats weighing 58-92 g were fed diets varying in casein content (C-1, C-2, or C-3) or modified wheat-supplemented-with-protein diet (mod. SW); 2) experiment II, rats weighing 40-70 g were fed a 10.5% casein diet (C-2, see footnote x, Table 5) or a wheat diet not supplemented with protein (W, Table 5); 3) experiment III, rats weighing 40-70 g were fed the modified SW diet (mod. SW, Table 5) or the casein diets (C-1, C-2, C-3, see footnote x, Table 5). After rats had been fed these diets 4, 8 and 12 weeks, 4 rats from each diet were sacrificed. They were anaesthetized by intra-peritoneal

Table 5. Formulation and comparison of casein (C-1, C-2, C-3) and modified supplemented and unsupplemented wheat diets (mod. SW and W) used for tissue studies

Diets	C-1	C-2	C-3	Mod. SW	W
Ingredients (%):					
Casein	5.2	10.5	21.0		
Wheat (Pitic 62)				64.0	88.8
Soybean meal				13.0	
Fishmeal				5.0	
Alfalfa	2.0	2.0	2.0	2.0	2.0
Sucrose	70.3	65.6	56.2	9.3	
Alphacel	13.1	12.6	11.5		
Vegetable oil	5.6	5.6	5.6	3.0	5.5
Defluorinated rock phosphate	2.2	2.2	2.2	2.2	2.2
Vitamin premix[x]	1.0	1.0	1.0	1.0	1.0
Mineral premix[y]	0.5	0.5	0.5	0.5	0.5
Chemical analysis:					
Nitrogen (%)	0.95	1.81	3.29	3.32	1.97
Crude fat	6.0	5.8	6.1	5.3	6.8
Gross energy (cals/g)	4347	4355	4510	4695	4632

[x]Contains per kg diet: vitamin A (7150 IU), vitamin D_3 (818 ICU), vitamin E (5.5 IU), vitamin B_{12} (11 µg), vitamin B_{58} premix (5.5 mg riboflavin, 11.0 mg pantothenic acid, 16.5 mg nicotinic acid, 275 mg choline chloride), DL-methionine (0.5 g), menadione (1.1 mg), Santoquin (1.1 mg) and penicillin - streptomycin (26.4 mg). In addition, the following supplements were added to the C-1, C-2 or C-3 diets fed to rats after the 8th week for experiment I, the 2nd week for experiment II and for all of experiment III: thiamin (1.25 mg), pyridoxine (1.20 mg), choline chloride (750 mg), potassium diphosphate (4.1 g) and magnesium sulfate (20 g)

[y]Contains per kg diet: manganese oxide (81.4 mg Mn), zinc oxide (11 mg Zn), ferrous sulfate (35.2 mg Fe), copper sulfate (6.6 mg Cu) and iodized salt (4.719 g)

Table 6. Percent essential amino acid requirements of growing rat and content of diets used for tissue studies

Amino acid	Requirement[1]	Diets				
		C-1	C-2	C-3	Mod. SW	W
Essential:						
Arginine		.26	.49	1.01	1.48	.62
Histidine	.30	.16	.28	.63	.44	.24
Isoleucine	.50	.29	.57	1.08	.76	.40
Leucine	.80	.54	.98	1.97	1.42	.91
Lysine	.90	.50	.87	1.79	1.09	.32
Methionine[2]	.60	.17	.30	.52	.35	.22
Phenylalanine[3]	.90	.29	.54	1.17	.87	.61
Threonine	.50	.25	.43	.90	.72	.40
Valine	.70	.35	.69	1.28	.90	.56
Total		2.83	5.15	10.35	8.03	4.28
Non-essential:						
Alanine		.22	.36	.68	.98	.54
Aspartic acid		.48	1.24	1.49	1.90	.82
Cystine[2]						.10
Glutamic acid		1.38	2.60	5.00	4.46	3.74
Glycine		.14	.24	.43	1.00	.60
Proline		.33	.54	2.13	.72	1.30
Serine		.27	.41	1.16	1.00	.61
Tyrosine		.25	.52	1.08	.62	.38
Total		3.07	5.91	11.97	10.68	8.09
Amino nitrogen (%):		0.76	1.41	2.88	2.57	1.54
% total nitrogen:		80.0	79.9	87.5	77.4	78.2

[1]Required essential amino acids according to National Research Council (1962)

[2]Partially destroyed by acid hydrolysis; cystine may supply 1/3 to 1/2 of methionine

[3]Tyrosine may spare 1/3 of requirement

injection of pentophenobarbital. At least 5 ml of blood were taken by syringe from the posterior vena cava and immediately centrifuged to obtain serum. Liver and gastrocnemii from both hind legs, as well as all other leg and thigh muscles were excised, immediately freed of all visible fat and connective tissue, dipped in physiological saline, blotted dry and frozen on dry ice. The weight of each sample was recorded.

Preparation of Samples

Deionized water was used in the preparation of all homogenates and in the isolation of nucleic acids. One gram of the recently thawed gastrocnemii, leg and thigh muscles or liver of each rat was homogenized in 6 ml of water by a polytron (Brinkman Instruments, model PT-10) homogenizer. This homogenate was further diluted to 20 ml with water and used in the isolation of RNA and DNA according to the modified Schmidt-Tannhauser method as described by Fleck and Munro (1962) and Wannemacher et al. (1965), respectively. The samples were prepared for analysis of free amino acids by precipitating the protein of the supernatant of 2 ml of the original homogenate with 1 ml of 10% sulfosalicylic acid, centrifuging at 39,000 x g for 20 minutes and bringing supernatant to volume of 6 ml with sodium citrate buffer at pH 1.8. A previous investigation indicated there was no appreciable difference in the levels of free amino acids

Table 7. Free amino acid means of a single liver homogenate after precipitation of protein by 3, 10, or 20% sulfosalicylic acid (SSA)

Amino acids (μmoles/100 g)	3% SSA	10% SSA	20% SSA
Essential:			
Histidine	11.2±0.09	11.5±0.07	10.5±0.06
Isoleucine	10.2±0.00	9.9±0.06	10.5±0.00
Leucine	22.4±0.11	22.8±0.10	22.6±0.02
Lysine	27.8±0.25	27.1±0.10	26.4±0.15
Methionine	8.7±0.02	9.2±0.00	8.5±0.07
Phenylalanine	7.1±0.21	9.3±0.07	8.8±0.09
Threonine	17.7±0.40	23.3±0.40	16.3±0.35
Valine	16.9±0.09	17.0±0.11	18.0±0.08
Total	122.1	133.2	121.7
Non-essential:			
Alanine	93.9±1.02	97.4±0.42	98.0±0.43
Aspartic acid	36.8±0.31	46.7±0.52	39.7±0.37
Cystine	1.9±0.05	10.9±0.43	5.2±0.52
Glutamic acid	75.7±0.51	76.7±0.16	81.1±0.20
Glycine	52.2±0.60	54.1±0.12	57.2±0.30
Proline	6.8±0.08	7.7±0.06	6.6±0.02
Serine	29.9±0.11	32.2±0.28	34.4±0.24
Tyrosine	7.5±0.07	8.2±0.04	8.4±0.02
Total	305.2	339.3	330.8
Total amino acids:	427.3	469.5	452.5

obtained from a single homogenate precipitated with 3, 10 or 20% sulfosalicylic acid (Table 7). Samples precipitated with 10% sulfosalicylic acid, however, were slightly higher than those obtained when the other two concentrations of sulfosalicylic acid were used.

Serum was prepared for free amino acid analysis by precipitating the protein in 1 ml of serum with 0.5 ml of 10% sulfosalicylic acid, centrifuging at 37,000 x g for 30 minutes and freezing the supernatant. Prior to analysis, the thawed serum was centrifuged again to remove any remaining precipitate.

The gastrocnemii of each individual rat were prepared for RNA, DNA, protein and free amino acid analysis as outlined for all tissue (except serum) in Fig. 3. Equal quantities of liver homogenate for every two out of four rats fed the various diets for a period of 4, 8 and 12 weeks were pooled before carrying out isolation procedures; similarily, serum samples were pooled. An equal quantity of each leg and thigh homogenate of all four rats on a specific diet for a specific period of time, also, was pooled.

Analyses

Determinations were carried out in duplicate on all samples. Ribonucleic acid and deoxyribonucleic acid were quantitated in experiments I and II by the orcinol (Dische, 1955) and diphenylamine (Giles and Myers, 1965) assays,

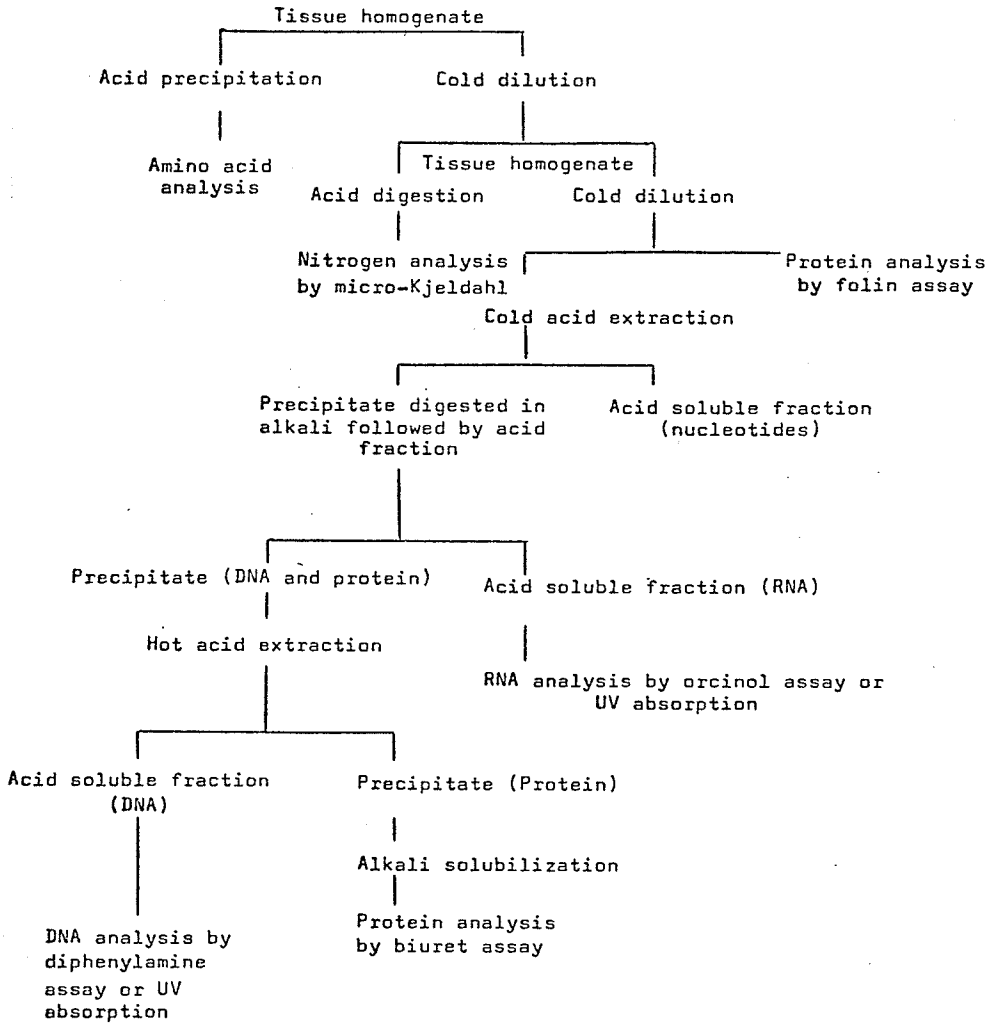

Fig. 3. Outline of methods used in tissue analysis for RNA, DNA, protein and free amino acids

respectively, as well as by ultraviolet absorption using a Unicam SP 800 spectophotometer. There was less variation between duplicates using the latter method so this was the only method used in the determinations of RNA and DNA in experiment III. In experiments I and II, protein determinations were made on 1) 1:50 homogenate by phenol procedure (Lowry et al., 1951), 2) 1:20 homogenate by micro-Kjeldahl (Horowitz, 1965) and 3) pellet left after isolation of DNA by buiret assay (Gornall et al., 1949); only the micro-Kjeldahl method was used for protein determinations in experiment III.

Free amino acid concentrations were determined by the automated amino acid analyzer (Beckman, Model 116). All amino acids except arginine (see chromatogram, Appendix A, Fig. 1) were eluted from a 56 cm column of UR-30 resin over a period of 5 hours; arginine was eluted from a 6.3 cm column of PA-35 resin over a period of 1 hour. Three sodium citrate buffers were used as follows: pH 3.17 (0.2N), pH 4.25 (0.2N) at 79 minutes and pH 6.3 (0.4N) at 139 minutes for the 56 cm column, and pH 5.28 (0.35N) for the 6.3 cm column, with a flow rate of 70 ml/min. Temperature was maintained at 55.3^{o}C.

A chromatogram of the physiological fluids, serum and gastrocnemii (Appendix A, Figs. 2 and 3), indicate definite resolution of most amino acids and non-amino

acids[1]. The aspartic acid peak, however, contains an unidentifiable compound (possibly methyl sulfoxide) while the serine peak contains asparagine and glutamine. The large peak before ornithine in muscle tissue (Appendix A, Fig. 3) probably contains all three methyl histidines.

Although elution of a lysine standard on both the 56 cm and 6.3 cm columns varied from 1-2%, a similar comparison for a tryptophan standard resulted in a higher value (6.9%) of this compound being eluted from the 56 cm column than from the 6.3 cm column. Determination of the amino acids from physiological fluids, however, gave higher lysine values when eluted from the 6.3 cm column than from the 56 cm column as other compounds were eluted at the same time as lysine on the 6.3 cm column. The lysine concentration, therefore, was calculated from values obtained when samples were analyzed on the 56 cm column.

All data was statistically analyzed. Methods described by Steel and Torrie (1960) were used.

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[1]Includes ornithine and citrulline (as well as ammonia and taurine) as they are not of nutritional significance even though classified biochemically as amino acids

RESULTS

The rats adjusted reasonably well to the use of tail cups within a day or two after their introduction. Because the sham tail cup was not as securely placed as the real tail cup held firmly over the anus, the sham tail cup was a source of annoyance to some of the rats.

Prevention of coprophagy in rats consuming a supplemented wheat (SW) diet or pure wheat (W) diet had no significant effect on the weight gain of rats over a period of 35 days, or on nitrogen balances at the two different stages of growth. Nor were the apparent and true amino acid availabilities from a pure wheat diet affected when consumed by 80 g and 300 g male rats, except in one instance. In the lighter, younger rats, a significant difference (Table 8) occurred in the true availability of lysine: 89% for rats with sham tail cups, 94% for control rats and 96% for those with real tail cups. This depression in the true availability of lysine ($P<0.01$) and that of tyrosine and phenylalanine ($P<0.10$) to the younger rats fitted with sham tail cups suggests that these rats possibly needed more time to adjust to the sham tail cups. Otherwise, these results indicate that the practice of coprophagy in control rats was not great enough to cause adverse effects when prevented, possibly because the diets were adequate in nutrients normally synthesized by the microflora.

Table 8. Percentage of the amino acids in pure wheat, and the mean and standard error of the true availability of amino acids to the growing rat as influenced by practice or prevention of coprophagy

Amino acids	Level in ration (%)	Pure wheat							
		Younger rats (80 g)				Older rats (300 g)			
		Control[1a]	Tail cup[2a]	Sham tail cup[3a]	Average[4a]	Control[1a]	Tail cup[1a]	Sham tail cup[1a]	Average[5a]
Essential									
Arginine	0.732	96.0	98.2	94.6	95.5±0.61	94.9	94.8	94.8	94.8±0.00
Histidine	0.311	97.3	96.6	96.1	97.0±0.52	96.3	96.5	96.2	96.3±0.00
Isoleucine	0.487	96.0	94.5	94.1	95.0±0.83	94.6	93.1	94.6	94.1±0.54
Leucine	0.981	95.6	96.1	94.0	95.0±0.80	95.9	94.2	94.8	95.0±0.64
Lysine	0.365	94.0	96.4	89.0*	92.4±2.27	89.9	90.3	90.7	90.3±0.31
Methionine	0.226	94.2	94.9	93.5	94.2±0.50	94.0	94.3	94.0	94.1±0.17
Phenylalanine	0.722	97.7	96.9	96.1	97.0±0.81	96.5	95.3	96.3	96.0±0.53
Threonine	0.403	95.7	95.4	94.0	95.0±0.73	95.1	92.1	95.4	94.2±1.50
Valine	0.614	95.8	94.8	93.7	94.9±0.88	92.7	93.6	94.0	93.4±0.40
Non-essential									
Alanine	0.517	92.3	93.6	90.6	92.0±0.87	91.9	89.0	91.6	90.8±0.86
Aspartic acid	0.748	92.2	92.4	87.9	91.5±0.81	93.2	89.4	92.2	91.6±1.20
Glutamic acid	5.719	98.4	98.4	97.8	98.2±0.44	98.5	97.8	98.3	98.2±0.42
Glycine	0.719	94.4	93.9	93.3	93.9±0.46	93.9	92.0	94.0	93.3±0.78
Proline	1.599	98.2	98.2	97.8	98.0±0.30	98.3	97.7	98.3	98.1±0.36
Serine	0.665	96.6	96.8	95.4	96.3±0.65	97.0	94.9	96.6	96.2±1.02
Tyrosine	0.403	96.1	95.3	90.7	94.1±2.01	94.6	92.7	94.1	93.8±0.62

[a]Feces of every two rats were pooled

[1]Four pooled samples; [2]Two pooled samples; [3]Three pooled samples; [4]Nine pooled samples; [5]Twelve pooled samples

*Different at $P<0.01$ within age group and amino acid

Availability Study

The availability of only 16 amino acids was determined as tryptophan was destroyed completely and S-containing amino acids were partially destroyed during acid hydrolysis. True amino acid availability from pure wheat fed to 80 g and 300 g rats was calculated according to the equation: % amino acid availability = [amino acid intake - (fecal amino acids - metabolic amino acids in feces from N-free diet) x 100]/amino acid intake. Metabolic amino acids were not considered in the calculation of apparent availability of amino acids. The resulting means and standard errors of true and apparent availability, as influenced by use of tail cups, are presented (Tables 8, 9).

True Availability

The average availability from wheat for each weight group was 90-98% (Table 8) of each amino acid considered. There were no significant differences in the average availability of each amino acid between weight groups. In both, glutamic acid and proline were the most available. They were followed closely by histidine, phenylalanine and serine in the lighter, younger rats and by histidine, serine and phenylalanine in the heavier, older rats. Aspartic acid followed by alanine and lysine were the least available to younger rats, while lysine followed by alanine and aspartic acid were the least available to the older rats.

Table 9. Percentage of the amino acids in pure wheat, and the mean and standard error of the apparent availability of amino acids to the growing rat as influenced by practice or prevention of coprophagy

Amino acids	Level in ration (%)	Pure wheat							
		Younger rats (80 g)				Older rats (300 g)			
		Control[1a]	Tail cup[2a]	Sham tail cup[3a]	Average[4a]	Control[1a]	Tail cup[1a]	Sham tail cup[1a]	Average[5a]
Essential									
Arginine	0.732	93.9	92.8	91.7	93.0±0.52	91.6	91.9	91.6	91.7±0.55
Histidine	0.311	93.2	93.8	93.9	93.6±0.74	92.8	93.4	92.4	92.9±0.59
Isoleucine	0.487	92.1	89.8	89.9	90.8±0.64	90.0	88.8	90.0	89.6±0.64
Leucine	0.981	92.5	92.4	91.5	92.1±0.37	91.7	91.0	91.6	91.4±0.51
Lysine*	0.365	87.5	88.3	82.2	85.9±1.05	81.2	82.5	81.6	81.8±0.82
Methionine	0.226	90.5	90.3	88.8	89.9±0.46	89.9	90.0	89.8	89.9±0.65
Phenylalanine	0.722	94.5	93.9	93.3	94.0±0.44	93.3	92.4	93.1	92.9±0.55
Threonine*	0.403	90.9	88.3	87.2	89.1±1.23	86.8	84.1	86.6	85.8±0.68
Valine	0.614	92.1	90.5	89.8	90.9±0.54	90.6	88.4	89.5	89.5±0.65
Non-essential									
Alanine	0.517	88.1	87.5	84.8	86.8±0.63	86.5	85.8	86.4	86.2±0.94
Aspartic acid	0.748	87.7	85.2	83.1	85.6±1.16	84.6	82.3	84.4	83.8±0.79
Glutamic acid	5.719	96.3	97.2	96.7	96.6±0.52	96.9	96.5	96.8	96.7±0.39
Glycine*	0.719	90.5	89.4	89.0	89.8±1.17	88.4	86.6	87.9	87.6±0.65
Proline	1.599	96.9	96.6	96.2	96.6±0.35	96.4	95.8	96.3	96.1±0.35
Serine	0.665	92.4	92.8	91.6	92.2±0.47	91.9	90.2	90.1	90.7±0.78
Tyrosine	0.403	91.1	90.5	88.7	90.2±0.62	89.4	87.6	89.2	88.7±0.64

[a]Feces of every two rats were pooled

[1]Four pooled samples; [2]Two pooled samples; [3]Three pooled samples; [4]Nine pooled samples; [5]Twelve pooled samples

*Different at $P<0.01$ within age group and amino acid

The type of diet (SW or W) consumed, prior to the availability study, by older rats had no significant effect on the availability of amino acids in pure wheat. There were significant differences, however, among the essential amino acids; lysine was the least available with histidine and phenylalanine the most available to both younger and older rats. Likewise, significant differences among the availabilities of non-essential amino acids were found; glutamic acid, proline and serine were more available than the other amino acids considered.

Apparent Availability

Apparent availability averages ranged from 83.8-96.6% (Table 9). Findings showed 1) the most and least available amino acids were the same as those for the true availability, 2) differences among essential and non-essential amino acids were similar to those for true availability values, 3) there were no significant differences between apparent availability values of the 80 g or 300 g rats for any particular amino acid. True availability values, however, were higher than apparent availability values by 1.4-8.3% (Table 10). The differences were greatest for lysine and other amino acids having the lowest true and apparent availability values; least differences were found for glutamic acid, proline and other amino acids having the higher values for both methods of measurement.

Table 10. Differences between the mean values (%) obtained for true and apparent availability of amino acids from pure wheat fed to younger (80 g) and older (300 g) rats

Amino acids (highest to lowest values)	True availability - apparent availability	
	Younger rats (80 g)[1a]	Older rats (300 g)[2a]
Glutamic acid	1.6	1.5
Proline	1.4	2.0
Histidine	3.4	2.4
Phenylalanine	3.0	3.1
Serine	4.1	5.5
Arginine	2.5	3.1
Leucine	2.9	3.6
Threonine	5.9	8.4
Isoleucine	4.2	4.5
Valine	4.0	3.9
Tyrosine	3.9	5.1
Methionine	4.3	4.2
Glycine	4.1	5.7
Lysine	6.5	8.5
Alanine	5.4	4.6
Aspartic acid	5.9	7.8

[a]Feces of every two rats were pooled

[1]9 pooled samples; [2]12 pooled samples

Generally, differences were greatest for the heavier, older rats - especially lysine, aspartic acid and threonine, possibly reflecting their resistance to proteolysis, their slower rate of absorption or an increase in the metabolic fecal output of these amino acids. For both groups of rats, however, the apparent digestibility of protein was 4% lower than the average apparent availability. A discrepancy in digestibility and availability values has been reported by de Muelenaere (1967a, 1967b), also. Nevertheless, apparent availability values reflect the true availability of many amino acids.

Tissue Study

The average weight of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW, W) diets are presented (Figs. 4, 5 and 6). Although little gain in weight was expected by rats fed the C-1 diet, lack of weight gain by rats fed the C-2 and C-3 diets (experiment I) became evident after 4 weeks (Fig. 4). By the end of the sixth week, a rat fed the C-2 diet had poor gait; subsequent sacrifice and histological examination of a brain section showed small haemorrhages. Continued lack of weight gain by rats fed the casein diets for seven weeks confirmed suspected dietary inadequacies. It was found the vitamin mixture used lacked thiamin and was inadequate in pyridoxine, choline, potassium, magnesium.

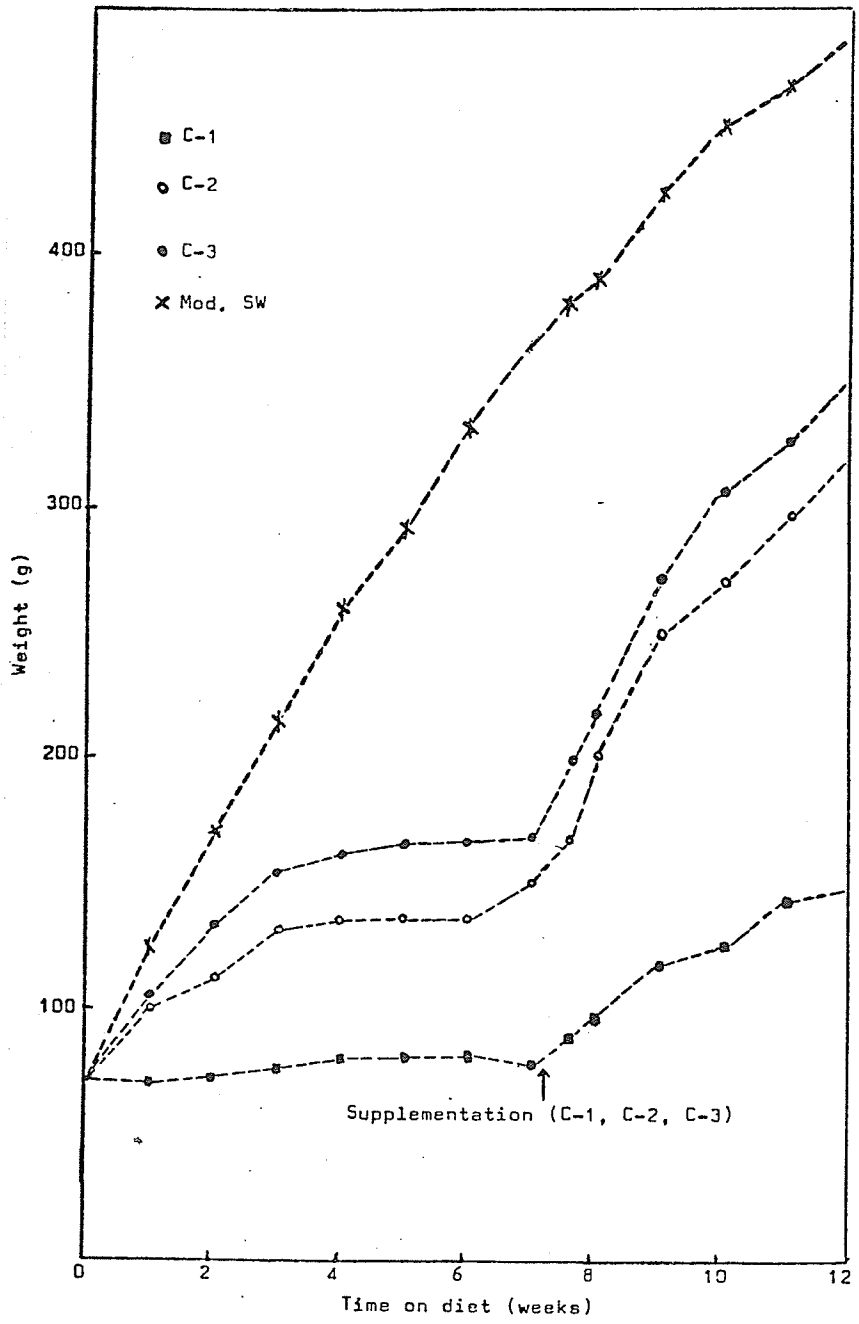

Fig. 4. Mean weight of rats fed casein (C-1, C-2, C-3) and modified supplemented wheat (mod. SW) diets for 12 weeks, experiment I

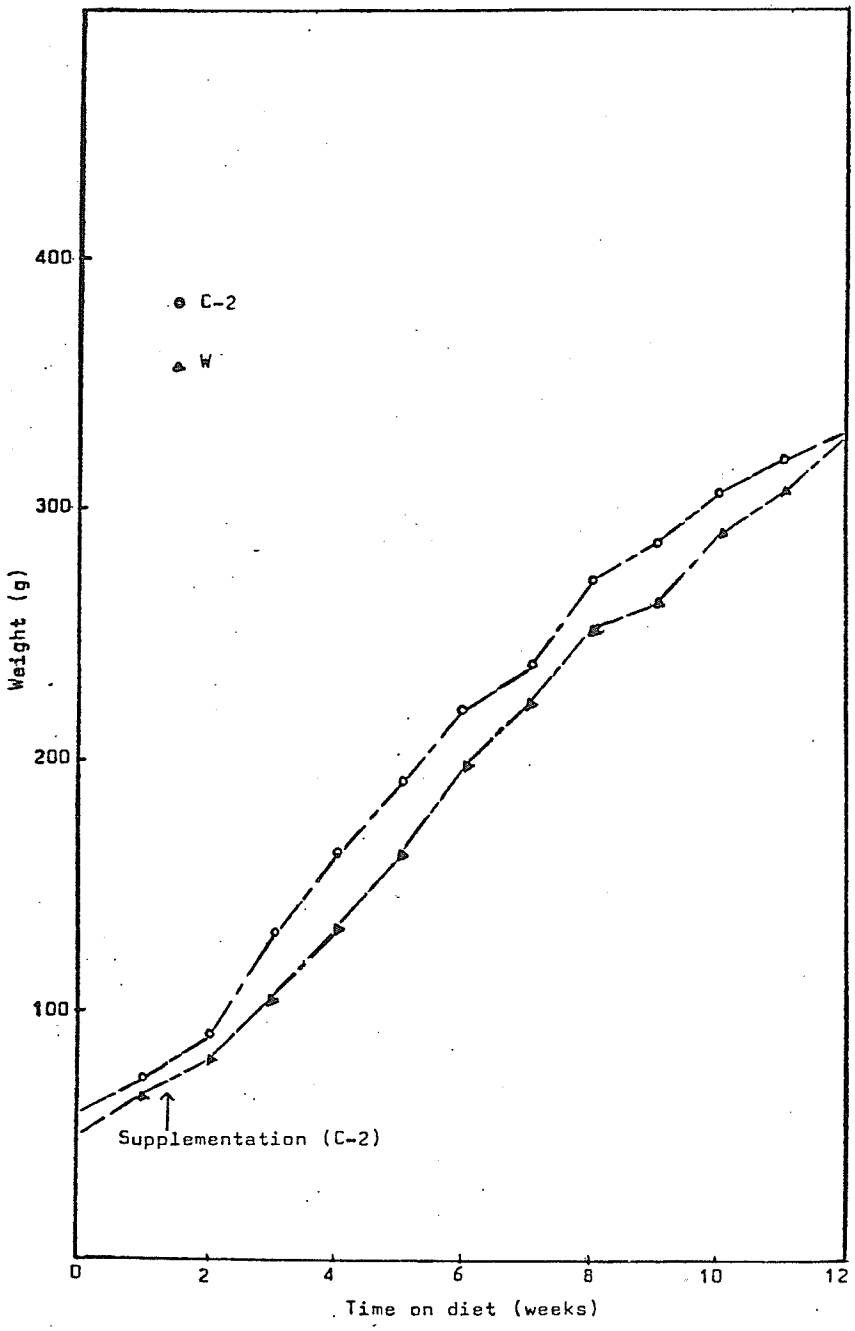

Fig. 5. Mean weight of rats fed casein (C-2) and wheat (W) diets for 12 weeks, experiment II

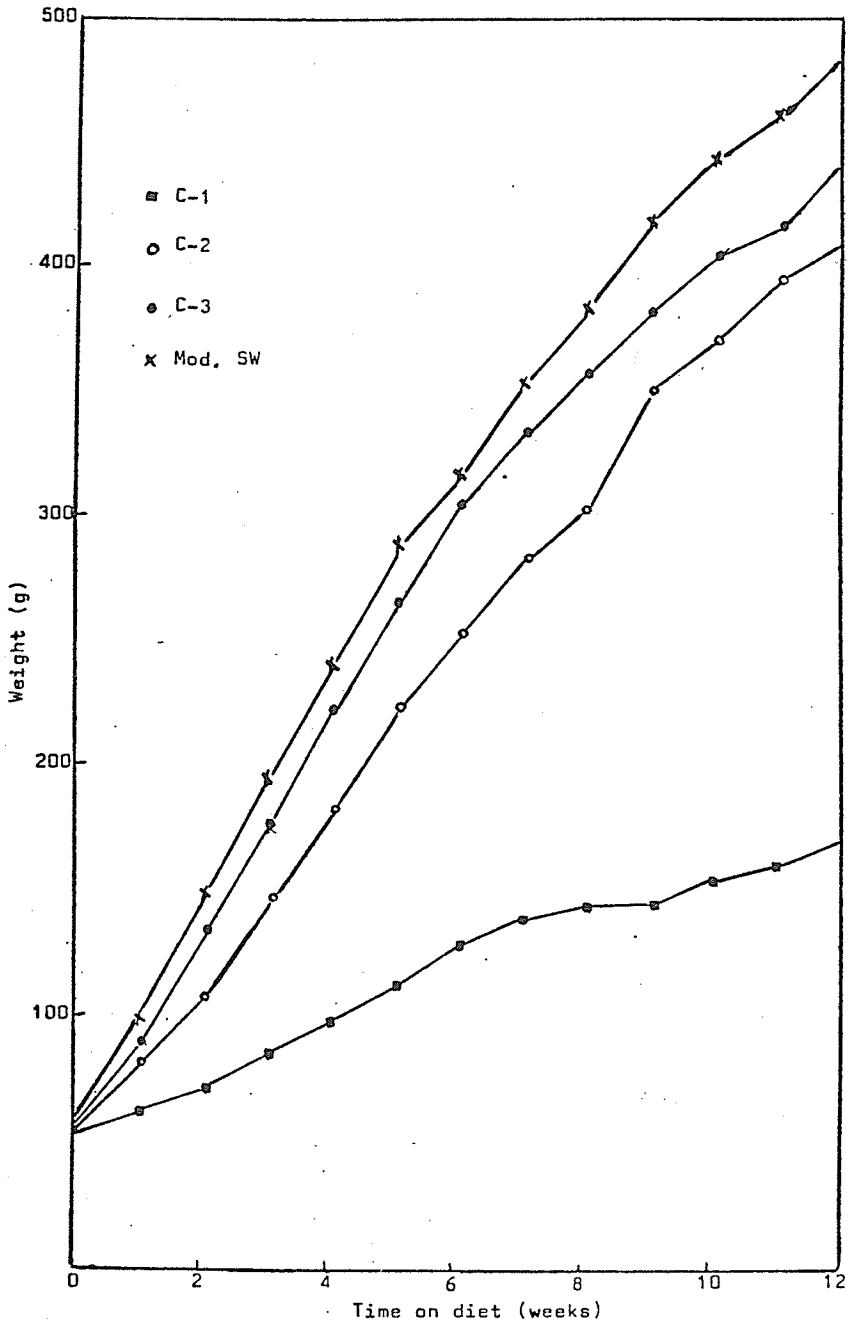

Fig. 6. Mean weight of rats fed casein (C-1, C-2, C-3) and supplemented wheat (mod. SW) diets for 12 weeks, experiment III

Accordingly, supplementation in the drinking water for the first few days and later in the food (Table 5), produced dramatic results by more balanced physical activity within an hour and by weight gain over a period of time (Fig. 4). In experiment II, rats were fed the inadequate C-2 diet for 10 days, only. On the basis of weight gain, rats fed the inadequate casein diets did not attain full recovery: 1) rats fed the C-3 diet for 12 weeks gained 281 g in experiment I compared with 385 g in experiment III, 2) rats fed C-2 diets gained 250 g, 274 g and 348 g in experiments I, II and III, respectively (Fig. 7), and 3) rats fed the C-1 diet gained 70 g in experiment I while a similar group in experiment III gained 115 g during the 12-week experimental period.

Weight gain of rats fed the other diets over the 12-week period were: 1) mod. SW diet, 421 g and 425 g in experiments I and III, respectively, 2) W diet, 276 g in experiment II. An average gain of 184 g by rats fed the W diet for 5 weeks was similar to the 170 g previously reported (Giovannetti et al., 1970). There was no significant difference in the weight gain of rats fed the C-2 or W diets in experiment II. Rats fed the C-1 diet in experiment III gained significantly less than rats fed the other diets. The weight gain of rats fed the mod. SW, C-2 and C-3 diets was greater than that of rats fed the C-1 diet for 84

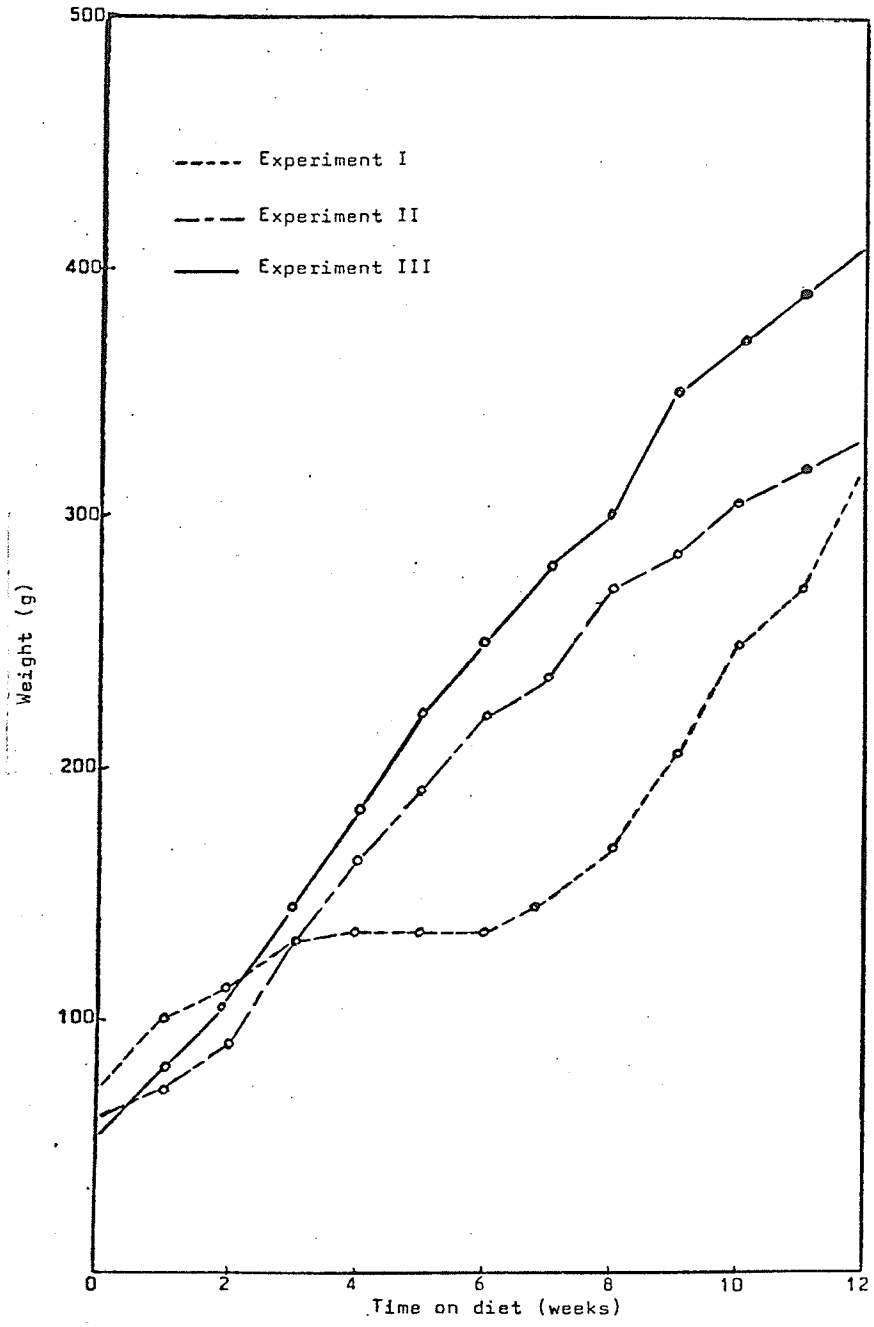

Fig. 7. Comparison of the mean weights of rats fed a casein (C-2) diet, with and without supplementation (footnote, Table 5) for 12 weeks, experiments I, II and III

days, but the weight gain of rats fed the mod. SW and C-3 diets was greater than C-2 as well as C-1 diets for 56 and 28 days. The means and standard errors for the weight of tissues in rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW or W) diet are presented (Appendix B, Tables I and II).

Other parameters were used to measure the effect of diets consumed 4, 8 or 12 weeks.

Nucleic Acids and Protein

The summary of mean values for DNA and RNA (Table 11) shows that the concentration of each nucleic acid in liver was at least twice that in the gastrocnemii; thigh and leg muscles of zero-time control rats and those fed the various diets 4 weeks, generally had slightly higher concentrations of DNA and RNA than did the gastrocnemii. In most instances, DNA and RNA of all tissues decreased over the 12-week period. The greatest decrease occurred between tissues of zero-time control rats (liver in experiment II but all tissues of experiment III) and that of rats fed the various diets for four weeks; generally, DNA and RNA concentrations in the tissues of rats fed the C-1 diet decreased the fastest. Remarkable, however, was the constancy of DNA concentrations after 12 weeks regardless of the diet consumed; values were 263-266 mg/100 g, 72-89 mg/100 g and 60-70 mg/100 g for liver, gastrocnemii, thigh and leg muscle,

Table 11. Summary of mean concentration of nucleic acids and RNA / DNA ratios in liver, gastrocnemii (GN), thigh and leg (TL) muscle of rats fed casein (C-1, C-2, C-3) or wheat (W, mod. SW) diets for 4, 8 or 12 weeks

Diet and time on diet		Liver			GN			TL		
		DNA (mg/100 g)	RNA (mg/100 g)	RNA/DNA ratio	DNA (mg/100 g)	RNA (mg/100 g)	RNA/DNA ratio	DNA (mg/100 g)	RNA (mg/100 g)	RNA/DNA ratio
Experiment II										
Zero-time controls		354	884	2.50						
C-2	4 wk	304	785	2.58	162	165	1.02	142	126	0.89
	8 wk	268	719	2.68	121	123	1.02	118	98	0.83
	12 wk	315	758	2.41	82	98	1.20	92	84	0.91
W	4 wk	296	736	2.55	101	142	1.40	158	136	0.86
	8 wk	250	656	2.62	92	96	1.04	83	109	1.31
	12 wk	291	701	2.41	90	90	1.00	73	81	1.11
Experiment III										
Zero-time controls		379	923	2.44	159	147	0.90	178	184	1.03
C-1	4 wk	248	593	2.39	103	103	1.00	122	112	0.92
	8 wk	251	617	2.46	78	95	1.23	84	97	1.15
	12 wk	263	663	2.52	78	89	1.14	70	80	1.14
C-2	4 wk	280	734	2.62	121	128	1.06	196	127	0.65
	8 wk	266	669	2.52	102	102	1.01	71	92	1.30
	12 wk	264	650	2.46	89	92	1.03	60	74	1.23
C-3	4 wk	290	766	2.64	114	136	1.19	175	141	0.80
	8 wk	282	701	2.48	88	100	1.14	71	92	1.30
	12 wk	266	730	2.74	72	95	1.32	61	78	1.28
Mod. SW	4 wk	279	810	2.90	108	131	1.21	145	112	0.77
	8 wk	291	725	2.49	80	91	1.14	80	74	0.92
	12 wk	266	656	2.47	78	83	1.06	69	66	0.96

respectively. The RNA/DNA ratios were lower in the tissues of rats sacrificed at the start of the experiment than they were for rats fed the diets 4, 8 or 12 weeks except for the liver of rats fed the C-2 and W diets for 12 weeks (experiment II) as well as rats fed the C-1 diet for 4 weeks and most thigh and leg muscle (experiment III), i.e. the thigh and leg muscle of rats fed the C-2 and C-3 diets for 4 weeks and rats fed the mod. SW diet 4, 8 and 12 weeks had ratios below those of control rats. Otherwise, there was no apparent trend upward or downward over the 12-week period in tissues of rats on a specific diet or in a particular tissue of rats fed a variety of diets.

A greater number of statistically significant decreases were apparent in gastrocnemii than in liver (Tables 13 and 14b). Since DNA decreases with age in terms of tissue weight (Miller, 1966), there is no explanation for the increase in DNA content in the liver of rats fed the C-2 diet from 8 to 12 weeks (Table 13). Nor are the DNA concentrations in gastrocnemii at 8 and 12 weeks (Table 15) in keeping with the constancy of DNA concentration (Thomson et al., 1953) regardless of diet. Liver concentration of RNA (Table 15), however, was influenced more by diet than was the gastrocnemii, for the livers of rats fed the mod. SW, C-3 and C-2 diets (4 weeks) and the mod. SW and C-3 diets (8 weeks) had significantly higher levels than did rats fed

Table 12. Summary of mean protein concentration in liver, gastrocnemii (GN), thigh and leg (TL) muscle of rats fed casein (C-1, C-2, C-3) or wheat (W, mod. SW) diets for 4, 8 or 12 weeks

Diet and time on diet		Liver			GN			TL		
		Protein (mg) 100 g	Protein (mg) mg DNA	Protein (mg) mg RNA	Protein (mg) 100 g	Protein (mg) mg DNA	Protein (mg) mg RNA	Protein (mg) 100 g	Protein (mg) mg DNA	Protein (mg) mg RNA
Experiment II										
Zero-time controls		17,156	48.5	19.4						
C-2	4 wk	14,625	48.1	18.6	20,516	126.6	124.3	21,750	153.2	122.6
	8 wk	13,937	52.0	19.4	20,468	169.2	166.4	22,875	193.8	233.4
	12 wk	16,625	52.8	21.9	23,097	281.7	235.7	21,375	232.3	254.5
W	4 wk	13,500	45.6	17.8	18,968	187.8	133.6	21,375	135.3	157.2
	8 wk	14,437	57.7	22.0	20,156	219.1	210.0	21,125	254.5	193.8
	12 wk	16,188	55.6	23.1	21,078	234.2	234.2	19,688	269.7	243.1
Experiment III										
Zero-time controls		18,468	48.7	20.0	16,687	104.9	113.5	18,438	103.6	100.2
C-1	4 wk	10,906	44.0	18.4	18,546	180.0	180.0	19,250	157.8	171.9
	8 wk	12,594	50.2	20.4	20,234	262.8	213.0	19,625	233.6	202.3
	12 wk	13,093	49.8	19.7	20,890	267.8	234.7	18,750	267.8	234.4
C-2	4 wk	13,750	49.1	18.7	18,781	155.2	146.7	19,500	99.5	153.5
	8 wk	15,187	57.1	22.7	20,375	201.7	199.8	19,312	272.0	209 9
	12 wk	16,250	61.6	25.0	20,125	226.1	211.8	21,375	356.2	288.8
C-3	4 wk	16,843	58.1	22.0	19,218	168.6	141.3	19,250	110.0	136.5
	8 wk	16,687	59.2	23.8	21,575	245.2	215.7	19,000	267.6	206.5
	12 wk	16,718	62.8	22.9	21,546	299.2	226.8	18,812	308.4	241.2
Mod. SW	4 wk	17,000	60.9	21.0	20,906	193.6	159.6	17,125	118.1	152.9
	8 wk	16,875	58.0	23.3	21,062	263.3	231.4	19,312	241.4	261.0
	12 wk	16,750	63.0	25.5	20,906	268.0	251.9	20,250	293.5	306.8

Table 13. Summary of significant decreases and increases in concentration of DNA, RNA and protein in liver and gastrocnemii of rats fed casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks - experiment II

Diet and time on diet	DNA		RNA		Protein	
	L	GN[1]	L	GN[1]	L	GN
Decreases						
C-2						
0-4 wk	*		*		*	
0-8 wk	*		**		*	
0-12 wk	*		**			
4-8 wk		**		**		
4-12 wk		**		**		
8-12 wk		**		**		
W						
0-4 wk	*		*		**	
0-8 wk	*		*		**	
0-12 wk	*		*			
4-8 wk	*	*		**		
4-12 wk		**		**		
Increases						
C-2						
8-12 wk	*				*	
W						
4-12 wk					**	*

[1]No zero-time control samples available for analysis

*Different at $P<0.05$; **Different at $P<0.01$

Table 14a. Summary of significant increases in concentration of RNA and protein in liver and gastrocnemii of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks - experiment III

Diet and time on diet	RNA		Protein	
	L	GN	L	GN
C-1				
0-8 wk				*
0-12 wk				*
4-12 wk	*		**	
C-2				
0-8 wk				*
0-12 wk				*
4-12 wk			*	
C-3				
0-8 wk				**
0-12 wk				**
4-8 wk				*
4-12 wk				*
Mod. SW				
0-4 wk				**
0-8 wk				**
0-12 wk				**

*Different at $P<0.05$; **Different at $P<0.01$

Table 14b. Summary of significant decreases in concentration of DNA, RNA and protein in liver and gastrocnemii of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks - experiment III

Diet and time on diet	DNA		RNA		Protein	
	L	GN	L	GN	L	GN
C-1						
0-4 wk	*	**	**	**	**	
0-8 wk	*	**	**	**	**	
0-12 wk	*	**	**	**	**	
4-8 wk		*				
4-12 wk		*				
C-2						
0-4 wk	+	**	**	**	**	
0-8 wk		**	**	**	**	
0-12 wk		**	**	**	**	
4-8 wk		*		*		
4-12 wk		**	*	**		
C-3						
0-4 wk	+	**	**			
0-8 wk		**	**	**		
0-12 wk		**	**	**		
4-8 wk		**		**		
4-12 wk		**		**		
8-12 wk		*				
Mod. SW						
0-4 wk	*	**	**			
0-8 wk	*	**	**	**		
0-12 wk	*	**	**	**		
4-8 wk		**	**	**		
4-12 wk		**	**	**		
8-12 wk			**			

*Different at $P<0.05$; **Different at $P<0.01$

+ Different at $P<0.10$

Table 15. Summary of significant changes in DNA, RNA and protein concentrations in liver and gastrocnemii of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diets for 4, 8 or 12 weeks - experiment III

Diet and time on diet	DNA		RNA		Protein	
	L	GN	L	GN	L	GN
4 weeks						
Mod. SW>C-1			**	*	**	
Mod. SW>C-2			*		**	
C-3>C-1			**	*	**	
C-3>C-2					**	
C-2>C-1			**	*	**	
8 weeks						
Mod. SW>C-1			*		**	
C-3>C-1			*		**	
C-2>C-1		**			*	
C-2>Mod. SW		**				
12 weeks						
Mod. SW>C-1					**	
C-3>C-1					**	
C-2>C-1		*			**	
C-2>C-3		**				
C-2>Mod. SW		*				

*Different at $P<0.05$

**Different at $P<0.01$

the C-1 diet.

Protein content (determined by micro-Kjeldahl) expressed as mg/100 g tissue or mg/mg DNA or mg/mg RNA (Table 12) was lower in liver than in muscle tissue; it was similar in the two types of skeletal muscle analyzed. The extent of protein lost from the liver after the start of the experiment varied with the nutritive value of the diet (Tables 13 and 14b), the greatest loss occurring in the livers of rats fed the C-1 diet. Although there was a consistent increase in the protein content of liver tissue (Tables 13 and 14a) from 4 weeks on, not all rats achieved levels as high as those of the zero-time control rats. The protein content of muscle rose significantly over control levels with highest levels being attained by rats fed the C-3 and mod. SW diets. These findings support the thesis of Waterlow and Stephen (1966) that low protein intake promotes loss of protein from the liver and decrease of protein synthesis in muscle tissue.

Free Amino Acids

Data for all identifiable ninhydrin-reacting substances extracted from the non-protein fraction of tissues obtained in experiments II and III are presented in Appendix C. The mean concentrations (mg/100 ml or 100 g) as well as the standard errors for all unpooled samples are included. Summaries of this data, converted to μmoles/100 ml or 100 g are found in Tables 16 and 17.

Table 16. Concentration of ninhydrin-reacting substances in serum (S), liver (L) and skeletal muscle (gastrocnemii, GN and thigh and leg, TL) of rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances (μmoles/100 ml or 100 g)	Control[1]		C-2 Diet												W Diet											
			4 wk				8 wk				12 wk				4 wk				8 wk				12 wk			
	L	GN	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL
Essential amino acids:																										
Arginine	0	39	8	0	18	24	10	0	14	10	18	0	20	13	12	0	19	25	22	0	10	10	22	0	18	19
Histidine	97	63	10	77	36	52	10	56	28	36	9	57	27	36	11	68	27	28	13	65	14	30	8	54	28	24
Isoleucine	96	24	14	40	15	20	12	28	20	22	12	28	18	23	11	43	9	19	8	36	13	15	9	33	19	20
Leucine	207	44	22	99	26	44	20	59	28	41	19	64	28	43	15	95	18	42	12	77	18	30	13	73	34	40
Lysine	306	139	84	230	146	225	63	124	67	132	68	129	73	95	20	139	-	83	16	98	-	27	19	110	-	70
Methionine	97	22	4	45	18	10	9	32	21	23	6	32	20	24	5	50	14	23	7	40	14	17	6	38	23	20
Phenylalanine	85	18	7	38	10	20	7	23	17	21	8	25	17	20	8	50	11	22	6	32	7	19	5	29	18	22
Threonine	173	110	44	147	122	140	63	98	100	128	56	84	113	100	34	109	79	80	32	89	52	60	28	63	75	63
Tryptophan	1	3	4	1	2	-	7	2	4	-	7	7	3	-	3	7	1	2	7	1	2	1	11	1	2	1
Valine	166	30	25	63	26	42	29	30	22	37	25	34	23	35	19	56	17	32	17	47	15	28	17	37	27	28
Non-essential amino acids:																										
Alanine	818	36	99	751	537	609	196	629	411	502	91	574	370	345	68	505	323	383	77	564	326	355	83	458	344	322
Aspartic acid	383	63	16	326	56	82	10	470	46	88	11	418	37	52	15	252	51	87	9	379	43	75	8	356	30	43
Cystine	4	-	1	34	-	-	4	4	-	-	5	8	-	-	1	5	-	-	4	0	-	-	8	0	-	-
Glutamic acid	604	301	87	971	570	674	86	597	309	449	72	684	252	347	87	748	468	483	66	425	279	290	55	468	215	274
Glycine	630	625	17	338	201	227	17	225	173	227	21	228	163	174	37	348	437	431	36	369	319	361	44	304	380	384
Proline	106	51	19	44	56	69	4	27	56	69	3	40	46	28	28	74	59	64	28	35	55	41	29	40	34	30
Serine	305	394	71	359	326	293	73	264	250	286	72	230	317	231	66	280	218	238	52	288	169	193	65	199	260	200
Tyrosine	76	18	14	50	22	33	13	25	29	31	10	26	16	25	9	41	14	23	8	30	11	22	10	28	20	23
Non-amino acids:																										
Ornithine	204	13	12	85	6	30	9	47	6	6	5	48	2	5	16	88	11	15	6	85	10	11	4	59	5	7
Citrulline	-	31	9	-	32	45	8	-	15	26	7	-	18	21	9	-	37	35	9	-	23	28	9	-	21	23
Ammonia	1118	-	101	1205	601	1093	99	945	819	869	91	849	2461	4635	91	998	1944	848	89	1021	2163	850	90	904	1016	827
Taurine	153	518	8	14	282	228	15	15	362	299	18	46	705	517	39	77	944	796	26	191	954	822	25	70	1200	886

[1]Zero-time control rats

Table 17a. Concentration of ninhydrin-reacting substances in serum (S), liver (L) and skeletal muscle (gastrocnemii, GN and thigh and leg, TL) of zero-time control rats and rats fed a casein (C-1) diet 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances (μmoles/100 ml or 100 g)	Control[1]				C-1 Diet											
					4 wk				8 wk				12 wk			
	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL
Essential amino acids:																
Arginine	50	–	30	38	7	–	24	26	9	–	10	16	4	–	9	14
Histidine	6	76	39	53	14	59	40	39	14	60	45	48	14	56	34	38
Isoleucine	10	58	17	23	8	28	15	18	9	20	12	14	9	38	13	22
Leucine	15	143	30	40	13	63	31	36	14	55	21	30	14	87	21	35
Lysine	36	245	146	177	83	164	157	206	66	177	152	112	90	229	45	93
Methionine	5	68	22	21	4	34	19	21	5	30	16	19	4	46	15	21
Phenylalanine	6	55	11	20	5	25	15	18	6	26	10	17	6	35	15	19
Threonine	16	113	73	64	19	67	68	51	34	94	81	74	34	119	83	103
Tryptophan	4	1	3	3	1	2	2	2	5	3	3	–	2	4	3	4
Valine	16	98	22	33	17	53	25	28	20	36	20	27	18	42	21	30
Non-essential amino acids:																
Alanine	45	590	333	348	103	634	469	467	119	789	474	459	140	704	407	435
Aspartic acid	9	328	66	113	8	417	42	58	9	342	37	53	17	307	38	54
Cystine	–	–	–	–	4	46	–	–	1	18	–	–	2	2	–	–
Glutamic acid	54	805	572	586	80	628	457	484	73	554	312	404	98	589	205	359
Glycine	30	462	602	694	16	254	194	194	20	285	210	202	19	247	252	238
Proline	17	84	55	53	36	28	52	65	31	36	41	45	30	44	37	61
Serine	35	233	222	214	79	332	388	398	87	365	408	327	90	337	344	389
Tyrosine	6	53	13	21	6	25	17	20	8	25	14	21	6	36	16	20
Non-amino acids:																
Ornithine	12	133	20	31	12	64	6	14	15	73	9	9	14	87	10	8
Citrulline	8	–	36	41	10	–	34	37	10	–	33	36	13	–	30	32
Ammonia	86	1070	4095	900	114	1245	2938	887	108	910	1820	981	131	940	1311	854
Taurine	23	250	911	732	16	18	407	317	11	16	314	265	14	14	231	178

[1]Zero-time control rats

Table 17b. Concentration of ninhydrin-reacting substances in serum (S), liver (L) and skeletal muscle (gastrocnemii, GN and thigh and leg, TL) of rats fed casein (C-2, C-3) or wheat (mod. SW) diet, 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances (μmoles/100 ml or 100 g)	C-2 Diet												C-3 Diet												Mod. SW Diet											
	4 wk				8 wk				12 wk				4 wk				8 wk				12 wk				4 wk				8 wk				12 wk			
	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL	S	L	GN	TL
Essential amino acids:																																				
Arginine	5	–	9	20	15	–	13	18	14	–	8	13	9	–	11	20	7	–	11	26	7	–	11	23	16	–	13	29	25	–	13	29	20	–	12	15
Histidine	10	68	35	44	9	54	30	32	8	61	22	26	8	89	23	44	7	78	25	39	8	61	20	26	9	68	19	49	7	54	18	23	8	52	18	20
Isoleucine	10	51	15	20	10	33	14	30	11	37	17	24	14	45	18	28	13	40	18	30	13	23	16	26	10	45	15	38	11	34	14	18	12	33	12	20
Leucine	15	121	27	43	17	76	21	45	17	76	26	41	24	106	33	50	22	95	27	55	23	63	25	49	15	111	23	67	16	76	20	32	18	71	21	35
Lysine	74	229	142	160	81	108	79	88	54	115	44	85	74	195	129	102	66	160	96	120	73	134	96	127	44	162	33	102	43	111	52	49	45	106	36	79
Methionine	6	54	16	24	7	40	13	21	7	51	12	25	7	48	14	26	8	58	17	26	6	8	14	28	6	52	15	42	7	39	9	19	6	36	13	20
Phenylalanine	6	48	13	24	6	34	9	22	7	32	12	20	8	43	13	24	8	38	12	27	7	25	10	23	6	41	9	30	6	30	10	16	7	26	9	16
Threonine	48	138	119	130	53	102	133	116	47	91	90	86	60	95	173	157	48	94	79	95	46	57	73	78	36	91	74	118	33	48	50	56	27	41	43	50
Tryptophan	11	9	3	2	12	3	2	2	8	–	4	6	9	–	4	1	7		3	2	2	2	5	2	5	2	4	3	12	1	4	3	8	3	2	0
Valine	25	78	27	55	24	38	26	35	27	38	28	36	35	73	38	51	32	67	29	54	34	49	24	47	21	58	19	40	22	40	20	20	23	35	16	29
Non-essential amino acids:																																				
Alanine	104	731	441	534	93	704	430	438	87	605	350	399	69	646	435	494	61	607	324	376	75	458	292	342	67	440	290	427	64	370	251	311	64	414	246	271
Aspartic acid	8	358	63	95	9	366	53	73	9	327	48	49	9	383	84	117	8	306	46	84	10	455	44	76	9	383	57	57	7	404	51	38	8	391	49	43
Cystine	4	17	–	–	5	46	–	–	6	10	–	–	4	–	–	–	2	8	–	–	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Glutamic acid	82	749	614	646	73	721	356	419	63	784	215	332	68	791	690	626	60	615	233	296	54	624	194	290	58	528	419	515	52	407	209	217	50	363	185	222
Glycine	19	327	245	266	15	207	193	171	17	244	151	161	11	284	138	133	11	236	98	123	15	173	102	112	34	319	398	485	32	256	252	239	28	219	211	219
Proline	32	73	58	63	36	43	59	46	33	29	45	70	42	80	103	113	36	48	45	53	38	38	45	64	29	45	40	40	32	35	31	33	28	32	23	32
Serine	70	386	267	318	62	276	297	242	62	187	188	203	42	186	189	220	40	167	141	168	50	148	148	160	45	199	127	184	41	137	114	108	41	108	100	126
Tyrosine	18	51	26	36	15	31	20	30	12	34	16	26	13	49	20	29	15	48	19	34	12	31	16	28	10	42	14	36	9	30	13	21	8	27	12	19
Ion-acting acids:																																				
Ornithine	15	93	4	14	4	59	6	4	6	48	8	7	11	56	6	7	7	51	11	6	5	45	6	4	15	89	10	15	8	63	6	6	8	53	4	5
Citrulline	11	–	24	38	7	–	21	23	5	–	4	17	9	–	24	26	7	–	13	14	6	–	13	14	8	–	21	22	8	–	14	11	8	–	12	18
Ammonia	100	1150	1324	1332	97	634	1452	1030	95	782	1055	877	104	1019	982	892	94	789	965	1060	94	715	937	824	90	536	988	820	89	492	918	667	101	525	955	712
Taurine	14	17	321	304	12	16	338	253	19	45	669	607	20	315	676	720	22	484	1320	1177	40	521	1240	1151	28	202	1018	1417	24	334	1140	1136	29	523	1200	1091

Generally the free amino acid concentrations (on basis of wet weight), like DNA and RNA, were much higher in liver than in skeletal muscle (Tables 16 and 17), with comparisons between liver and muscle similar to those of Schurr et al. (1950); levels in thigh and leg muscle, however, usually were slightly higher than in gastrocnemii. With the exception of tryptophan, most ninhydrin-reacting substances were lower in serum than in other tissues. In many instances, however, the higher concentrations of tryptophan were found in serum. Although no measurable amount of cystine was detected in skeletal muscle (Appendix A, Fig. 3), it was present in serum (Appendix A, Fig. 2) and highest in liver. High levels of ornithine were found in the liver also but arginine and citrulline were not in sufficiently high concentration for detection. Taurine and ammonia were highly concentrated in skeletal muscle. These differences are most evident when depicted as bar graphs (Figs. 8 and 9), with liver and tissue values interpreted on the basis of mg of DNA. Concentration changes for diets consumed 4, 8 or 12 weeks are also noticeable.

In serum (Figs. 8a and 9a), most obvious are the high concentrations of the three essential amino acids - lysine, threonine and valine. During the 12 week period, values for lysine and threonine were more variable for rats fed the C-1, C-2 and W diets than they were for those fed

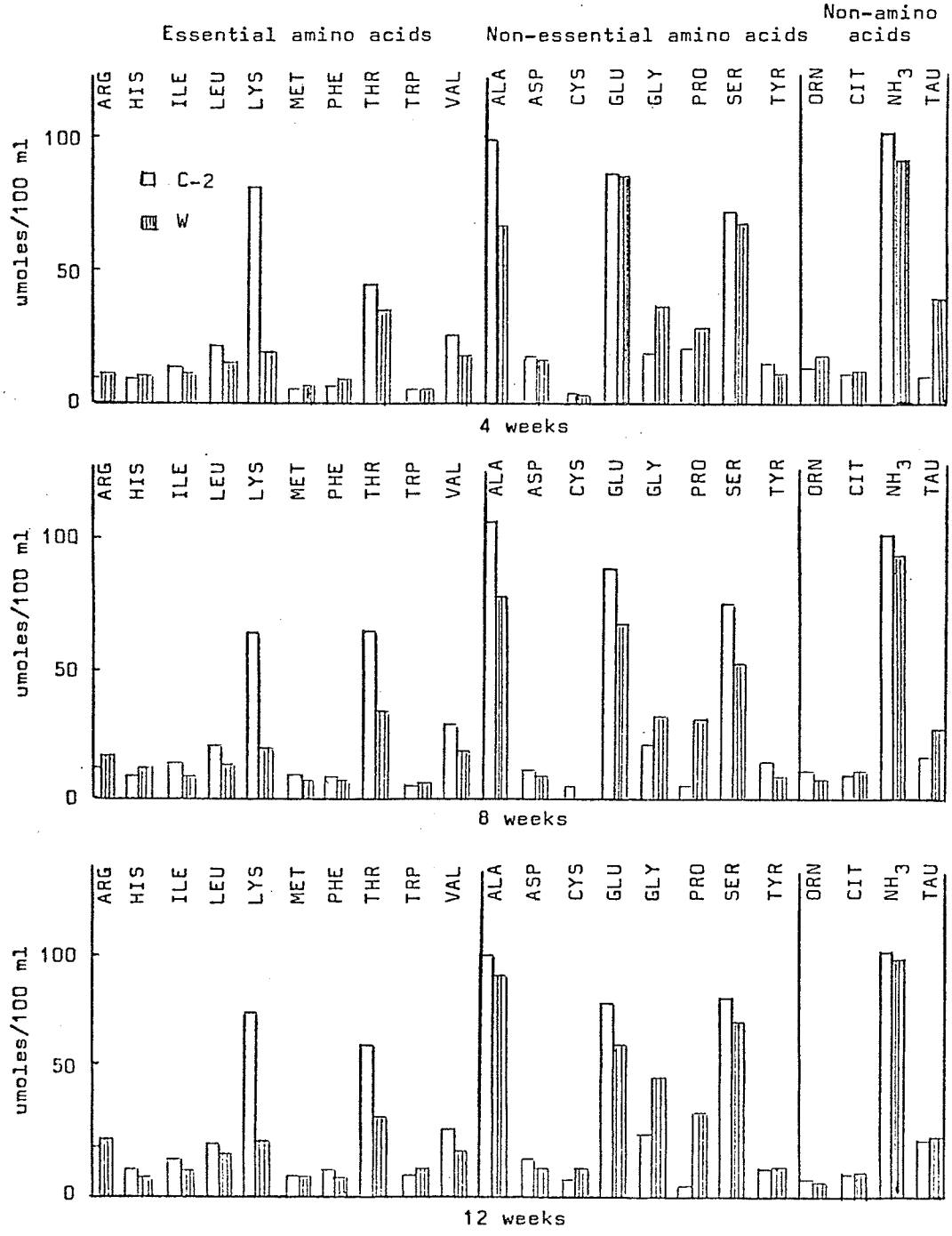

Fig. 8a. Mean concentration of ninhydrin-reacting substances in serum of rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

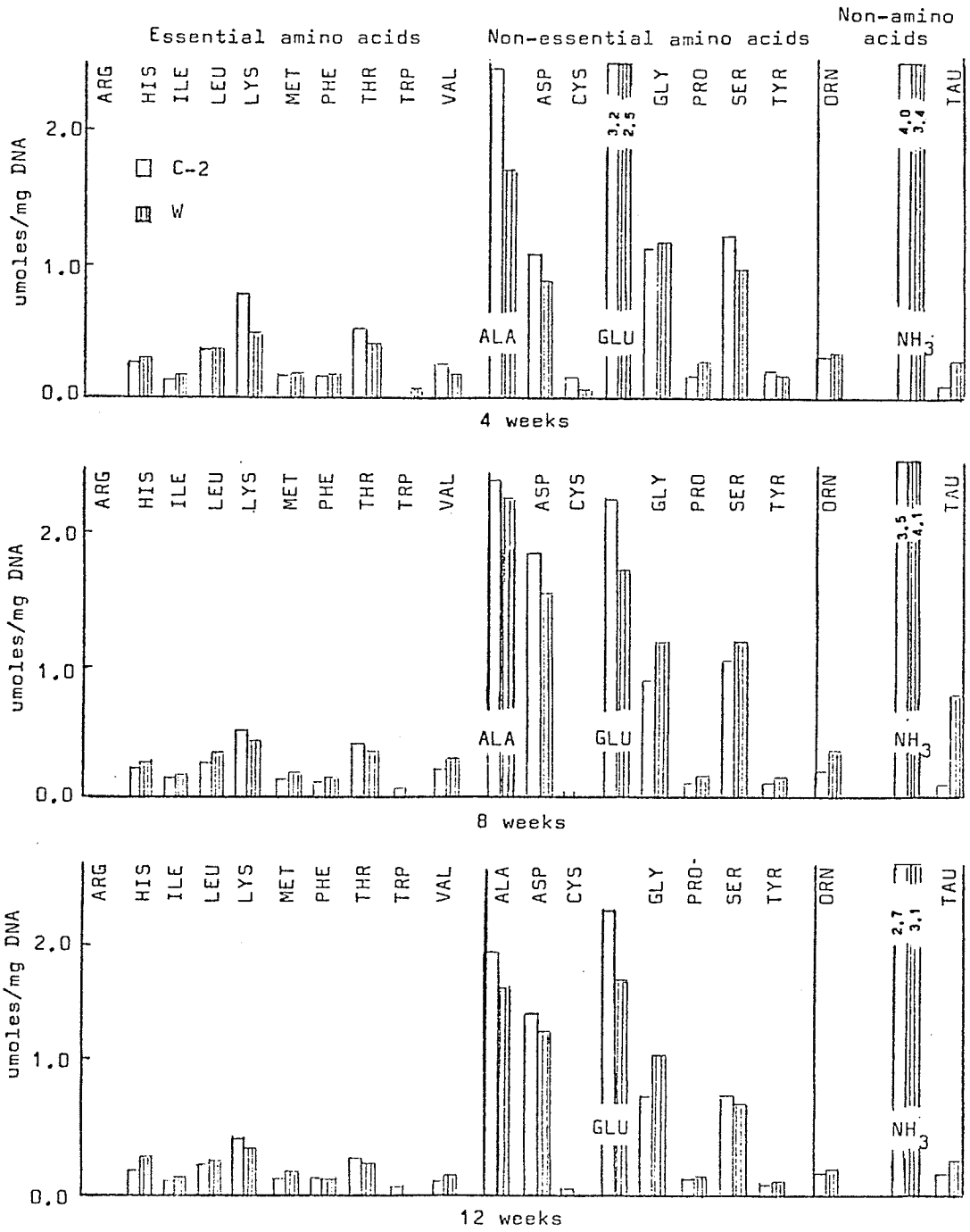

Fig. 8b. Mean concentration of ninhydrin-reacting substances in liver of rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

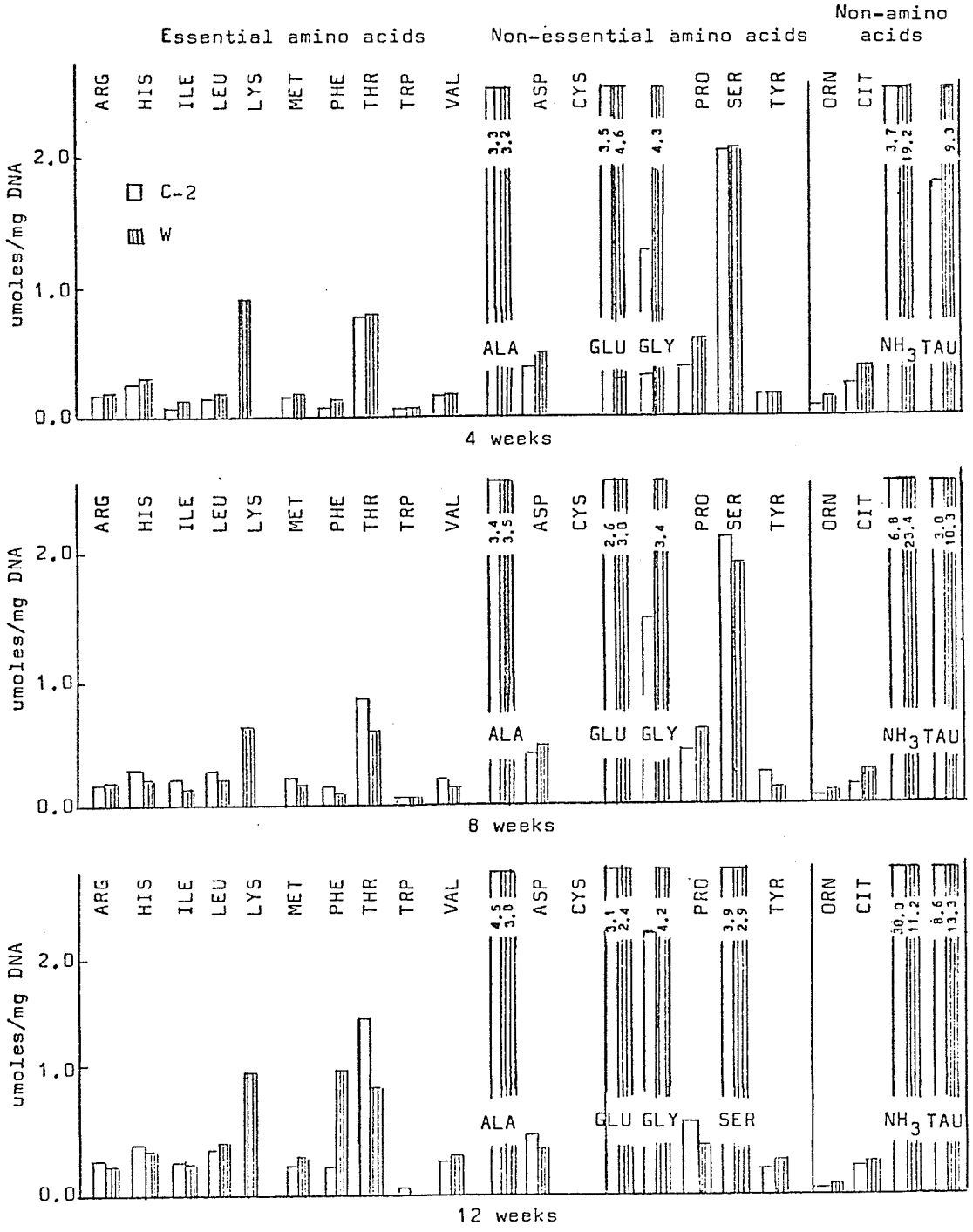

Fig. 8c. Mean concentration of ninhydrin-reacting substances in gastrocnemii of rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

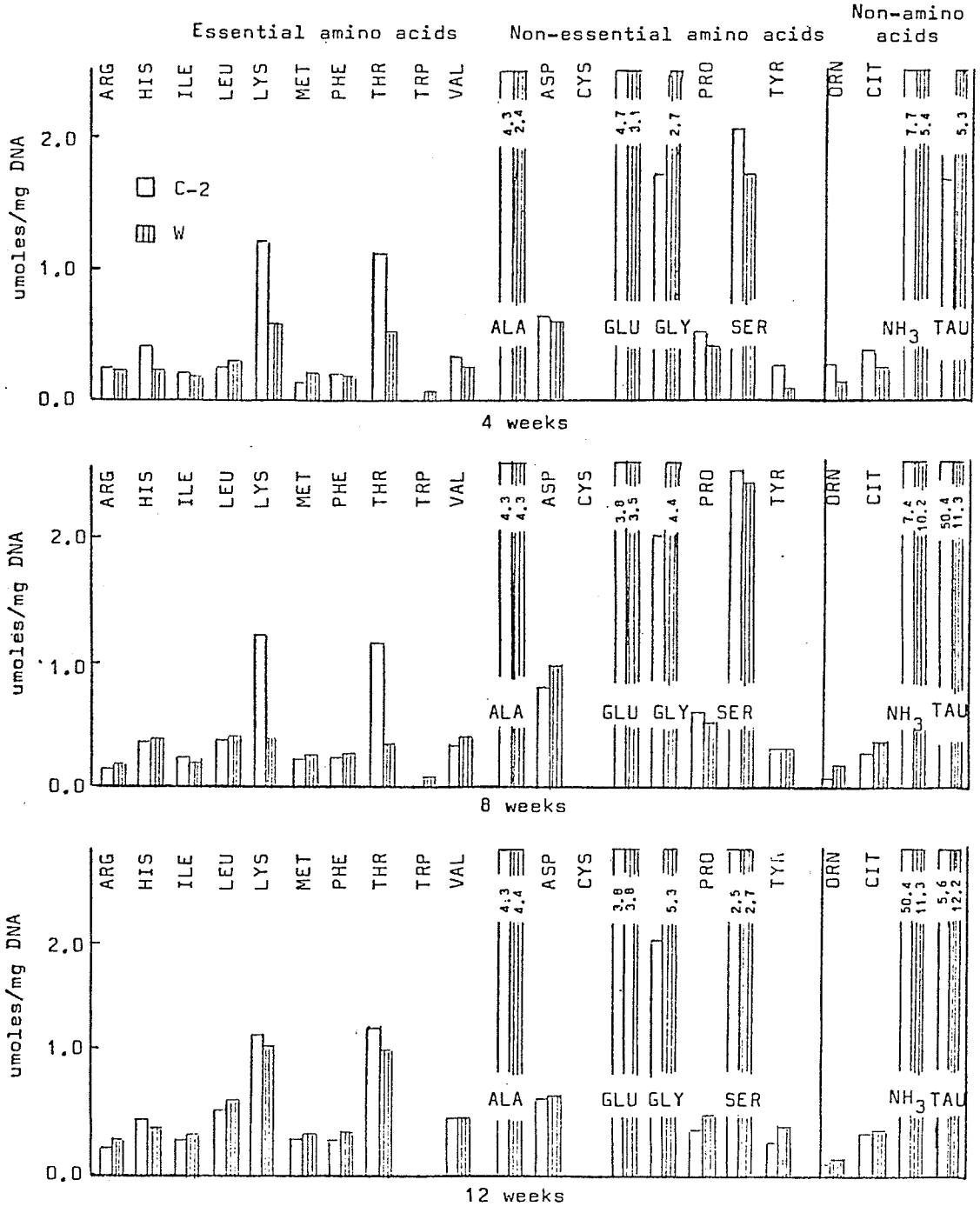

Fig. 8d. Mean concentration of ninhydrin-reacting substances in thigh and leg muscle of rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

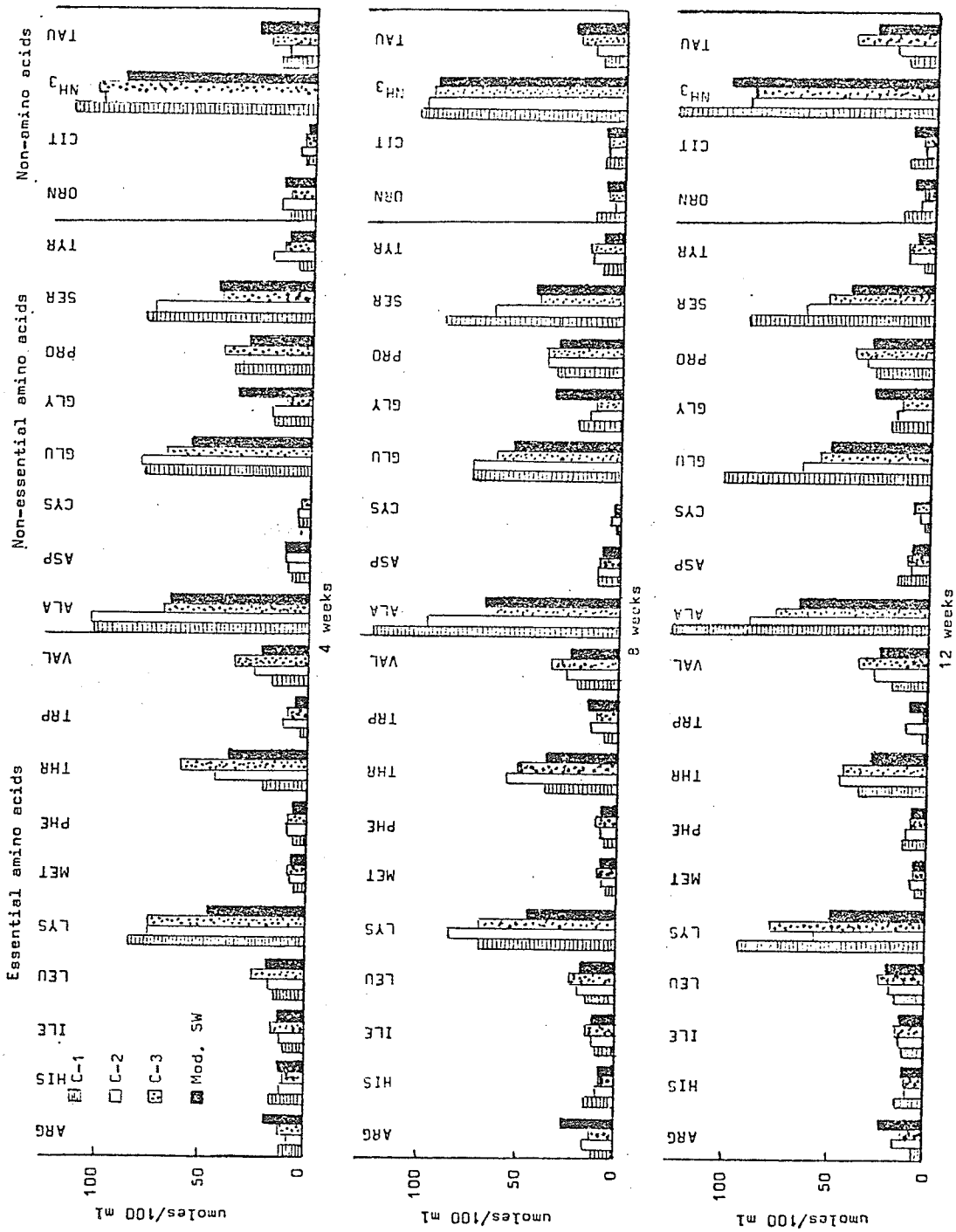

Fig. 9a. Mean concentration of ninhydrin-reacting substances in serum of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

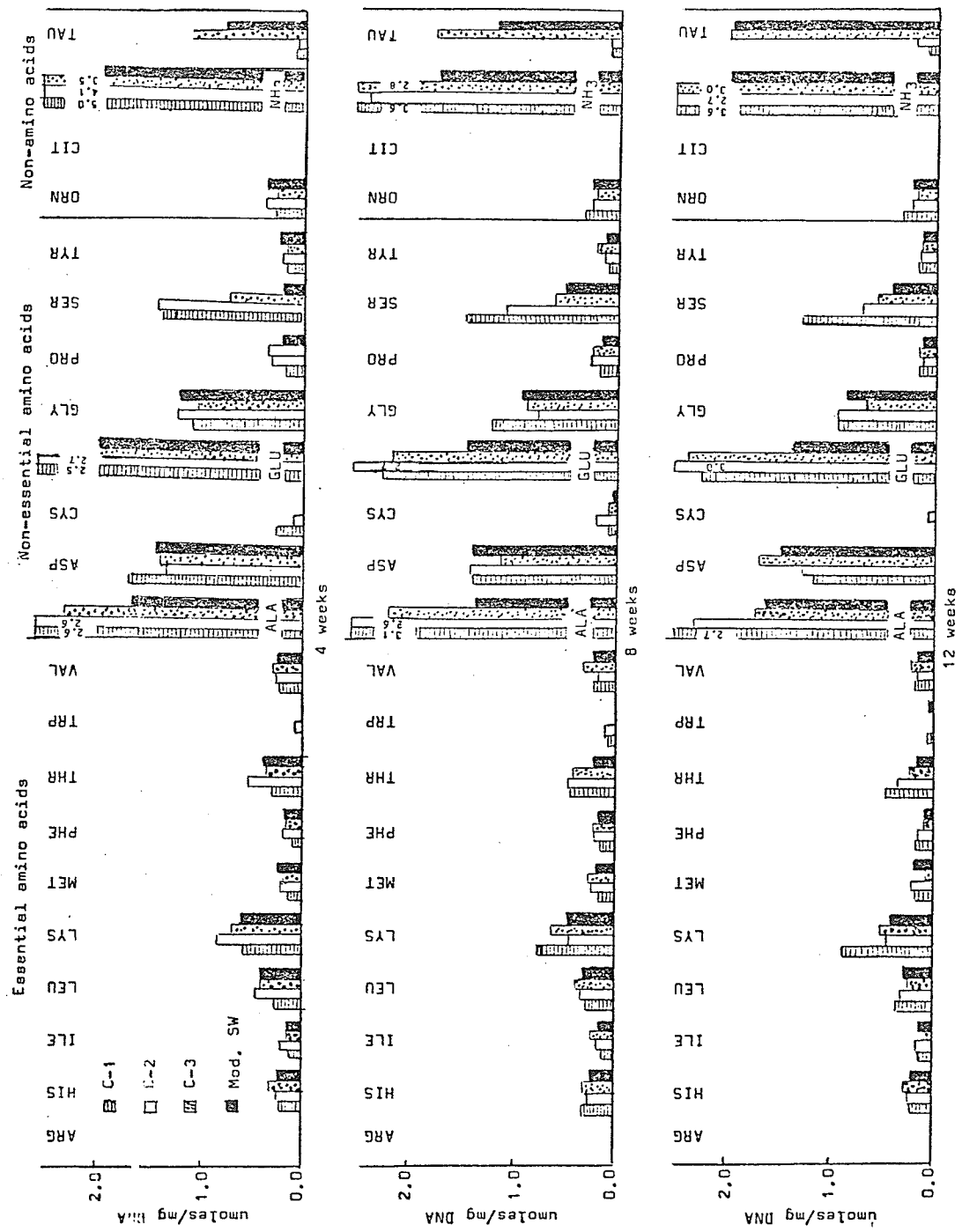

Fig. 9b. Mean concentration of ninhydrin-reacting substances in liver of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

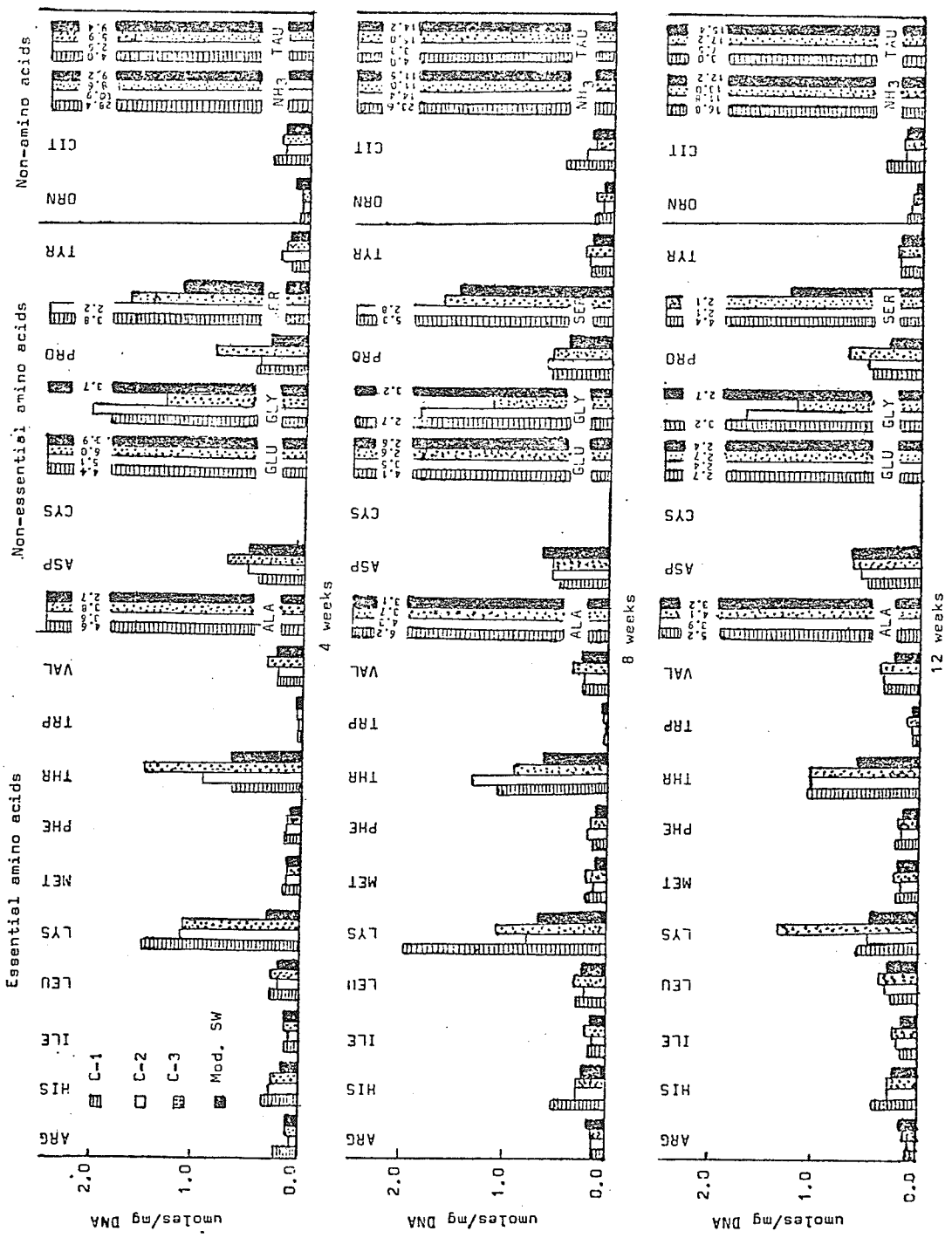

Fig. 9c. Mean concentration of ninhydrin-reacting substances in gastrocnemii of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

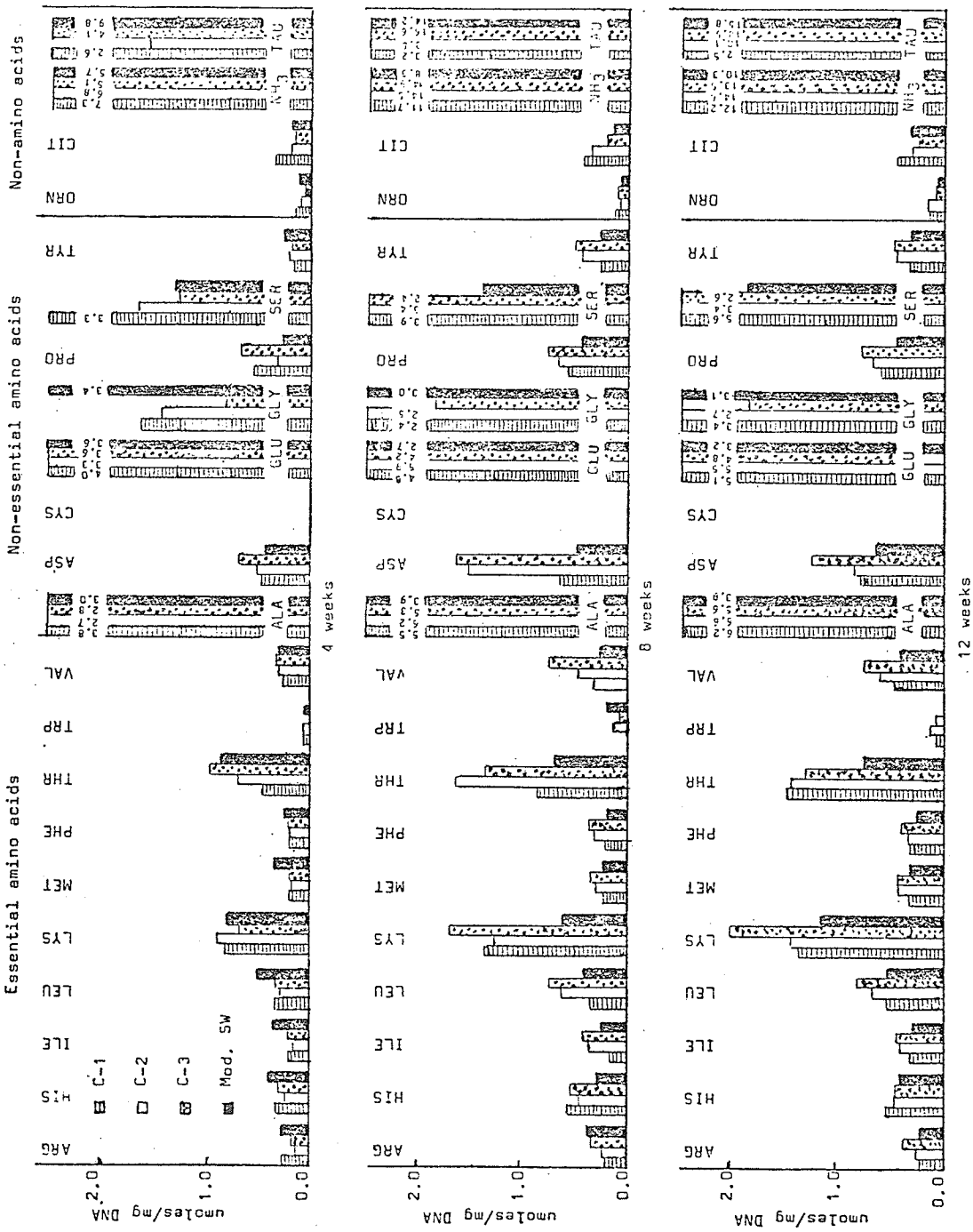

Fig. 9d. Mean concentration of ninhydrin-reacting substances in thigh and leg muscle of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

the C-3 and mod. SW diets. Alanine, glutamic acid and serine were the most highly concentrated of the non-essential amino acids of serum, while ammonia was the most concentrated for non-amino acids. Lowest in concentration were histidine, methionine, phenylalanine and tryptophan of the essential amino acids and either ornithine or citrulline of the non-amino acids.

Evaluation of liver (Figs. 8b and 9b) and skeletal muscle (Figs. 8c, 8d and 9c, 9d) concentration values for free amino acids and non-amino acids on the basis of μmoles/mg DNA, generally showed liver values less concentrated than either gastrocnemii or thigh and leg muscle. Tryptophan in liver was present in only trace amounts but levels of aspartic acid were higher than found in muscle. Glycine values were much higher in muscle, however, than those found in liver. Like free amino acids in serum, the intracellular free amino acids and non-amino acids were most highly concentrated as 1) lysine, threonine, leucine and valine of the essential amino acids, 2) alanine, glutamic acid and serine of the non-essential amino acids, and 3) ammonia of the non-amino acids. In addition, 1) histidine values were higher or as high as those for valine in the liver and even higher than valine in the muscle, 2) high glycine values of muscle varied greatly with diet, and 3) taurine was markedly higher in muscle than in any other tissue.

The results of statistical analysis 1) to determine how diet affected the concentrations of ninhydrin-reacting substances in serum, liver, gastrocnemii, thigh and leg muscle, 2) to note changes resulting from consumption of diets over a period of time, 3) to detect differences existing between the three types of tissue, are included in Appendix D. A summary showing the effect of diet is presented (Tables 19 and 20), also.

Many ninhydrin-reacting substances were not affected by either diet or the length of time rats consumed the diet. In experiment II (Table 18), the diets had no significant effect on 3, 3 and 2 essential amino acids and 4, 4 and 3 non-essential amino acids of serum, liver and gastrocnemii, respectively; the duration of time rats were fed these diets had even less effect on the serum for 8 of the essential amino acids, and all of the non-essential amino acids except cystine remained statistically unchanged regardless of the diets consumed by the rats. Findings for experiment III (Table 18) also indicate that serum is less affected by the type of diet consumed and the extent of the feeding period than are liver and gastrocnemii. In both experiments, the diets used had the greatest influence on the free amino acid and non-amino acid concentrations of the gastrocnemii. However, the liver concentrations (experiment III) were greatly influenced by diet, also.

Table 18. Summary of non-significant changes of ninhydrin-reacting substances in serum (S), liver (L) and gastrocnemii (GN) of rats fed a casein (C-1, C-2, C-3) or a wheat (mod. SW, W) diet for 4, 8 or 12 weeks

Ninhydrin-reacting substances	Experiment II						Experiment III					
	S		L		GN		S		L		GN	
	Diet	Time[1]	Diet	Time	Diet	Time[1]	Diet	Time	Diet	Time	Diet	Time
Essential amino acids:												
Arginine					×		×	×				
Histidine		×	×									
Isoleucine		×						×			×	×
Leucine		×						×				×
Lysine									×			
Methionine	×	×	×				×				×	
Phenylalanine	×	×						×			×	×
Threonine		×	×									
Tryptophan	×	×			×	×						×
Valine		×										
Non-essential amino acids:												
Alanine	×	×										
Aspartic acid		×	×		×		×	×	×	×		
Cystine			×	×								
Glutamic acid	×	×					×		×			
Glycine		×					×		×	×		
Proline		×			×	×	×	×				
Serine	×	×	×	×								
Tyrosine	×	×	×									
Non-amino acids:												
Ornithine	×				×		×					
Citrulline		×						×				
Ammonia	×	×	×	×								
Taurine			×	×								

[1]No comparisons made with zero-time controls

Table 19. Comparison of ninhydrin-reacting substance concentrations in serum, liver and gastrocnemii of rats fed diets containing casein (C-2) or wheat (W) for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances	Serum		Liver		Gastrocnemii	
	Diet	Time[1]	Diet	Time[1]	Diet	Time[1]
Essential amino acids:						
Arginine	W>C-2	8*				
Histidine	W>C-2	4*			C-2>W	4*, 8*
	C-2>W	12*				
Isoleucine	C-2>W	12*	W>C-2	4*, 8*	C-2>W	4*, 8*
Leucine	C-2>W	8, 12*	W>C-2	8*	C-2>W	4*, 8*
Lysine	C-2>W	4, 8, 12	C-2>W	8*	C-2>W	4, 8, 12
Methionine					C-2>W	4, 8
Phenylalanine			W>C-2	8*	C-2>W*	8*
Threonine	C-2>W	8*			C-2>W	4, 8, 12
Tryptophan			W>C-2	4*		
Valine	C-2>W	8*, 12*	W>C-2	8*	C-2>W	4, 8
Non-essential amino acids:						
Alanine			C-2>W	12*		
Aspartic acid	C-2>W	8*				
Cystine	C-2>W	8*				
Glutamic acid			C-2>W	12		
Glycine	W>C-2	8*, 12*	W>C-2	8*	W>C-2	4, 8*, 12
Proline	W>C-2	8	W>C-2	4*		
Serine					C-2>W	4, 8
Tyrosine					C-2>W	4, 8*
					W>C-2	12
Non-amino acids:						
Ornithine			W>C-2	8*		
Citrulline	W>C-2	12*			W>C-2	8*
Ammonia					C-2>W	12
					W>C-2	4, 8
Taurine	W>C-2	4, 12			W>C-2	4, 8, 12

[1]Length of time (weeks) on diet

*Different at $P<0.05$; otherwise, different at $P<0.01$

Table 20a. Comparison of free essential amino acid concentrations in serum, liver and gastrocnemii of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW[1]) for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances	Serum		Liver		Gastrocnemii	
	Diet[2]	Time[3]	Diet[2]	Time[3]	Diet[2]	Time[3]
Arginine					C-1>SW*, C-3, C-2	4
Histidine	C-1>C-2, SW, C-3	4, 8*	C-3>C-1, C-2, SW	8*	C-1, C-2, C-3>SW	4*
					C-2>SW	8
					C-1>C-2, C-3, SW	8, 12
Isoleucine	C-3>C-2, SW, C-1	4*	C-2, SW, C-3>C-1	4*		
Leucine	C-3>C-2, SW, C-1	4*	C-2, SW, C-3>C-1	4*	C-3>C-2, SW	4*
			C-2>C-3, SW	4	C-1>SW	4
Lysine	C-1>C-2, SW	12*			C-1, C-2, C-3>SW	4*
					C-1>C-2, SW	8
Methionine			C-3>C-2, SW, C-1	8*		
			C-2, SW>C-1	8		
Phenylalanine	C-3>SW, C-2, C-1	4*	C-2, C-3, SW>C-1	4*		
	SW>C-1	4	C-3>SW, C-1	8*		
			C-2>C-1	8		
Threonine	C-3, C-2, SW*>C-1	4*	C-2>C-1, SW	4*	C-3>C-2, SW, C-1	4*
	C-3>SW	4*	C-2, C-1, C-3>SW	8*	C-2>SW, C-1	4
	SW>C-1	4	C-1, C-2>SW	12*	C-2>SW, C-3, C-1	8
					C-1, C-3>SW	8
					C-2, C-1, C-3>SW	12
Tryptophan	C-2>SW, C-1	4*	C-2, C-1>SW, C-3	8*	C-3, C-2>C-1, SW	12*
	C-3>C-1	4				
Valine	C-3>C-2, SW, C-1	4*	C-3>SW, C-2, C-1	8	C-3>C-2, C-1, SW	4*
	C-2>C-1	4			C-2>SW	4
	C-3>SW, C-1	12*				

[1] Only SW used in body of table

[2] When possible diets listed according to concentration of amino acid in tissue e.g. Histidine - highest in serum of rats fed C-1 diet, lowest in rats fed C-3 diet

[3] Length of time (weeks) on diet

* Different at $P<0.05$; otherwise, different at $P<0.01$

Table 20b. Comparison of free non-essential amino acid concentrations in serum, liver and gastrocnemii of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW[1]) for 4, 8 or 12 weeks, experiment III

Non-essential amino acids	Serum		Liver		Gastrocnemii	
	Diet[2]	Time[3]	Diet[2]	Time[3]	Diet[2]	Time[3]
Alanine	C-1>C-3, SW, C-2*	8	C-2, C-3*, C-1*>SW	4	C-1, C-2, C-3>SW	4*
	C-2>C-3, SW	8	C-1, C-2, C-3*>SW	8	C-1, C-2, C-3*>SW	8
	C-1>C-2, C-3, SW	12	C-1, C-2>SW	12	C-2>C-3	8
	C-2>SW	12*	C-1>C-3	8*, 12	C-1, C-2>SW	12
			C-2>C-3	12*	C-1>C-3	8, 12
Aspartic acid					C-3>C-1, SW*	4
Cystine	C-2>C-1, SW	8*	C-2>C-1, C-3, SW	8		
			C-1>C-3*, SW	8		
Glutamic acid	C-2, C-1>C-3, SW	4*			C-3, C-2>SW	4
					C-3, C-2*>C-1	4
					C-2, C-1>C-3, SW	8
					C-2>C-1	8*
Glycine	SW>C-2, C-1, C-3	4, 8, 12*			SW, C-2, C-1*>C-3	4
	C-2>C-3	4*			SW>C-1, C-2	4
	C-1>C-3	8			C-2>C-1	4*
					SW, C-1, C-2>C-3	8
					SW>C-2	8*
					C-1, SW*>C-3	12
					C-1>C-2	12*
Proline			C-3>SW, C-1	4*	C-3>C-1, SW	4
			C-2>C-1	4*	C-2>C-1*, SW	8
Serine	C-1>C-3, SW	4	C-2, C-1>SW, C-3	4*	C-1>C-2, C-3, SW	4, 8, 12
	C-2>C-3	4, 12*	C-1>C-2, C-3, SW	8	C-2>SW	4, 8, 12
	C-2>SW	4, 12	C-2>C-3, SW	8	C-3>SW	4
	C-1>SW, C-3	8*	C-1>C-2, C-3, SW	12	C-2>C-3	4, 8
	C-1>C-2, C-3, SW	12				
Tyrosine	C-2, C-3>C-1	4*, 8, 12	C-2, C-3>C-1	4	C-2>C-1, SW	4, 8
	C-2>SW	4*, 12*	C-3>C-2, SW, C-1	8*	C-2>C-3	4*
	C-3>SW	12			C-3>SW	4*, 8
					C-3>C-1	8

[1] Only SW used in body of table

[2] Diets listed according to concentration of amino acid in tissue

[3] Length of time (weeks) on diet

* Different at $P<0.05$; otherwise, different at $P<0.01$

Table 20c. Comparison of free non-amino acid concentrations in serum, liver and gastrocnemii of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW[1]) for 4, 8 or 12 weeks, experiment III

Non-amino acids	Serum		Liver		Gastrocnemii	
	Diet[2]	Time[3]	Diet[2]	Time[3]	Diet[2]	Time[3]
Ornithine			C-2>C-1, C-3	4*	SW>C-2	4*
			SW>C-3	4*		
Citrulline	C-1>C-3, SW, C-2	12			C-1>C-2, C-3, SW	4*, 8, 12
					C-2>C-3, SW	8
Ammonia	C-1>SW, C-2, C-3	12	C-1>C-2, SW	8*	C-1>C-2, SW, C-3	4
			C-3>SW	8*	C-1, C-2>C-3, SW	8*
					C-2>C-3, SW	4*, 8*
Taurine	C-3>C-1, C-2	8*, 12	C-3>C-1	8*, 12	C-3, SW>C-1, C-2	4, 8, 12
	SW>C-1, C-2	8*	C-3>C-2	8*	SW>C-3	4
	SW>C-1	12*	SW>C-1, C-2	8*, 12	C-2>C-1	12

[1]Only SW used in body of table

[2]When possible diets listed according to concentration of amino acid in tissue

[3]Length of time (weeks) on diet

*Different at $P<0.05$; otherwise, different at $P<0.01$

1. Dietary effects

The summary for experiment II (Appendix D, Table I or Table 19) shows rats fed the C-2 diets had higher concentrations of 1) six essential amino acids in serum, 2) eight essential amino acids in gastrocnemii than did rats fed the W diet. The latter rats, however, showed higher levels for all but one essential amino acid (lysine) in the liver than did the group of rats fed the C-2 diet. Lysine, also, was higher in the serum and gastrocnemii of rats fed the C-2 diets than in those consuming the W diet for 4, 8 and 12 weeks; in fact, no lysine was detectable in the gastrocnemii of rats fed the W diet. Significantly higher, too, were the levels of methionine in gastrocnemii of rats fed the C-2 diet 4 and 8 weeks but not after 12 weeks. Actually, fewer differences in the free amino acid content of liver and gastrocnemii occurred after consumption of diets for 12 weeks, as the ninhydrin-reacting substance levels in similar tissues of rats fed the W diet increased more than those of rats fed the C-2 diet (Table 16 or 19).

Of the non-essential amino acids, rats fed the C-2 diet had higher levels of aspartic acid and cystine in serum (8 weeks), alanine and glutamic acid in liver (12 weeks) and alanine, serine and threonine in gastrocnemii (4 and 8 weeks) than did the W diet-fed rats. All glycine concentrations, however, were higher in the serum, liver and gastrocnemii of

rats fed the W diets than those fed the C-2 diets. Proline was also higher in serum and liver of the rats fed the W diet. Most of the non-amino acids, especially taurine, were more highly concentrated in the serum and gastrocnemii than the liver of rats fed the W diet.

Within the various tissues analyzed (experiment III, Appendix D, Tables II or Table 20a), significant differences in free essential amino acid concentrations also resulted when four different diets were fed to rats 4, 8 or 12 weeks. As in experiment II, response to the diets fed varied within a particular tissue over a period of time. A particular effect often persisted throughout the 12-week period, i.e. valine in the serum of rats fed the C-3 diet for 4 and 12 weeks was more highly concentrated than that of rats fed the other diets even though there was no difference between serum levels of valine in rats fed C-2 and C-3 diets (12 weeks). On the other hand, the essential amino acid, threonine, showed the greatest variation in gastrocnemii as rats fed the C-3 diets for 4 weeks had significantly higher levels of this amino acid than did rats fed the other diets; however, after 8 weeks rats fed the C-2 diet had the highest levels and by 12 weeks a similar concentration of threonine was found in the gastrocnemii of rats fed C-1, C-2 and C-3 diets which were all significantly higher than rats fed the mod. SW diets. More commonly, however, similar responses to diet

were reflected as a low concentration of an amino acid, i.e. rats fed the mod. SW diet for 4, 8 and 12 weeks had lowest levels of threonine in the liver and histidine in gastrocnemii; the lowest level of threonine in rats fed the mod. SW diet, however, was found in gastrocnemii (12 weeks). In general, the lower concentrations of essential amino acids were found in tissues of rats fed either the C-1 or mod. SW diets, except for arginine in gastrocnemii and histidine and lysine in the serum and gastrocnemii of rats consuming the C-1 diet for 4, 8 or 12 weeks. In many instances, higher levels of essential amino acids were found in the tissues of rats fed the C-3 diet but often there were no significant differences in these concentrations compared to rats consuming the C-2 or mod. SW diets.

Concentration values for non-essential amino acids (Appendix D, Tables II or Table 20b) showed a preponderance of high levels in the tissues of rats fed the C-1 or C-2 diets, e.g. alanine, cystine, serine, tyrosine (except at 8 weeks in liver) and glutamic acid in serum at 4 weeks and in gastrocnemii at 8 weeks; usually lowest values were found in rats fed the mod. SW diet. Aspartic acid, glutamic acid and proline were most highly concentrated in gastrocnemii of rats fed the C-3 diet 4 weeks. Highest levels of glycine were found in the serum and gastrocnemii of rats, fed the mod. SW diets for 4, 8 or 12 weeks. No significant

difference in concentration of glutamic acid or glycine was found in the liver of rats fed the four diets used in experiment III (Table 18 or 20b).

The summary of non-amino acids (Appendix D, Tables II or Table 20c) shows the ornithine levels in serum were also similar regardless of the diet consumed. Citrulline and ammonia, however, were highest in rats fed the C-1 diet, with ammonia showing higher levels in the serum, liver and gastrocnemii after 4, 8 and 12 weeks consumption, respectively; again, lowest values were found in rats fed the mod. SW diet. Taurine was most prominent in the serum, liver and gastrocnemii of rats fed either the mod. SW or C-3 diets, mostly after 8 or 12 weeks.

2. Effect of diet with age

The data (Appendix D, Tables III and IV) indicate that 1) more significant changes occurred in the liver and gastrocnemii than in the serum, 2) most of these significant changes occurred between the start of the experiment and after rats had consumed the diets 4 weeks, and 3) in liver and muscle the significant changes mostly involved a decrease in concentration of the various amino acids. Most of the significant increases, however, were exhibited in the serum of rats studied in experiments II and III (Appendix D, Tables IIIa, IVa).

Unfortunately, no data for serum were available to

determine the changes that probably took place between the start of the experiment and after consumption of the C-2 and W diets (experiment II). In experiment III, in the serum of rats fed the C-1 diet, 1) only cystine, serine and ammonia increased after consumption of the diet for 4 weeks, 2) threonine, alanine and serine after 8 weeks and 3) threonine, alanine, cystine, serine and ammonia after 12 weeks. Concentration increases in threonine, valine, alanine, cystine, glutamic acid, serine and tyrosine in the serum of rats fed the C-2 diet for 4 weeks persisted throughout the experiment. Similar serum increases were noted for histidine, lysine, methionine, threonine of C-3 fed rats as well as histidine, lysine, threonine and valine in rats fed the mod. SW diet. There was an increase in the concentration of serum alanine, regardless of the diet fed. Significant increases in serum of rats from 4-8 weeks, 4-12 weeks and 8-12 weeks occurred in 1) the arginine, cystine and taurine values of rats fed the C-2 diets (experiment II), 2) the threonine and alanine values of rats fed the C-1 diet, as well as the valine, methionine and histidine values of the rats fed the C-2, C-3 or mod. SW diets, respectively (experiment III). Only lysine and ornithine decreased between 4, 8 or 12 weeks in rats fed the C-2 diet (experiment II); similar decrease in serum lysine did not occur in rats fed the C-2 diet although ornithine followed the same pattern. Decreases, however, were

more prominent in the serum of rats of experiment III.

In liver (experiments II and III, Appendix D, Tables IIIb, IVb) most of the significant changes consisted of decreases from the start of the experiment to 4, 8 or 12 weeks later. Consistent decreases occurred in most essential amino acids of experiment II, with the exception of tryptophan and threonine as well as aspartic acid and glutamic acid; not all essential amino acid concentrations in experiment III, however, showed a decrease but leucine, phenylalanine and valine in most rat livers decreased. Between 4-8 or 4-12 weeks most decreases in essential amino acids occurred in the liver of rats fed the C-2 diet in experiment II and in the liver of rats fed the C-3 and mod. SW diets as well as the C-2 diet in experiment III. Among the non-amino acids, most significant decreases occurred between 0-4, 8 or 12 weeks for 1) ornithine for all rats, 2) ammonia in rats fed the mod. SW diet, and 3) taurine for rats fed the C-1 and C-2 diets.

Concentration of ninhydrin-reacting substances in the gastrocnemii did not have as many increases as serum or as many decreases as liver. Nor were the essential amino acid concentrations in experiment III involved in as many changes from period to period as they were in other tissues. In experiment II (Appendix D, Table IIIc), significant increases occurred in 4 and 6 essential amino acids of rats

fed the W diet between 4-12 weeks and 8-12 weeks, respectively; decreases, however, included 4 or 5 essential amino acids in rats fed the C-2 diet and 8 in rats fed the W diet for various periods after the start of the experiment to 4, 8 or 12 weeks later. Between 4-8 weeks and 4-12 weeks, lysine and histidine decreased in rats fed the C-2 diet while arginine, histidine and threonine decreased between 4-8 weeks in rats fed the W diet. Most of the non-essential amino acids decreased throughout the experiment, especially aspartic acid, glutamic acid, glycine and serine. The concentration of the non-amino acids, ornithine and citrulline, also were reduced, mostly in rats fed the C-2 diet.

Only the concentrations of two essential amino acids, threonine and valine, increased in the gastrocnemii of rats in experiment III (Appendix D, Table IVc); alanine, proline, serine and tyrosine increased, also, mostly between 0-4 weeks or 0-8 weeks. There was a persistent increase in the taurine content of gastrocnemii in rats fed the C-2 and/or C-3 diets. Arginine decreased significantly for all rats except those fed the C-1 diet between the start of the experiment and after four weeks on the diet; lysine decreased between 0-4 weeks, 0-8 weeks and 0-12 weeks in rats fed the mod. SW diets and between 4-12 weeks and 8-12 weeks in rats fed the C-1 diet. Glutamic acid, glycine and all the non-amino acids decreased almost without exception for all diets

throughout most of the experimental period. These extensive changes, as significant increases or decreases, indicate that the gastrocnemii is indeed a metabolically active tissue.

3. Comparison of tissues

When serum, liver and gastrocnemii were analyzed statistically (Appendix D, Tables V and VI), all free amino acids except three, and ornithine were more highly concentrated in the liver than the serum of zero-time control rats. In addition, the essential amino acids - arginine, histidine, leucine, phenylalanine and valine, non-essential amino acids - aspartic acid, glutamic acid, tyrosine and ornithine were in higher concentration in the liver than in the gastrocnemii of zero-time control rats. There was a highly significant difference ($P<0.01$) between histidine, leucine, glutamic acid, glycine and serine of serum and gastrocnemii of these rats, with concentrations of lysine, threonine, tyrosine, ornithine, citrulline and taurine being higher in the gastrocnemii than in the serum, at a less significant level ($P<0.05$). The highest concentration of arginine was found in the gastrocnemii of control rats. There was no difference in the tryptophan or ammonia content of tissues.

Tryptophan, however, was higher 1) in the serum than in the liver of rats fed the C-2 diet (4 weeks, experiment II; 8 and 12 weeks, experiment III), C-3 diet (4, 8 and 12

weeks) and W diet (12 weeks); 2) in the serum than in gastrocnemii of rats fed the C-2 diet (4 weeks, experiment II; 12 weeks, experiment III), the C-3 diet (8 weeks) and W diet (12 weeks); 3) in the gastrocnemii than the liver of rats fed the C-2 (12 weeks, experiment III) and C-3 diets (8 weeks). Of particular interest also are the higher lysine values for serum than for gastrocnemii of rats fed the W diet (4 and 8 weeks). While the arginine content of serum and gastrocnemii was higher than that of the almost arginine-devoid liver, the serum content of rats fed the W (8 weeks) and mod. SW diets (12 weeks) was higher than that found in the gastrocnemii, with the latter being higher than the serum for rats fed the C-1 and C-2 diets (4 weeks) as well as the W diet (12 weeks).

Cystine, also, was higher in the serum than in the 1) liver of rats fed the C-2 diet (8 weeks), C-3 (4 weeks), W diet (8 weeks), 2) gastrocnemii in rats fed the C-3 diet (4 weeks), W diet (8 and 12 weeks). Although citrulline levels in serum were higher than the trace amounts found in liver, citrulline was most highly concentrated in skeletal muscle. In fact, all the degradative products of protein metabolism in the tissues analyzed, were more highly concentrated in the gastrocnemii than in either the liver or serum. By far the greatest concentrations of free amino acids per gram of tissue, however, were found in the livers of most

rats.

4. Overall status of free amino acids in tissues

The total amino acid content of tissues other than serum was lower for the rats consuming the various diets over a period of time than it was for the rats sacrificed at the start of the experiment (Tables 21 and 22). The lower concentrations of most individual amino acids in serum and the higher concentrations in the liver noted previously, were reflected in the total amino acid content of tissues. The slightly higher individual amino acid level for thigh and leg muscle than for gastrocnemii was found also in the total amino acid content, except for rats consuming the C-1 diet (12 weeks). The total amino acid concentration of tissues decreased over a period of time with the exception of levels of amino acids in the serum and liver of rats fed the C-1 diet. This increase in total amino acids was found as an increase in both the essential and non-essential amino acids, the essential to non-essential amino acid ratio (E/N ratio) indicating that the increase in the essential amino acids was not as great as in the non-essential amino acids.

The higher E/N ratio of serum than other tissues of rats fed the various diets indicate serum contained a higher proportion of essential amino acids than any other tissue. Data for E/N ratios of zero-time control rat livers (0.42, experiment II and 0.34, experiment III) suggest a

Table 21. Summary of concentrations (μmoles/100 ml or 100 g) of essential (E) and non-essential (N) amino acids (E:N ratio), total amino acids (% alanine, aspartic acid, glutamic acid, glycine, serine - AAGGS), non-amino acids, total ninhydrin-reacting substances - NRS (% taurine) in serum, liver, gastrocnemii, thigh and leg of rats fed casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

Variable	Serum						Liver						
	C-2			W				C-2			W		
	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	148	225	226	136	140	138	1229	741	453	459	618	485	438
N amino acids	324	313	285	310	275	302	2927	2867	2240	2007	2252	2092	1852
E:N ratio	0.46	0.72	0.79	0.44	0.51	0.46	0.42	0.20	0.21	0.42	0.27	0.23	0.24
Total amino acids	472	538	511	446	416	440	4156	2868	2693	2666	2870	2577	2290
AAGGS (%)	61.4	54.1	52.2	61.2	56.7	57.9	65.9	76.0	81.1	80.0	74.2	78.6	77.9
Non-amino acids	130	125	117	147	130	129	1475	1305	1007	943	1163	1297	1033
Total NRS	602	664	627	593	546	568	5631	4913	3699	3609	4033	3874	3323
Taurine (%)	1.3	2.3	2.9	6.5	4.6	4.4	2.7	0.2	0.3	1.2	1.8	4.9	2.1

Variable	Gastrocnemii							Thigh and leg					
		C-2			W			C-2			W		
	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	491	419	321	342	196	145	244	577	450	388	355	238	307
N amino acids	1812	1767	1275	1201	1571	1203	1282	1987	1652	1022	1710	1338	1276
E:N ratio	0.27	0.24	0.25	0.28	0.12	0.12	0.19	0.29	0.27	0.38	0.21	0.18	0.24
Total amino acids	2303	2186	1596	1543	1767	1348	1526	2564	2102	1410	2065	1576	1583
AAGGS (%)	75.6	77.2	74.5	73.8	84.7	84.3	80.4	73.5	73.8	68.6	78.5	80.8	77.2
Non-amino acids	562	922	1201	3185	2936	3150	2242	1396	1200	5178	1695	1711	1745
Total NRS	2864	3108	2797	4728	4702	4498	3768	3961	3301	6588	3760	3286	3328
Taurine (%)	18.0	9.0	12.9	14.9	20.0	21.2	31.8	5.7	9.0	7.8	21.1	25.0	26.6

[1] Zero-time controls
[2] Weeks

Table 22a. Summary of concentrations (μmoles/100 ml or 100 g) of essential (E) and non-essential (N) amino acids (E:N ratio), total amino acids (% alanine, aspartic acid, glutamic acid, glycine, serine - AAGGS), non-amino acids, total ninhydrin-reacting substances - NRS (% taurine) in serum and liver of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

	Serum												
		C-1			C-2			C-3			Mod. SW		
Variable	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	120	172	180	196	210	235	199	248	218	220	168	182	174
N amino acids	198	332	349	403	337	308	289	258	233	262	252	237	227
E:N ratio	0.61	0.52	0.52	0.48	0.62	0.76	0.69	0.96	0.94	0.84	0.67	0.76	0.77
Total amino acids	318	504	529	599	547	543	488	506	451	482	420	419	401
AAGGS (%)	55.0	56.8	58.4	60.9	51.7	46.4	42.8	39.4	39.6	42.3	50.6	46.9	47.6
Non-amino acids	129	152	144	172	141	120	125	143	130	144	140	130	146
Total NRS	447	656	673	771	688	664	613	648	582	626	559	548	548
Taurine (%)	5.0	2.3	1.6	1.7	2.0	1.8	3.1	3.0	3.8	6.3	5.0	4.4	5.3

	Liver												
		C-1			C-2			C-3			Mod. SW		
Variable	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	856	496	500	656	796	458	501	694	630	423	630	433	403
N amino acids	2555	2364	2413	2264	2693	2395	2220	2419	2034	1926	1954	1638	1553
E:N ratio	0.34	0.21	0.21	0.29	0.30	0.19	0.22	0.29	0.31	0.22	0.32	0.26	0.26
Total amino acids	3411	2859	2913	2920	3489	2853	2721	3113	2664	2349	2584	2071	1956
AAGGS (%)	70.8	79.2	80.1	74.7	73.1	79.7	78.8	73.5	72.4	79.0	72.2	75.9	76.4
Non-amino acids	1523	1327	999	1042	1265	710	875	1390	1324	1287	827	889	1101
Total NRS	4934	4186	3912	3961	4754	3563	3597	4504	3988	3636	3411	2961	3056
Taurine (%)	5.0	0.4	0.4	0.3	0.3	0.4	1.2	7.0	12.1	14.3	5.9	11.2	17.1

[1] Zero-time controls
[2] Weeks

Table 22b. Summary of concentrations (μmoles/100 ml or 100 g) of essential (E) and non-essential (N) amino acids (E:N ratio), total amino acids (% alanine, aspartic acid, glutamic acid, glycine, serine - AAGGS), non-amino acids, total ninhydrin-reacting substances - NRS (% taurine) in gastrocnemii, thigh and leg of rats fed casein (C-1, C-2, C-3) or wheat (mod. SW) diet 4, 8 or 12 weeks, experiment III

	Gastrocnemii												
		C-1			C-2			C-3			Mod. SW		
Variable	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	391	396	372	260	406	341	263	457	316	296	224	209	183
N amino acids	1853	1618	1496	1298	1714	1409	1012	1659	905	841	1345	922	826
E:N ratio	0.21	0.24	0.25	0.20	0.24	0.24	0.26	0.28	0.35	0.35	0.17	0.23	0.22
Total amino acids	2244	2014	1868	1298	2120	1750	1275	2116	1221	1136	1569	1131	1009
AAGGS (%)	79.5	76.8	77.1	79.9	76.9	76.0	74.5	72.6	68.9	68.6	82.2	77.6	78.4
Non-amino acids	5062	3385	2176	1582	1672	1817	1746	1689	2309	2195	2038	2079	2171
Total NRS	7306	5400	4044	3140	3772	3567	3022	3804	3530	3332	3608	3210	3181
Taurine (%)	12.4	7.5	7.7	7.3	8.4	9.4	22.1	17.7	37.3	37.2	28.2	35.5	37.7

	Thigh and leg												
		C-1			C-2			C-3			Mod. SW		
Variable	Control[1]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	448	445	357	379	520	411	351	504	473	428	519	265	285
N amino acids	2029	1687	1510	1545	1957	1426	1239	1733	1133	1071	1745	967	932
E:N ratio	0.22	0.26	0.24	0.24	0.26	0.29	0.28	0.29	0.42	0.40	0.30	0.27	0.31
Total amino acids	2471	2132	1867	1924	2477	1837	1590	2237	1606	1499	2264	1232	1217
AAGGS (%)	78.8	75.1	77.3	76.1	75.0	73.4	71.8	71.0	65.1	65.3	73.7	74.1	72.3
Non-amino acids	1705	1254	1292	1072	1688	1311	1508	1707	2257	1992	2274	1820	1826
Total NRS	4108	3386	3158	2996	4165	3147	3099	3944	3863	3492	4537	3052	3043
Taurine (%)	17.8	9.3	8.3	5.9	7.2	8.0	19.5	18.2	30.4	32.9	31.2	37.2	35.8

[1] Zero-time controls
[2] Weeks

higher concentration of essential amino acids in the liver than either gastrocnemii or thigh and leg muscle which had similar ratios. Most of the ratios for livers of rats consuming the diets 4, 8 or 12 weeks were more like those for skeletal muscle than they were for control rats. The ratios for serum of rats fed the less nutritionally-adequate C-1 and W diets remained consistently lower than ratios for serum of other rats; usually, the higher E/N ratios applied to the rats fed the more adequate diets, C-2, C-3 and mod. SW, also. On the whole, however, the non-essential amino acids account for a greater part of the total amino acids and ninhydrin-reacting substances found free in tissues.

Alanine, aspartic acid, glutamic acid, glycine and serine (AAGGS) account for over 50% of the total amino acids. In this study, AAGGS values were similar to those of Ryan and Carver (1963), Kaplan and Shimizu (1963), Scharff and Wool (1964) and made up 75.6% of the total amino acid content of gastrocnemii in control rats sacrificed at the start of experiment II. In controls for experiment III, 78.8% of total amino acids in thigh and leg muscle consisted of AAGGS; values for serum and liver were 55.0% and 70.8%, respectively. The lowest percentages of these amino acids were found in most tissue analyses of rats fed the C-3 diet; the highest percentages were found in the liver of C-2 diet-fed rats and in the skeletal muscle of W diet-fed rats (experiment II).

Non-amino acids contributed about 25-33% of the total ninhydrin-reacting substances in all tissues of zero-time control rats except for the gastrocnemii in experiment III which contained approximately 66% of the total ninhydrin-reacting substances. The non-amino acid content contained high levels of taurine. Taurine in control rats contributed the following to the total ninhydrin-reacting substances: 5% in serum, 2.7-5.0% in liver, 12.4-18.0% in gastrocnemii, and 17.8% in thigh and leg muscle. Concentrations in the livers of rats fed the C-1 and C-2 diets, however, were lower than the taurine content of the serum for the same rats, but the taurine content in liver of rats fed the C-3 and mod. SW diets exceeded the serum values for the same rats. Liver values for rats fed the C-2 and W diets (experiment II) were lower than those of serum and were highest in skeletal muscle. In experiment III, taurine content of gastrocnemii and thigh and leg muscle of rats fed the C-1 and C-2 diets was half that of controls while rats fed the C-3 and mod. SW diets had a taurine content about twice as large as that found in zero-time control rats.

On the whole, data obtained for tissue of rats fed the C-2 diet in experiment III agree well (Table 23) with that found for rats fed the C-2 diet in experiment II, despite some of the differences noted above.

Table 23. Summary of concentrations (μmoles/100 ml or 100 g) of essential (E) and non-essential (N) amino acids (E:N ratio), total amino acids (% alanine, aspartic acid, glutamic acid, glycine, serine - AAGGS), non-amino acids, total ninhydrin-reacting substances - NRS (% taurine) in serum, liver, gastrocnemii, thigh and leg of rats fed casein (C-2) diet 4, 8 or 12 weeks, experiments II and III

Variable	Serum C-2						Liver C-2						
	Exp. II			Exp. III			Controls[1]		Exp. II			Exp. III		
	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	Exp. II	Exp. III	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	148	225	226	210	235	199	1229	856	741	453	459	796	458	501
N amino acids	324	313	285	337	308	289	2927	2555	2867	2240	2207	2693	2395	2220
E:N ratio	0.46	0.72	0.79	0.62	0.76	0.69	0.26	0.34	0.20	0.21	0.42	0.30	0.19	0.22
Total amino acids	472	538	511	547	543	488	4156	3411	2868	2693	2666	3489	2853	2721
AAGGS (%)	61.4	54.1	52.2	51.7	46.4	42.8	65.9	70.8	76.0	81.1	80.0	73.1	79.7	78.8
Non-amino acids	130	125	117	141	120	125	1475	1523	1305	1007	943	1265	710	875
Total NRS	602	664	627	688	664	613	5631	4934	4913	3699	3609	4754	3563	3597
Taurine (%)	1.3	2.3	2.9	2.0	1.8	3.1	2.7	5.0	0.2	0.3	1.2	0.3	0.4	1.2

Variable	Gastrocnemii C-2								Thigh and leg C-2					
	Controls[1]		Exp. II			Exp. III			Exp. II			Exp. III		
	Exp. II	Exp. III	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]	4[2]	8[2]	12[2]
E amino acids	491	391	419	321	342	406	341	263	577	450	388	520	411	351
N amino acids	1812	1853	1767	1275	1201	1714	1409	1012	1987	1652	1022	1957	1426	1239
E:N ratio	0.27	0.21	0.24	0.25	0.28	0.24	0.24	0.26	0.29	0.27	0.38	0.26	0.29	0.28
Total amino acids	2303	2244	2186	1596	1543	2120	1750	1275	2564	2102	1410	2477	1837	1590
AAGGS (%)	75.6	79.5	77.2	74.5	73.8	76.9	76.0	74.5	73.5	73.8	68.6	75.0	73.4	71.8
Non-amino acids	562	5062	922	1201	3185	1672	1817	1746	1396	1200	5178	1688	1311	1508
Total NRS	2864	7306	3108	2797	4728	3792	3567	3022	3961	3301	6588	4165	3147	3099
Taurine (%)	18.0	12.4	9.0	12.9	14.9	8.4	9.4	22.1	5.7	9.0	7.8	7.2	8.0	19.5

[1]Zero-time control rats
[2]Weeks

DISCUSSION

This discussion deals with some controversial aspects in the determination of amino acid availabilities by the fecal analysis method. In addition, emphasis is placed on interpretation of the tissue study data. No attempt is made, however, to compare serum and liver free amino acid values found on this long term study (i.e. 28, 56 and 84 days) with the many short term studies (i.e. several hours to seven days duration) found in the literature; but where applicable, similar changes in levels of free amino acids, especially with reference to the limited number of longer-term studies of 28 days duration, will be noted. Rather, the aim is to determine whether free amino acids of developing tissue, i.e. serum, liver or skeletal muscle, reflect the amino acid intake; and if so, to what extent are deficiencies and excesses reflected in these tissues.

Availability Study

Determination of the availability of amino acids from protein is a measure of their potential utilization in a biological system. *In vitro* assays measure the rates at which amino acids are released by proteolytic enzymes whereas *in vivo* assays determine the amino acid levels of undigested protein in relation to that of ingested protein.

To date, all methods used give only an estimate of the true availability of amino acids. In particular, the fecal analysis method has been criticized because the role of the gastrointestinal tract in mammalian protein metabolism is not well understood.

Accurate measurement of the metabolic fecal amino acids has been a problem; many techniques have been used to determine its importance. Mitchell and Bert (1954) showed metabolic fecal nitrogen was independent of the level of dietary protein by extrapolation of a series of nitrogen levels to zero-protein intake level. This concept has been upheld by Nasset (1965) also, for the mixture resulting from the approximate seven-fold dilution of ingested labeled protein by endogenous protein (Nasset and Ju, 1961) was hydrolyzed in the lumen to yield an amino acid pool with a relatively constant molar ratio regardless of the type and amount of protein ingested. Other findings, however, indicate that endogenous nitrogen secretions increased or decreased with the level of protein in the diet (Albanese, 1959; Twombly and Meyer, 1961; Snook, 1965).

The contribution of the microflora as such and their influence on the fecal amino acid concentrations has also been questioned. Since Kuiken (1952) did not find a significant change in the availability of amino acids to sulfathiozole-fed rats, he concluded that bacterial synthesis and

degradation had little effect on endogenous levels of amino acids and proteins. Gustaffson and Fitzgerald (1960), however, found the numbers of lacto-bacilli and possibly other micro-organisms in intestinal contents and feces of rats were simultaneously reduced by coprophagy. In this study, no significant difference was observed in the level of amino acids available whether or not the rats wore tail cups.

Of greater importance perhaps is the release into the lumen of desquamated cells (Nasset, 1965). No doubt, this is promoted by the amount of dry matter in the diet. Mitchell (1924) found incorporation of fiber in the diet increased the proportion of metabolic fecal nitrogen to dry matter consumed. Maynard and Loosli (1969) determined the relationship to be approximately 100 mg of metabolic fecal N per 100 g of dry matter consumed by rat, pig and man. In the present study, the N-free diet consumed at an average level of 9.5 g daily by the 80 g rats and 23 g by the 300 g rats, resulted in an average daily metabolic fecal N production of 13 mg and 32 mg, respectively. Whereas the observed mean level of metabolic fecal N was higher (39% for 80 g rats and 50% for 300 g rats) than predicted by Maynard and Loosli, a linear relationship (Fig. 10) occurred between the amount of diet consumed and the amount of metabolic fecal N excreted by the rats in all three tail cup treatments. Though the amount of metabolic fecal N excreted by the three

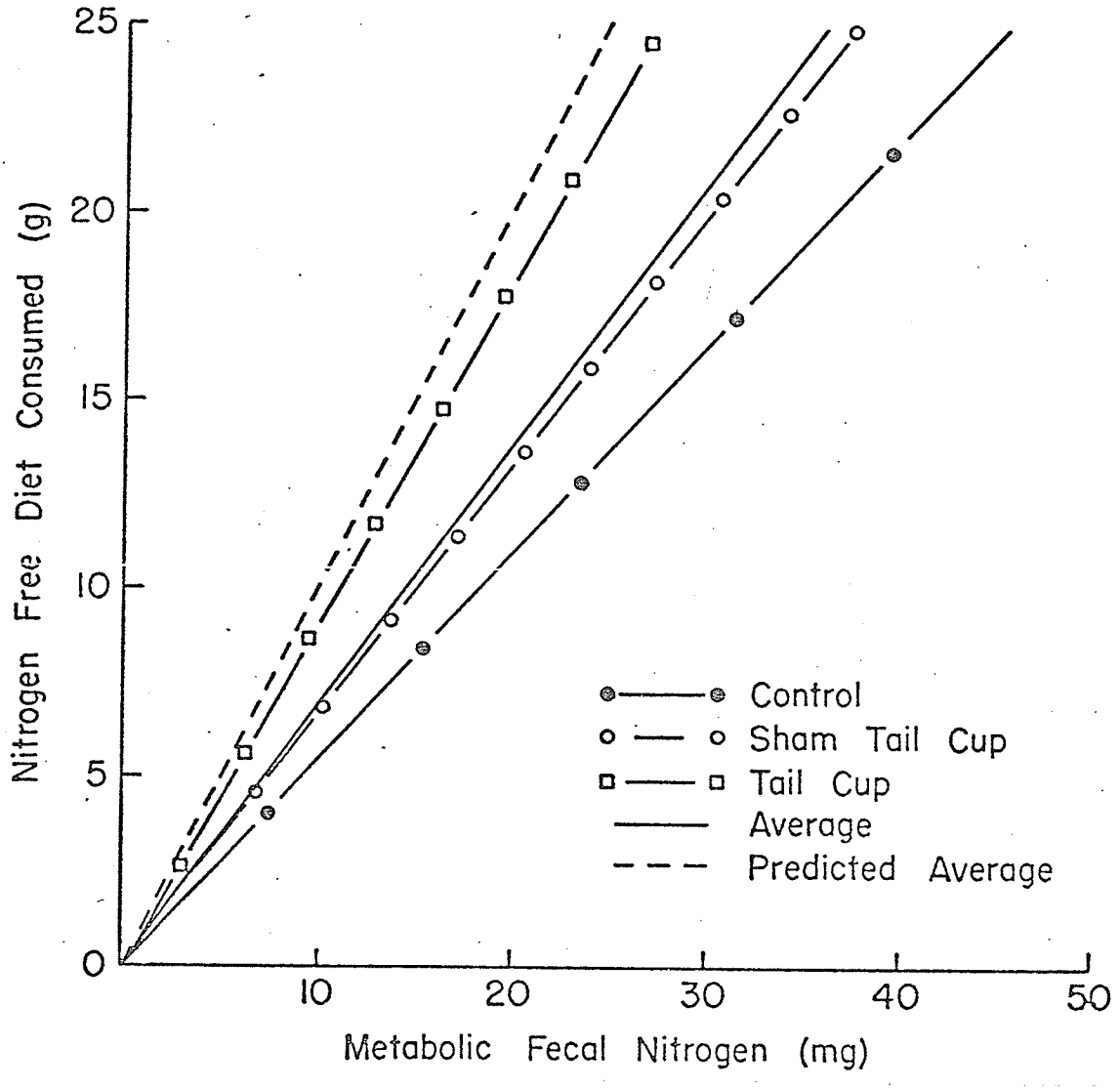

Fig. 10. The relationship between the amount of nitrogen free diet (g) consumed and the amount of fecal N (mg) produced

groups did not differ statistically, that excreted by rats with real tail cups was essentially the same as the average level predicted from the amount of diet consumed. The low intake of N-free diet accounts for low estimates of fecal N and about a 3% depression in the calculated availability of amino acids. An increase in metabolic fecal N results, however, when indigestible material in the diet is high. Meyer (1956) found a linear relationship between the cellulose content of diet and the metabolic fecal N. A significant difference in production of metabolic fecal N resulted when diets containing 15% to 30% cellulose were compared with those containing no cellulose. On this basis, the use of a N-free diet containing a cellulose content of 20% in this study would give a higher fecal N excretion than wheat and an estimated 3% increase in the calculated availability of amino acids. Despite these problems in establishing precise true availabilities, comparisons of the availability of different amino acids are unchanged.

The availability of essential amino acids from wheat in this study compares favorably with values obtained by Kuiken and Lyman (1948) although methods for determining metabolic fecal N and amino acids differed. The values for all of the essential amino acids except threonine (3.2% lower) were from 0.4% to 1.3% higher than found in this study. The higher availability values usually found by the fecal

analysis method are possibly the result of larger excretions of metabolic fecal nitrogen (amino acids) from low protein or nitrogen-free diets than from diets of higher protein content. Thus, in the use of the best techniques presently available for the determination of metabolic fecal nitrogen, there must be acceptance of the questionable assumption that protein content of the diet does not influence endogenous nitrogen content.

The comparison between the apparent and true availability values in this study indicate the contribution made by endogenous amino acids. Despite the discrepancies in absolute availability values, however, the determination of apparent digestibility may serve satisfactorily in the estimation of available amino acids, particularly in the determination of the least available ones. This suggests a reassessment of the contribution to animals of the amino acids from wheat diets, i.e. a reduction of lysine by 11-14%, valine and methionine by 6-10%, threonine by 5-11% and so on in younger rats, and similarily for older rats, if actual values of the amino acids absorbed are desirable.

Tissue Study

Without consideration of the availability of amino acids, however, Table 6 indicates 1) all essential amino acids of the C-1 diet and all except arginine, leucine and

possibly phenylalanine in the W diet were inadequate, 2) histidine, lysine, methionine, phenylalanine, threonine and valine of the C-2 diet as well as the methionine content of the C-3 diet and mod. SW diet may have been inadequate for the growing rat. The methionine content, however, probably was greater than indicated by analysis as S-containing amino acids are partially destroyed during acid hydrolysis. Accordingly, the diets rank as follows from the most to the least nutritionally adequate: C-3, mod. SW, C-2, W, C-1.

Nucleic Acids and Protein in Developing Tissues

In keeping with the findings of Enesco and Leblond (1962) and Winick and Noble (1965) it might not be erroneous to suspect hyperplasia was not complete in all tissues of rats fed the C-2, W and C-1 diets, especially that of skeletal muscle since the rats were placed on these dietary regimen at 24 or 25 days of age. However, evidence that hyperplasia was not restricted to a significant extent in liver or skeletal muscle and that these tissues realized their full complement of cells with each diploid nucleus containing 6.2 μg DNA, is indicated by the lack of statistical differences among the DNA content of liver or that of most gastrocnemii of rats fed the various diets (Table 15). This is based on the assumption that rats fed the C-3 and mod. SW diets are adequate or near-adequate in all essential nutrients for the growing rat. Using these rats as a

guide-line, less weight gain over the experimental period and greater protein loss between the start of the experiment and four weeks later (Table 12) indicate restricted hypertrophy in liver of rats fed the C-1 and C-2 diets. The statistically higher values of DNA (Table 15), however, obtained in the gastrocnemii of rats fed the C-2 diet for 8 and 12 weeks than that in rats fed the other diets suggests greater restriction in the size of cells in the gastrocnemii of rats fed the C-2 diet and consequently more cells and more DNA per gram of tissue. Even higher DNA levels were found in gastrocnemii of rats fed the C-2 diet in experiment II (Table 11); no doubt, this was partially the result of the diet fed the first ten days of the experiment. The 78, 72 and 78 mg of DNA per 100 g gastrocnemii of rats fed the C-1, C-3 and mod. SW diets, respectively, is lower than the 89 mg of DNA per 100 g gastrocnemii of rats fed the C-2 diet in experiment III for the same length of time. Thus, although diet does not affect the DNA content of a cell it indirectly influences the DNA content of tissues by restricting or promoting the development of cells.

Since Figure 1 shows DNA content (mg/g tissue) decreases with development, a similar decrease in our study may indicate less increase in cell number than in cell size. On this basis, the greatest increase in cell size occurred in the livers of most rats between 24 and 52 days

of age (Table 11); in most instances, this period of fastest cell development in the gastrocnemii, thigh and leg muscles of rats fed the C-2, C-3, mod. SW diets and to a limited extent the C-1 diet continued until the rats were 80 days old.

The protein/DNA ratios (Table 12), however, indicate a reappraisal of such an interpretation for a 10% and 6% decrease in these ratios of liver for rats fed the C-1 diet and to a lesser extent rats fed the W diet for 4 weeks, respectively, suggest a loss of protein and an accumulation of 1) fat, possibly because of inadequate intake of choline resulting from reduced consumption of food, or 2) water as a result of hypoproteinemia or the retention of the pre-restricted diet level of extracellular fluid. Lack of normal development of liver tissue of rats fed the C-1 and W diets for 4 weeks was also reflected in the size of the organ (Appendix A, Tables I and II); livers of rats fed 1) the C-1 diet weighed 5.9 g, 2) the W diet weighed 6.2 g compared with the more normal weight of 11.5 g for rats fed the C-3 diet 4 weeks. In addition, the livers of rats fed the C-1 diets for 4 weeks were pinkish in color and fragile to touch. Rats fed the C-2 diet, however, may be considered a border-line case as there was only a slight increase in the protein/DNA ratio in rats fed from 24 to 52 days of age. The greatest accumulation of protein (Table 12) occurred in

livers of rats fed 1) the C-1 and C-2 diets from 52 to 80 days of age and 2) W diets from 80 to 108 days of age; rats fed the C-3 and mod. SW diets stabilized the protein content of their liver, at approximately 1700 mg/100 g liver, by the time they were 52 days old.

No loss of protein (Table 12) during the first four weeks of the study was evident in the gastrocnemii; in fact, there was an average increase of 15% over control values after rats consumed the diets 4 weeks. Rats fed the C-3 and mod. SW diets, however, accumulated protein in the gastrocnemii faster than did rats fed the C-1, C-2 and W diets, with a tapering off when rats were 80 days old. The accumulation of protein in the thigh and leg muscle over control values was much less than that shown by gastrocnemii.

An increase in protein content, however, is preceded by an increase in RNA content (Fig. 11). Like protein, the synthesis and catabolism of RNA is influenced by the quality and quantity of protein in the diet. Data (Table 12) indicate the more adequate the diet, the less quickly RNA levels were reduced; in fact, the RNA liver level of rats fed the C-1 diet was lowest at 4 weeks (52 days old). This suggests the possible reduction in RNA by the activation of ribonuclease as a result of an inadequate supply of amino acids. The protein content of muscle, however, as well as the protein/RNA ratios (Table 12) indicate that there was a more

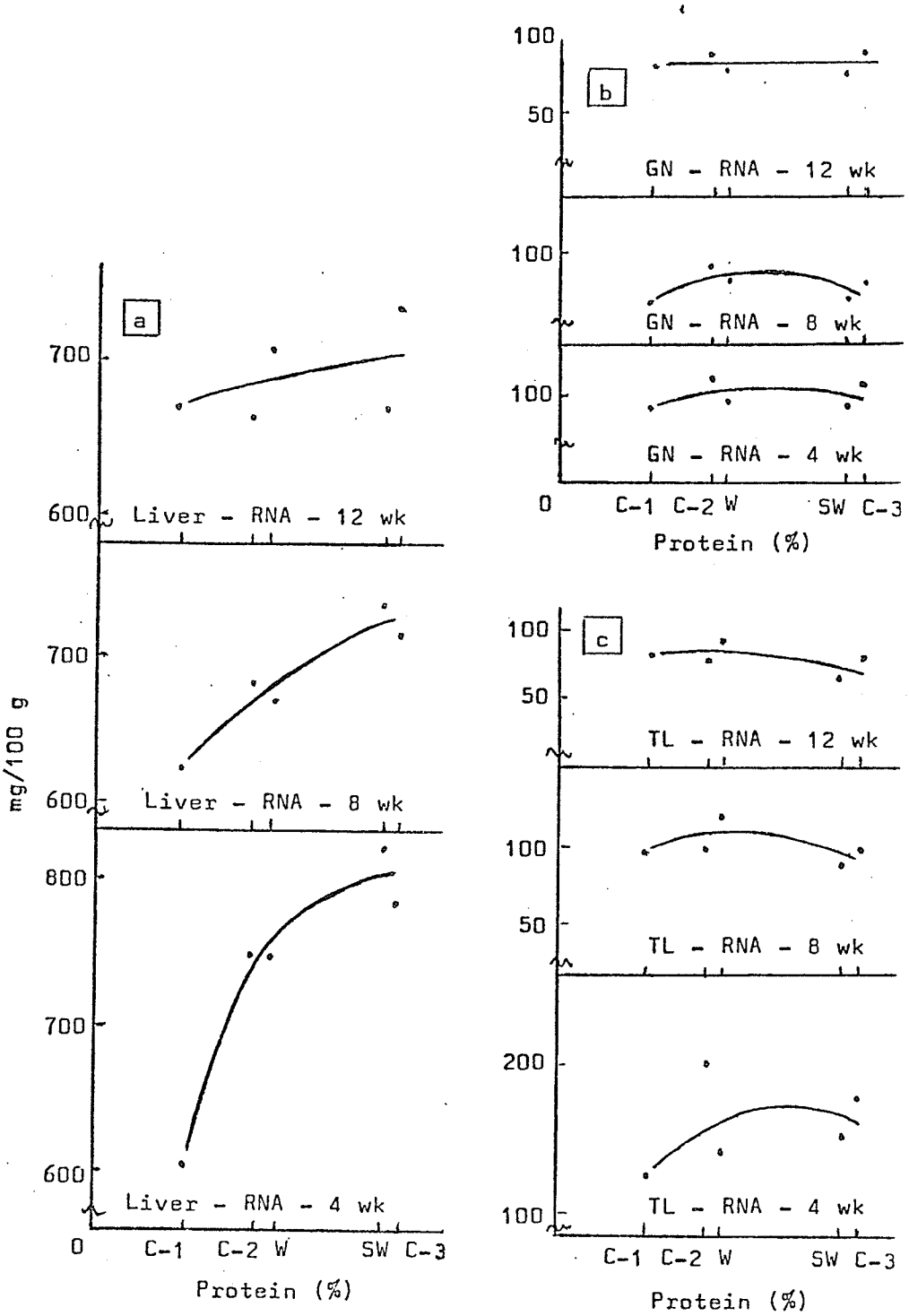

Fig. 11. Effect of protein intake on RNA (mg/100 g) in a) liver, b) gastrocnemii - GN, c) thigh and leg - TL of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW, W) diet 4, 8 or 12 weeks

normal accumulation of protein in these tissues than there was in liver, an increase in the ratio with age showing a greater accumulation of protein than of RNA.

Present evidence, therefore, suggests that the diet significantly influenced normal development of the tissues. Hypertrophy was inhibited in rats fed C-1, W and possibly C-2 diets with rats fed the C-1 diet developing the least. Although the tissues of rats fed the C-1 diet decreased in size, liver was the most affected for there was 1) a decrease rather than increase in protein content, 2) more rapid than normal decrease in DNA and RNA content, and 3) smaller, pinkish, fragile livers. Data (Table 11) and a more normal-looking liver after 8 weeks consumption of the diets suggest some adaptation and/or physiological aging.

A better understanding of the mechanisms involved in the processes of depletion and repletion indicate more frequent sampling of rats to determine 1) polysome pattern, 2) ribonuclease activity and 3) concentration of DNA, RNA and protein. Consideration might also be given to the 1) fat content of livers and the possible extent of lipogenesis by measurement of the activity of glucose-6-dehydrogenase, acetyl carboxylase or citrate cleavage enzyme, 2) type and magnitude of edema that may be involved, by analysis of serum protein and thiocyanate space, 5 g of protein or less per 100 ml of serum with low albumin/globulin ratio

indicating a true protein malnutrition edema and a thiocyanate space of approximately 30% of body weight indicating a hunger edema.

Free Amino Acids in Developing Tissues

Protein metabolism is intimately connected with amino acid metabolism; a change in one, therefore, should be reflected in the other. As Miller (1969) indicated an increase in amino acid pools during protein synthesis, reduction in levels of free amino acids might be expected during protein catabolism. Thus, the effect of diet on free amino acid levels of tissues possibly reflect the protein status of these tissues. Much emphasis (McLaughlan and Morrison, 1968; Rogers and Harper, 1968; Snyderman et al., 1968; Wannemacher and Allison, 1968) has been placed on the close relationship of serum amino acids and those ingested in a single meal. Recently, Munro (1970) reported there is evidence that changes in amino acid pools of serum and other tissues persist when proteins low in one or more essential amino acid are fed over a period of time.

In this long term study, the various tissues responded differently to the diets. With few exceptions, changes in the serum were less extreme than those in the liver, but usually more extreme than those in skeletal muscle. These prominent changes in all tissues persisted throughout the experiment in some instances; but more often, they were

modified to the extent that significantly different responses to different diets were minimized or no longer existed. That some of these changes, however, are not solely influenced by dietary protein alone, is recognized for the decline in food intake by rats fed the deficient diets may be responsible for some of the changes.

1. Serum

As indicated previously, several investigators have demonstrated that ingestion by rats of a diet low in one amino acid results in a reduction of that amino acid alone in the serum (Almquist, 1954; Baker et al., 1966; Clark et al., 1966; Gray et al., 1960; Longnecker, 1961; McLaughlan, 1964; Steele et al., 1950; Swendseid et al., 1963b, 1966; Wu, 1954). No such effect can be expected in this study for the inadequate diets, C-1, C-2 and W, had more than one deficient amino acid; lysine was the most limiting in the W diet and methionine in the C-2 diet as well as perhaps in the more adequate diets, C-3 and mod. SW diets. It is interesting to note that although analysis of the mod. SW diet (Table 6) revealed all other amino acids were adequate as compared with NRC recommended allowances, the lower content of all essential amino acids except arginine in the mod. SW diet compared with those of the C-3 diet were reflected as such in serum (Table 24a). The average serum non-essential amino acid pattern also followed closely

Table 24a. Percentage change in the levels of free amino acids and non-amino acids in serum of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW, W) diet 4, 8 or 12 weeks

Ninhydrin-reacting substances	C-1			C-2			C-3			Mod. SW			W*		
	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12
Essential amino acids:															
Arginine	+ 42	+ 86	- 22	0	+206	+172	+ 82	+ 44	+ 48	+214	+400	+302	+134	+336	+342
Histidine	+140	+128	+141	+ 61	+ 46	+ 33	+ 38	+ 18	+ 38	+ 48	+ 20	+ 33	+ 81	+113	+ 30
Isoleucine	- 22	- 14	- 13	+ 1	+ 1	+ 2	+ 37	+ 28	+ 28	- 1	+ 2	+ 11	+ 6	- 19	- 15
Leucine	- 15	- 12	- 6	+ 1	+ 13	+ 13	+ 55	+ 43	+ 49	- 2	+ 3	+ 18	0	- 19	- 15
Lysine	+127	+ 79	+145	+103	+123	+ 48	+101	+ 80	+100	+ 20	+ 18	+ 23	- 45	- 66	- 47
Methionine	- 13	0	- 11	+ 27	+ 41	+ 37	+ 37	+ 56	+ 18	+ 20	+ 47	+ 22	+ 4	+ 54	+ 20
Phenylalanine	- 12	- 4	+ 1	- 4	+ 8	+ 15	+ 27	+ 37	+ 23	+ 6	- 2	+ 22	+ 33	- 2	- 19
Threonine	+ 21	+113	+109	+197	+229	+191	+276	+201	+186	+127	+105	+ 71	+110	+ 98	+ 76
Tryptophan	- 94	+ 6	- 47	+153	+174	+ 95	+118	+ 58	- 42	+ 6	+169	+ 97	- 31	+ 58	+153
Valine	+ 3	+ 22	+ 13	+ 54	+ 49	+ 64	+115	+ 99	+111	+128	+ 36	+ 39	+ 16	+ 4	+ 5
Non-essential amino acids:															
Alanine	+126	+162	+208	+128	+105	+ 92	+ 51	+ 33	+ 64	+ 47	+ 40	+ 40	+ 49	+ 68	+ 82
Aspartic acid	- 19	- 9	+ 77	- 12	- 13	- 13	- 7	- 13	+ 6	- 9	- 26	- 17	+ 56	- 7	- 17
Cystine	+307	+120	+200	+430	+490	+610	+370	+250	+800	--	--	--	+ 60	+350	+830
Glutamic acid	+ 48	+ 35	+ 81	+ 51	+ 35	+ 15	+ 25	+ 10	- 1	+ 6	- 5	- 8	+ 60	+ 21	+ 1
Glycine	- 50	- 37	- 40	- 40	- 52	- 47	- 65	- 65	- 54	+ 7	+ 1	- 13	+ 18	+ 3	+ 39
Proline	+111	+ 80	+ 77	+ 87	+111	+ 94	+145	+113	+119	+ 69	+ 84	+ 61	+ 62	+ 61	+ 70
Serine	+124	+147	+154	+ 98	+ 75	+ 74	+ 19	+ 12	+ 42	+ 26	+ 16	+ 16	+ 86	+ 46	+ 83
Tyrosine	- 4	+ 32	- 2	+196	+150	+103	+120	+152	+100	+ 72	+ 54	+ 37	+ 45	+ 33	+ 62
Non-amino acids:															
Ornithine	+ 4	+ 24	+ 21	+ 27	- 63	- 53	- 9	- 42	- 60	+ 25	- 30	- 44	+ 33	- 47	- 63
Citrulline	+ 30	+ 27	+ 61	+ 40	- 15	- 44	+ 12	- 12	- 22	- 7	- 3	- 5	+ 17	+ 17	+ 8
Ammonia	+ 31	+ 24	+ 51	+ 15	+ 12	+ 10	+ 20	+ 8	+ 8	+ 3	+ 3	+ 17	+ 4	+ 2	+ 4
Taurine	- 31	- 52	- 40	- 37	- 46	- 16	- 13	- 1	+ 25	+ 24	+ 7	+ 30	+ 70	+ 12	+ 12

*Compared with control values for experiment II

the content of the C-3 and mod. SW diets. For the most part, levels of most of these amino acids in the serum of rats fed the C-3 and mod. SW diets are reasonably constant from 4-12 weeks in relation to those of control levels at the start of the experiment. This suggests the rate of protein synthesis and catabolism throughout the period are also relatively constant.

Most changes with age, however, were seen in rats fed the lower protein diets, C-1 and W. Although half of the serum essential amino acids, that decreased initially below control levels, in rats fed the C-1 diet, progressively increased with age, those higher than control levels varied greatly, i.e. arginine decreased, histidine remained somewhat the same, lysine fluctuated while threonine and valine continued to increase. This large increase in serum lysine ($^{+}127\%$ to $^{+}79\%$ to $^{+}145\%$) and histidine ($^{+}140\%$ to $^{+}128\%$ to $^{+}141\%$) beyond that of controls, also seen by Longnecker and Hause (1961), Richardson et al. (1965), Swendseid et al. (1966), Adibi (1968) when they fed low protein diets, indicate their accumulation beyond that required for the limited synthesis of protein possible with the feeding of such an inadequate diet.

Again, some of the amino acids in the serum of rats fed the W diet increased with time while all others decreased; arginine, histidine, methionine, threonine and valine

were consistently higher than control values. In fact, it was not surprising to find that the most deficient amino acid of the diet, lysine, constantly about 50% below control levels. The more depressed the lysine values became, the higher was the rise in methionine, the second most limiting amino acid and conversely when lysine became less depressed; this suggests that protein synthesis was limited by the amino acid in shortest supply. The non-essential amino acid pattern of serum in rats fed the C-1 and W diets differed; the C-1 diet containing lower levels of all non-essential amino acids than the W diet promoted higher levels of most non-essential amino acids almost consistently except for glycine. The increasing alanine levels in both indicate partial degradation of other amino acids while the varying glutamic acid levels in the serum of rats on the C-1 and W diets suggest fluctuations in the degree of transamination.

Falling mid-way between the adequate and the inadequate diets was the C-2 diet. Striking was the similarity of serum isoleucine, leucine and phenylalanine with those of the mod. SW diet, even though the content of these amino acids in the latter diet were about 50% higher than those found in the C-2 diet. Still there was a resemblance to the serum of C-1 diet-fed rats as the higher histidine and lysine levels still persisted. The non-essential amino acids in the serum of rats fed the C-1 and C-2 diets were

similar.

Common to the serum of all rats was 1) the high arginine level, ranging from 42 to 400% above that of control rats except in two instances, 2) the higher concentrations of histidine, especially in the serum of rats fed the C-1 diet, and 3) the similarity of methionine content despite the great variation of this amino acid in the diet. Tryptophan was the amino acid that varied most, not only in the serum of rats fed different diets but also over a period of time in rats fed the same diet. No doubt, these changes were magnified by the difficulty in reading, with accuracy, such low levels as 0.7-11.8 μmoles/100 ml serum.

The non-amino acids varied greatly from diet to diet and from period to period, but only relatively low concentrations were found in the serum. Taurine was in highest concentration in the serum of rats fed the mod. SW diet. This suggests that the mod. SW diet was not deficient in S-containing amino acids.

2. Liver and serum

The liver presents a different picture for all essential and non-essential amino acids except tryptophan, alanine, aspartic acid, cystine and serine, were usually less concentrated than control levels. It is in the metabolically active liver that we meet what some refer to as "the first line of defense" against the post-absorption influx of serum

amino acids. The liver might also be looked upon as a processing plant; it disposes of a large influx of free amino acids by degradation into small molecules or synthesis into protein. A linear relationship exists between the protein content of the liver (Table 12) and its free amino acid content (Table 24b); the more inadequate the diet, the greater was the loss of liver protein and the greater was the decrease in free amino acids, i.e. most essential amino acids in liver of rats fed the C-1 and W diets fell about 50% below that of control values by 4 weeks except for 1) less depressed levels of lysine and histidine as in the serum of rats fed the C-1 diet, 2) histidine and threonine in rats fed the C-1 and C-2 diets, and 3) the more depressed valine level in rats fed the W diet. It took rats fed the other diets 12 weeks to reach the same level of reduction, part of which could be the process of physiological aging when amino acids are in lower concentration in the tissues.

With age, however, and possible adjustment to diet after 52 days of age, the protein content of the liver increased. In addition, 1) all essential amino acids except arginine and valine in the liver of rats fed the C-1 diet became less depressed below control levels while in serum there was an increase in most essential and non-essential amino acids suggesting a mobilization of amino acids through protein catabolism elsewhere or an increase in the consumption

Table 24b. Percentage change in the levels of free amino acids and non-amino acids in liver of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW, W) diet 4, 8 or 12 weeks

Ninhydrin-reacting substances	C-1			C-2			C-3			Mod. SW			W		
	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12
Essential amino acids:															
Arginine	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Histidine	- 23	- 21	- 26	- 11	- 29	- 21	+ 16	+ 3	- 20	- 11	- 30	- 32	- 30	- 33	- 45
Isoleucine	- 51	- 65	- 34	- 12	- 44	- 36	- 23	- 31	- 60	- 22	- 42	- 43	- 56	- 63	- 67
Leucine	- 57	- 62	- 40	- 16	- 47	- 47	- 26	- 34	- 57	- 23	- 47	- 51	- 55	- 63	- 65
Lysine	- 33	- 28	- 7	- 7	- 56	- 54	- 21	- 35	- 46	- 34	- 55	- 57	- 55	- 69	- 65
Methionine	- 50	- 56	- 33	- 21	- 42	- 26	- 30	- 34	- 88	- 24	- 43	- 48	- 49	- 59	- 62
Phenylalanine	- 56	- 54	- 38	- 13	- 40	- 42	- 22	- 32	- 55	- 26	- 45	- 53	- 42	- 63	- 67
Threonine	- 41	- 18	+ 5	+ 21	- 10	- 20	- 17	- 18	- 50	- 20	- 58	- 62	- 37	- 49	- 64
Tryptophan	+200	+237	+387	+1012	+312	- 88	- 75	- 88	+187	+125	- 13	+237	+942	+ 14	+ 14
Valine	- 46	- 44	- 57	- 21	- 62	- 61	- 26	- 32	- 51	- 41	- 60	- 65	- 67	- 72	- 78
Non-essential amino acids:															
Alanine	+ 7	+ 33	+ 19	+ 24	+ 19	+ 2	+ 9	+ 2	- 23	- 26	- 38	- 30	- 39	- 31	- 44
Aspartic acid	+ 26	+ 4	- 7	+ 9	+ 11	- 1	+ 16	- 7	+ 38	+ 16	+ 23	+ 18	- 35	- 1	- 8
Cystine	+ 46	+ 18	+ 2	+ 17	+ 46	+ 10	0	+ 8	0	0	0	0	+ 38	-100	-100
Glutamic acid	- 23	- 32	- 27	- 7	- 11	- 3	- 2	- 24	- 23	- 35	- 50	- 55	+ 23	- 30	- 23
Glycine	- 45	- 39	- 47	- 30	- 56	- 48	- 39	- 49	- 63	- 32	- 45	- 53	- 45	- 42	- 52
Proline	- 67	- 58	- 49	- 14	- 49	- 66	- 6	- 43	- 55	- 47	- 59	- 63	- 31	- 67	- 63
Serine	+ 42	+ 56	+ 44	+ 65	+ 18	- 20	- 21	- 29	- 37	- 15	- 42	- 54	- 9	- 6	- 35
Tyrosine	- 53	- 54	- 32	- 3	- 41	- 36	- 7	- 10	- 41	- 22	- 44	- 50	- 47	- 61	- 64
Non-amino acids:															
Ornithine	- 53	- 45	- 35	- 27	- 56	- 64	- 58	- 62	- 67	- 33	- 53	- 61	- 57	- 59	- 71
Citrulline	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Ammonia	+ 16	- 16	- 13	+ 7	- 41	- 27	- 5	- 27	- 34	- 50	- 54	- 51	- 11	- 9	- 20
Taurine	- 93	- 94	- 95	- 94	- 94	- 93	+ 26	+ 93	+110	- 20	+ 33	+109	- 50	+ 24	- 55

of the diet, 2) most free amino acids of liver and serum in rats fed the W diet decreased while protein content of the liver increased by 12 weeks to the level of rats fed the more adequate diets, even though most of the liver essential amino acids were depressed to 65% (78% for valine) below that of control values. Nevertheless, there was a striking resemblance between the free amino acids of liver in rats fed the C-1 and W diets for 4 and 8 weeks except for the less depressed levels of lysine, methionine, threonine and valine in the liver of rats fed the C-1 diet; after 12 weeks consumption of these diets, there was a greater accumulation of many free amino acids in the livers of rats fed the C-1 diet even though protein content was far below that found in the livers of other rats.

Liver levels of free essential amino acids in rats fed the C-2 diet were more like those of rats fed the C-3 and mod. SW diets than were those of rats fed the C-1 and W diets. Like all rats except those fed the C-1 diet, there was a decrease in most amino acids as well as ornithine and ammonia over a period of time, indicating a more efficient utilization of amino acids for protein synthesis. Also, the decreasing content of methionine, lysine and other essential amino acids and the increasing levels of taurine in the livers of rats fed the C-3 and mod. SW diets suggest degradation of methionine beyond the need for synthesis.

The levels of free amino acids found in liver tissue suggest restricted protein synthesis may have been limited by an inadequate supply of 1) all essential amino acids in the case of the C-1 diet-fed rats, 2) lysine and methionine in W diet-fed rats, and 3) methionine chiefly in the C-2 diet-fed rats; low taurine values in these diets give further support that methionine was certainly not in abundant supply in the livers of rats fed the C-1, C-2 and W diets.

Compared with serum, however, the methionine content of liver (Table 20a) was significantly different in rats fed diets 8 weeks, it being highest in livers of rats fed the C-3 and lowest in those fed the C-1 diet. Thus, while there were many significant differences in free amino acid content of liver, they were not always the same as those found in serum. These differences, however, persisted for a longer period in the liver for after eight weeks of feeding there were six significant differences in essential amino acid levels in liver of rats fed the various diets in experiment III compared to only one in serum. Again, there was great fluctuation in tryptophan values.

3. Skeletal muscle, liver and serum

The role of skeletal muscle in amino acid metabolism is a controversial one. Pawlak and Pion (1967) reported muscle was sensitive to a deficiency as rats fed a wheat

gluten diet showed a depression in lysine similar to that of serum after feeding a wheat-flour diet 18 and 35 days. On the other hand, Sheffner and Bergeim (1952) fed a high lysine-containing peptone by stomach tube to fasting rats and found values for arginine and lysine increased greatly in the muscle but lysine alone increased significantly in the liver; supplementation with phenylalanine resulted in no change in serum liver or muscle levels but methionine supplementation caused an increase in the plasma and muscle with a lower increase in the liver. Clark (1966), however, found changes in the free amino acids of liver tissue appeared to have a higher correlation with the alterations observed in plasma amino acid concentrations than did muscle tissue when they fed amino acid mixtures lacking either leucine, isoleucine, valine or threonine for three days.

The critical factor here may be one of time. It is not realistic to expect significant changes in skeletal muscle during a short-term study if the liver plays its role in modifying serum amino acid fluctuations; nor should one expect to find significant changes in metabolically stable tissues such as brain (Denton et al., 1950).

The only known study on muscle that was of longer duration than the present one was that carried out by Wannemacher and Allison (1968). They fed rats, weighing 200-250 g, 1) a protein-free diet for 1, 3, 7, 21, 42 or

100 days, or 2) varying levels of casein and wheat gluten for 28 days, and then determined the free amino acids of serum, liver and abdominal muscle by automated ion-exchange chromatography. Generally, the concentration of serum free amino acids in our study agree with the findings of Wannemacher and Allison (1968) for the more inadequate the diet the more diminished and augmented were the serum essential and non-essential amino acids, respectively. In Wannemacher and Allison's study, there was an initial increase in the serum free amino acids of rats fed a 13% casein diet; most free amino acids in rats fed a wheat gluten diet, however, were lower than those of a protein free diet, lysine being markedly decreased even when other essential amino acids increased with increased consumption of the diet. Although most of the essential amino acids in the liver of rats fed the C-1 and W diets decreased to 50% of normal control values, not all individual amino acids responded in the same manner as did the free amino acids in the liver of rats depleted by 10% of their body protein.

Our data, however, do not support the finding of Wannemacher and Allison (1968) that the higher the dietary protein, the higher the levels of liver free amino acids. However, values for liver free amino acids of rats fed the C-2 and W diets were in keeping with Wannemacher and Allison's finding that rats fed the wheat gluten had a lower

free lysine liver content than did animals fed an equivalent amount of casein protein. Unlike data on skeletal muscle in this study, free amino acids of abdominal muscle were not reduced below control level until rats had lost one-third of their body protein when they decreased only by 15%. As in liver, the free amino acids in the abdominal muscle of rats consuming a 25% casein diet (Wannemacher and Allison, 1968) increased to their maximal level; and, although essential amino acids increased as rats consumed a higher level of wheat gluten protein, non-essential amino acids were not increased beyond those of the protein-free diets. The free amino acid pattern of the abdominal muscle in Wannemacher and Allison's study did not reflect the lysine deficiency of the wheat gluten.

In this study, generally, the response of skeletal muscle varied with the extent of the inadequacy of the diet. Concentration changes from those of control values in the gastrocnemii of rats fed the C-1 and C-2 diets (Tables 24c, 24d) were less extreme than those found in the liver of the same rats (Table 24b); the less depressed levels for histidine and lysine seen in serum and liver persisted through eight weeks of feeding but lysine decreased considerably by 12 weeks. Free amino acids in the muscle tissue of rats fed the more adequate diets, C-3 and mod. SW, attained their extremes of concentration more quickly than did liver and

Table 24c. Percentage change in the levels of free amino acids and non-amino acids in gastrocnemii of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW, W) diet 4, 8 or 12 weeks

Ninhydrin-reacting substances	C-1			C-2			C-3			Mod. SW			W		
	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12
Essential amino acids:															
Arginine	- 19	- 67	- 71	- 71	- 56	- 71	- 62	- 64	- 65	- 57	- 56	- 59	- 53	- 74	- 55
Histidine	+ 4	+ 16	- 12	- 10	- 24	- 43	- 16	- 35	- 48	- 51	- 53	- 54	- 57	- 78	- 56
Isoleucine	- 11	- 27	- 23	- 10	- 20	+ 1	+ 5	+ 5	- 6	+ 36	+ 19	+ 23	- 59	- 48	- 20
Leucine	+ 3	- 19	- 29	- 21	- 28	- 23	+ 11	- 10	- 15	- 23	- 33	- 30	- 59	- 59	- 24
Lysine	+ 7	+ 4	- 70	- 3	- 46	- 70	- 12	- 35	- 34	- 78	- 65	- 76	-100	-100	-100
Methionine	- 14	- 16	- 30	- 24	- 39	- 48	- 36	- 24	- 34	- 33	- 60	- 42	- 38	- 33	+ 5
Phenylalanine	+ 34	- 16	+ 34	+ 14	- 17	+ 3	+ 18	+ 9	- 8	- 17	- 8	- 18	- 37	- 59	- 1
Threonine	- 7	+ 11	+ 14	+ 63	+ 83	+ 23	+137	+ 8	0	+ 1	- 32	- 41	- 29	- 54	- 32
Tryptophan	- 23	+ 9	- 10	+ 3	- 20	+ 45	+ 32	- 17	+ 58	+ 35	+ 16	- 20	- 54	- 47	- 18
Valine	+ 14	- 7	- 2	+ 27	+ 19	+ 27	+ 78	+ 33	+ 11	- 12	- 9	- 26	- 43	- 51	- 9
Non-essential amino acids:															
Alanine	+ 45	+ 46	+ 26	+ 36	+ 33	+ 8	+ 34	0	- 10	- 11	- 23	- 24	- 11	- 10	- 5
Aspartic acid	- 37	- 45	- 43	- 4	- 20	- 27	+ 28	- 30	- 33	- 14	- 23	- 26	- 19	- 32	- 53
Cystine	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Glutamic acid	- 21	- 46	- 65	+ 7	- 38	- 63	+ 20	- 60	- 67	- 27	- 64	- 68	+ 55	- 8	- 29
Glycine	- 68	- 65	- 59	- 60	- 68	- 75	- 78	- 84	- 94	- 34	- 59	- 65	- 30	- 49	- 40
Proline	- 6	- 25	- 33	+ 5	+ 7	- 18	+ 87	- 19	- 18	- 17	- 44	- 58	+ 16	+ 8	- 34
Serine	+ 74	+ 83	+ 55	+ 20	+ 34	- 16	- 15	- 37	- 34	- 43	- 49	- 55	- 45	- 57	- 34
Tyrosine	+ 28	+ 1	+ 22	+ 90	+ 47	+ 18	+ 48	+ 41	+ 15	+ 3	- 2	- 9	- 25	- 39	+ 13
Non-amino acids:															
Ornithine	- 70	- 52	- 47	- 82	- 68	- 59	- 69	- 46	- 71	- 48	- 67	- 79	- 18	- 30	- 62
Citrulline	- 7	- 10	- 19	- 35	- 43	- 63	- 34	- 65	- 64	- 43	- 61	- 66	+ 20	- 24	- 31
Ammonia	- 29	- 56	- 68	- 68	- 65	- 75	- 77	- 77	- 78	- 76	- 78	- 77	*	*	*
Taurine	- 56	- 66	- 75	- 65	- 63	- 27	- 26	+ 44	+ 36	+ 11	+ 25	+ 31	+ 82	+ 84	+131

*No control value with which to compare them

Table 24d. Percentage change in the levels of free amino acids and non-amino acids in thigh and leg muscle of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW, W) diet 4, 8 or 12 weeks

Ninhydrin-reacting substances	C-1			C-2			C-3			Mod. SW			W		
	4	8	12	4	8	12	4	8	12	4	8	12	4	8	12
Essential amino acids:															
Arginine	- 32	- 59	- 64	- 48	- 53	- 65	- 47	- 33	- 41	- 23	- 23	- 60	- 34	- 74	- 49
Histidine	- 26	- 9	- 29	- 18	- 40	- 51	- 17	- 27	- 51	- 8	- 58	- 63	- 47	- 44	- 56
Isoleucine	- 23	- 40	- 7	- 11	+ 31	+ 5	+ 22	+ 31	+ 11	+ 66	- 23	- 15	- 17	- 37	- 11
Leucine	- 10	- 26	- 12	+ 7	+ 13	+ 2	+ 24	+ 37	+ 22	+ 67	- 20	- 37	+ 5	- 24	0
Lysine	+ 16	- 37	- 48	- 10	- 51	- 53	- 43	- 32	- 28	- 43	- 73	- 56	- 54	- 85	- 61
Methionine	- 2	- 12	- 2	+ 11	- 1	+ 19	+ 25	+ 22	+ 30	+100	- 12	- 3	+ 7	- 20	- 3
Phenylalanine	- 8	- 15	- 2	+ 21	+ 13	+ 1	+ 23	+ 37	+ 16	+ 52	- 17	- 16	+ 10	- 2	+ 13
Threonine	- 21	+ 14	+ 59	+101	+ 80	+ 33	+143	+ 47	+ 21	+ 83	- 14	- 22	+ 23	- 7	- 3
Tryptophan	- 50	- 88	+ 31	- 50	- 25	+ 81	- 75	- 50	- 50	0	0	-100	- 50	- 81	- 82
Valine	- 16	- 18	- 7	+ 68	+ 8	+ 9	+ 57	+ 64	+ 44	+ 22	- 39	- 13	- 1	- 13	- 13
Non-essential amino acids:															
Alanine	+ 34	+ 32	+ 25	+ 53	+ 26	+ 14	+ 42	+ 8	- 2	+ 22	- 11	- 23	+ 10	+ 2	- 8
Aspartic acid	- 49	- 54	- 53	- 16	- 35	- 57	+ 3	- 26	- 33	- 50	- 66	- 63	- 18	- 51	- 54
Cystine	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Glutamic acid	- 18	- 32	- 39	+ 10	- 29	- 44	+ 6	- 50	- 51	- 13	- 63	- 63	- 18	- 51	- 54
Glycine	- 73	- 71	- 66	- 62	- 75	- 77	- 81	- 83	- 84	- 31	- 66	- 69	- 38	- 48	- 45
Proline	+ 21	- 15	+ 14	+ 18	- 14	+ 31	+112	- 1	+ 20	- 26	- 39	- 40	+ 21	- 23	- 45
Serine	+ 86	+ 53	+ 82	+ 48	+ 13	- 5	+ 3	- 22	- 26	- 14	- 50	- 42	+ 4	- 10	- 7
Tyrosine	- 6	- 3	- 8	+ 67	+ 41	+ 19	+ 35	+ 56	+ 29	+ 66	- 4	- 10	+ 8	+ 2	+ 9
Non-amino acids:															
Ornithine	- 56	- 71	- 76	- 57	- 86	- 77	- 79	- 82	- 97	- 54	- 82	- 85	- 52	- 67	- 78
Citrulline	- 11	- 12	- 22	- 7	- 44	- 59	- 38	- 67	- 67	- 47	- 73	- 56	- 14	- 31	- 45
Ammonia	- 2	+ 8	- 6	+ 47	+ 14	- 3	- 1	+ 17	- 9	- 9	- 26	- 21	- 6	- 6	- 9
Taurine	- 57	- 64	- 76	- 59	- 66	- 18	- 2	+ 60	+ 57	+ 93	+ 55	+ 48	+ 8	+ 12	+ 21

then remained relatively constant. In most cases, rats fed the W diet, like those fed the C-3 and mod. SW diets, reached their maximum extremes by 4 weeks; however, they fluctuated more during the weeks that followed, with the net effect being an increase in concentration.

Other than tryptophan, however, lysine was the essential amino acid that varied most in concentration. Highest levels of lysine were found at four weeks in the gastrocnemii of rats fed the C-1 diet while lowest levels were shown by rats fed the wheat diets, mod. SW and W. In fact, no lysine was found in the gastrocnemii of rats fed the W diet for 4, 8 and 12 weeks. Lysine levels in the muscle of rats fed the C-1, C-2 and mod. SW diet, however, were reduced to about 70% below control levels after consuming the diet for 12 weeks.

This, by far, represents the most striking response to variations in the levels of absorbed amino acids. It is all the more remarkable because the thigh and leg muscle did not give a similar response. Although amino acid values of thigh and leg muscle, in general, were only slightly higher than those of the gastrocnemii (Tables 16 and 17c, 17d), the amino acids in the thigh and leg muscle of rats fed the C-1 diet, compared with zero-time levels, were 1) more reduced than the gastrocnemii and 2) less reduced than most other amino acids of rats fed the other diets (Tables

24c and 24d). For the most part, however, lysine values for the wheat diets, mod. SW and W, were definitely more depressed in the gastrocnemii than in the thigh and leg muscle, the free lysine values for the latter being 54, 85 and 61% below those of controls at 4, 8 and 12 weeks, respectively.

These findings suggest that the gastrocnemii is not truly representative of skeletal muscle. It may have a greater requirement for lysine than the thigh and leg muscle either because there is a greater degree of protein synthesis or degradation of lysine required for, or resulting from, a specialized function of this muscle. As a result of a preliminary study (Pawlak and Pion, 1967) showing threonine as well as lysine in low concentration of hind-leg muscles of rats fed wheat gluten diets for 18 days, Pawlak and Pion (1968a, 1968b) fed graded amounts of lysine or threonine in wheat flour diets to 100 g Wistar rats for 14 days and found that only when the diet was increased to 0.8% of lysine or 0.42% of threonine was there maximal growth with a large increase in the threonine content of blood and muscle or a larger increase in the lysine content of muscle than of blood. These hind-leg muscles, like serum (Morrison et al., 1961) may require sufficient supply of an amino acid to satisfy its needs for protein synthesis before it shows an increase in concentration. This present study,

however, showed the gastrocnemii as being more sensitive to a definite deficiency of one amino acid in the W diet than it was to a general decrease in all amino acids, i.e. C-1 and C-2 diets.

Since no known amino acid deficiencies in skeletal muscle other than lysine and threonine have been reported, a study to determine the gastrocnemii, thigh and leg muscle response to other amino acids in another grain as well as a corresponding amino acid mixture is suggested. The control diet might consist of the grain supplemented to the recommended level(s) for growing rats, giving consideration to the availability of amino acids from the grain. Supplementation of the grain with graded levels of the most limiting amino acid and sampling the serum and various muscles as did Pawlak and Pion (1967, 1968a, 1968b) may give further information on whether present requirements are precise. Frequent sampling during the more acute growth phase, i.e. when rats weigh 50 to 150 g is indicated by the large changes that occurred from 0 to 28 days in this study. At least five rats per treatment and individual analysis would give a more statistically accurate picture of the depletion and repletion process; multiple regression analyses of the resulting effects of 1) diet over a period of time, or 2) graded levels of the diet, might reveal some interesting information and supply some prediction equations.

Little is known of the role skeletal muscle plays in protein metabolism. To determine whether synthesis and catabolism vary between feeding and fasting states as they do in liver tissue, suggests the use of labelled compounds and incorporation studies at frequent intervals in a long-term study. This labelled compound might well be the amino acid most limiting and if a comparison is made between control diets and test diets, some light might also be thrown on the effects of physiological aging.

Of course, elucidation of the detailed mechanisms involved in the growth and development of even one tissue opens up a new frontier requiring many life-times of delving at the intermediary metabolism level. A start could be made by tracing the metabolism of one amino acid at a time as well as the enzymes involved with its synthesis and degradation as there is lack of information on such in tissues other than liver. That certain hormones such as insulin, are required for incorporation of amino acids into protein as well as for transporting amino acids with glucose to supply the components and energy, respectively, further complicates the picture.

4. An overview

Amino acids are added to body fluids by intestinal absorption, synthesis, or breakdown of tissue protein while they are removed for synthesis of tissue protein and other

uses, or degraded via the urea cycle. The size of the amino acid pool represents the balance between these processes. Although the total amino acid pool is small, representing only about 0.5% of the total amino acids in the animal body (Munro, 1970), it turns over very rapidly in serum, liver and other internal organs but more slowly in muscle proteins. The significance of free amino acids in muscle is of utmost importance since muscle is the single largest tissue of the body, making up 45% of its content. It is the free amino acid pools of muscle that are called upon in time of nutritional stress; it is not by increased degradation of protein that muscle amino acid pools contribute to the needs of more metabolically active tissues but according to Waterlow and Stephen (1966, 1967, 1968) reduced synthesis is responsible for its role as a provider of the missing amino acids.

While incorporation studies to determine the rate of protein synthesis and degradation as well as quantitation of free amino acids and protein of tissues are perhaps desirable for an accurate picture, evaluation of the nutritional value of diets by measurement of tissue amino acid pools and protein content is possible. As a readily available criteria of measurement, amino acid pools of serum have held much attention with controversial results. For the most part, however, dietary excesses and deficiencies are reflected in serum free amino acids. Generally, it was found in this

study that most amino acids of adequate diets were reflected accurately in the serum but low protein diets having more than one amino acid inadequately supplied distorted the picture considerably with high levels of histidine, lysine and non-essential amino acids. Nor was methionine in the various diets accurately represented in the serum for there were no significantly different levels even though it was the most limiting in the casein diets. Tryptophan, however, was in highest concentration in serum even though it is in the liver that aromatic transaminases are most active.

Free amino acid patterns of liver are known to differ from those of plasma. It is surprising, however, that in certain instances such as that of methionine, dietary inadequacies are better noted in liver than in serum. On the whole, however, the changes in the free amino acid pools of liver are more dramatic than are those of serum. The data of this study suggest there are great reductions with age as levels of most amino acids decreased greatly below zero-time control rats even with the adequate diets, mod. SW and C-3. Levels of branched-chain amino acids, isoleucine, leucine and valine are usually higher in liver as there is poor utilization in this tissue, being used only for protein synthesis as deaminases for branched-chain amino acids are confined to muscular tissue.

To date, free amino acids of skeletal muscle have

not been given much attention. Only a few of the limited number of studies conducted specify what muscle was used. This detail may be of importance for while there was much similarity in the protein content and free amino acid concentrations in the muscles of hind legs used in this study, it was in the gastrocnemii that the most limiting amino acid showed its most dramatic results. Our findings, therefore, agree with those of Rogers and Harper (1968) in that free amino acids of skeletal muscle reflect the serum free amino acid patterns which in turn are influenced by those in the diet; in fact, this study showed free amino acids of muscle as a better indicator of deficiencies than those of the serum. And, so, in keeping with the findings of Pawlak and Pion (1968a, 1968b) free amino acid levels of skeletal muscle, especially those of the gastrocnemii, act as a sensitive index of excesses and deficiencies. They might, therefore, be the most valuable criteria for determination of requirements, evaluation of a diet or the nutritional status of an individual, even though muscle biopsies pose more of a problem than the sampling of blood.

SUMMARY

Male weanling rats of Wistar strain were used to determine the biological availability of amino acids from pure wheat when coprophagy was prevented in rats weighing 80 g and 300 g. Similar rats were used to observe the effect of diets, varying in casein content (C-1, C-2 or C-3) or wheat content (W), one of which was modified by supplementation with protein (mod. SW), for 4, 8 or 12 weeks, on the free amino acid levels of the serum as well as the concentration of DNA, RNA, protein and free amino acids of liver and skeletal muscle.

The true and apparent availability of sixteen amino acids were not affected by the prevention of coprophagy except for the true availability of lysine in lighter, younger rats. This significant decrease in availability to younger rats fitted with sham tail cups was possibly the result of their non-adjustment to the tail cup. Otherwise, no adverse effects from coprophagy prevention were noted possibly because coprophagy was not practised to any great extent by control rats.

The true availability of amino acids for both weight groups was similar, ranging from 90-98%, with glutamic acid and proline being most available and aspartic acid, alanine or lysine the least available. Of the essential amino acids, however, lysine was significantly less available with

histidine and phenylalanine the most available to both younger and older rats. Although apparent availability averages were lower by 1.4-8.4% than true availability averages, the similar trends noted suggest that apparent availability determinations reflect true availability values.

In the tissue study, rats fed diets that varied in amino acid and protein content (6% in C-1, 12% in C-2 and W, 21% in C-3 and 20% in mod. SW) had weight gains as follows over the 12-week period: 115 g for C-1, 276 g for W, 385 g for C-3, 348 g for C-2, 425 g for mod. SW. Although the weight gain of rats fed the mod. SW, C-2 and C-3 was greater than that of rats fed the C-1 diet for 12 weeks, only that for rats fed the mod. SW and C-3 diets was greater than the C-2 and C-1 diets for 4 or 8 weeks.

With age, the nucleic acid concentrations decreased, the greatest decrease taking place between the start of the experiment and four weeks later; the decrease was fastest in tissues of rats fed the C-1 diet. By 12 weeks, however, DNA values were 263-266 mg/100 g liver, 72-89 mg/100 g gastrocnemii and 60-70 mg/100 g thigh and leg muscle irrespective of the diet consumed. On the other hand, RNA values were influenced by diet, significantly so in the liver, for rats fed the mod. SW, C-3 and C-2 diets for 4 weeks and those fed the mod. SW and C-3 diets for 8 weeks had higher liver RNA levels than did rats fed the C-1 diet. Also influenced by

diet was the protein content of liver: the lower the protein content of the diet, the greater was the loss of protein from the start of the experiment to 4 weeks later, and the slower the protein accumulation between 4 to 12 weeks. However, there was no protein loss in skeletal muscle but an increase above zero-time control values which was less in thigh and leg muscle than in gastrocnemii.

Although protein content was lower in the liver than in skeletal muscle, the concentration of most amino acids, like nucleic acids, was higher in the liver. Notable exceptions were: 1) the higher serum tryptophan and cystine content, and 2) the non-measurable amounts of arginine and citrulline in the liver. Generally, thigh and leg muscle free amino acids were higher than those of the gastrocnemii but cystine was not detectable in either of them; characteristic of skeletal muscle was the high taurine and ammonia content. Amino acids, whether extracellular or intracellular were most highly concentrated as lysine, threonine, leucine, valine or histidine of the essential amino acids, and alanine, glutamic acid and serine of the non-essential amino acids.

The concentration of most ninhydrin-reacting substances were affected by diet and by the length of time rats consumed the diet; statistically, serum was the least and gastrocnemii the most affected. In general, the essen-

tial amino acids were less concentrated in tissues of rats fed either the C-1 or mod. SW diets, except for arginine in gastrocnemii, histidine and lysine in serum and gastrocnemii of rats consuming the C-1 diet for 4, 8 or 12 weeks. On the whole, essential amino acids were more highly concentrated in the tissues of rats that consumed the C-3 diet but often these concentrations were not statistically different from those found in rats fed the C-2 or mod. SW diets. Non-essential amino acids were higher in the tissues of rats fed the C-1, C-2 and W diets and lowest in rats fed the mod. SW diet; highest concentrations of aspartic acid, glutamic acid and proline were found in the gastrocnemii of rats fed the C-3 diet 4 weeks but glycine was highest in serum and gastrocnemii of rats fed the mod. SW diet 4, 8 or 12 weeks. Of the non-amino acids, ornithine of serum was not influenced significantly by diet but citrulline and ammonia were highest in all tissues of rats fed the C-1 diet while taurine was prominent in the more mature rats fed the mod. SW or C-3 diets.

Over the 12-week experimental period, most changes occurred between the start of the experiment and four weeks later; these changes were exhibited mostly as decreases, the greatest number of decreases taking place in the liver. Most significant increases, however, occurred in the free amino acid concentrations of serum. Although the ninhydrin-

reacting substances of gastrocnemii neither showed as many increases as the serum nor as many decreases as the liver, the large number of significant changes indicate this muscle is indeed metabolically active.

In fact, the findings of this study indicate that intracellular amino acid pools are as sensitive as extracellular free amino acids to fluctuations in amino acid intake. Observed in liver and skeletal muscle as well as serum were 1) increases in a few essential and many non-essential amino acids as a result of restricted protein synthesis by rats fed an inadequate supply of many essential amino acids from the C-1 diet, 2) decreases in non-essential amino acids involved with transamination and increases of degradative products of urea cycle or taurine when most amino acids are in more than adequate supply for protein synthesis, e.g. rats fed the C-3 and mod. SW diets, 3) changes associated with adaptation, over a period of time, to a diet in which all but one or two amino acids were adequate for the growing rat, i.e. rats fed the C-2 and W diets.

Actually, the intracellular rather than the extracellular amino acid pools may be more sensitive to actual excesses and deficiencies of specific amino acids in the diet. Free methionine levels in the liver indicated more accurately than other tissues the methionine content in the diets; this was also reflected in the free taurine content

of the liver, it being lowest in rats fed the C-1 diet, second lowest in rats fed the W diet and highest in rats fed the C-3 and mod. SW diets. Although the lysine content of diets has been reflected in the free amino acid pools of skeletal muscle its complete absence in the gastrocnemii of rats fed the W diet 4, 8 or 12 weeks indicate the sensitivity of this muscle to the lysine content of the diet. These studies, therefore, indicate the need for further investigations into specific tissue requirements for amino acids.

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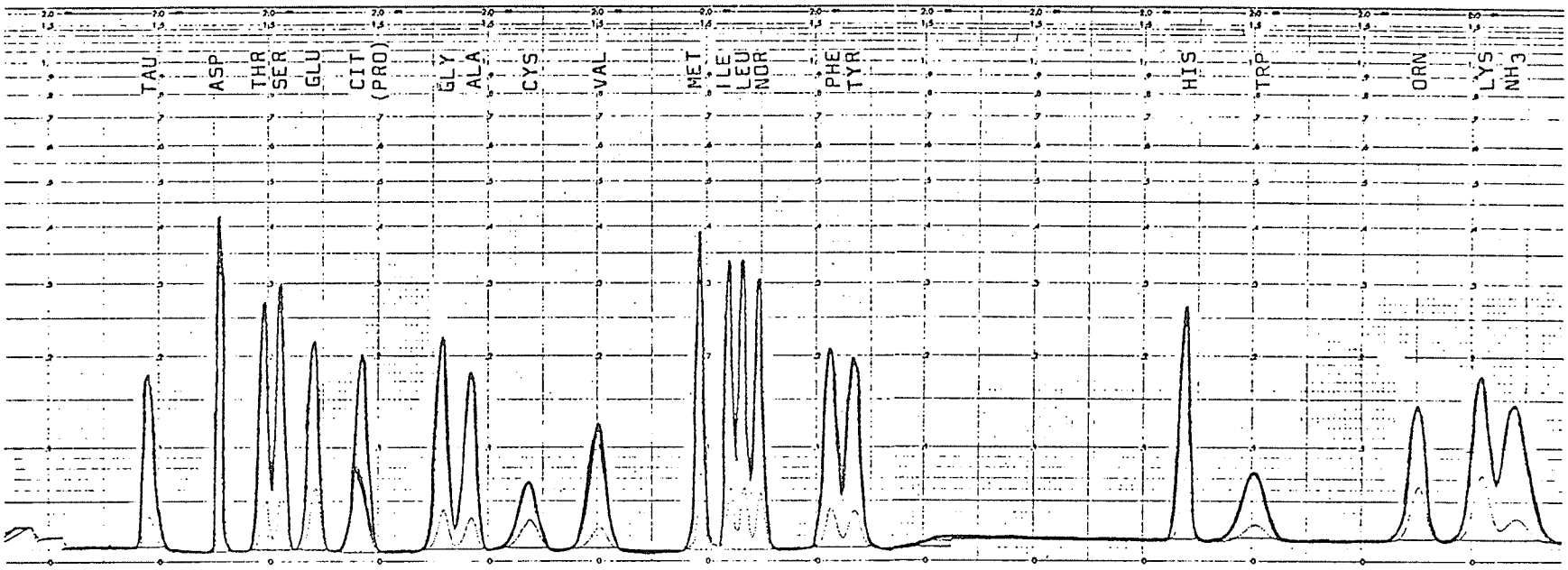
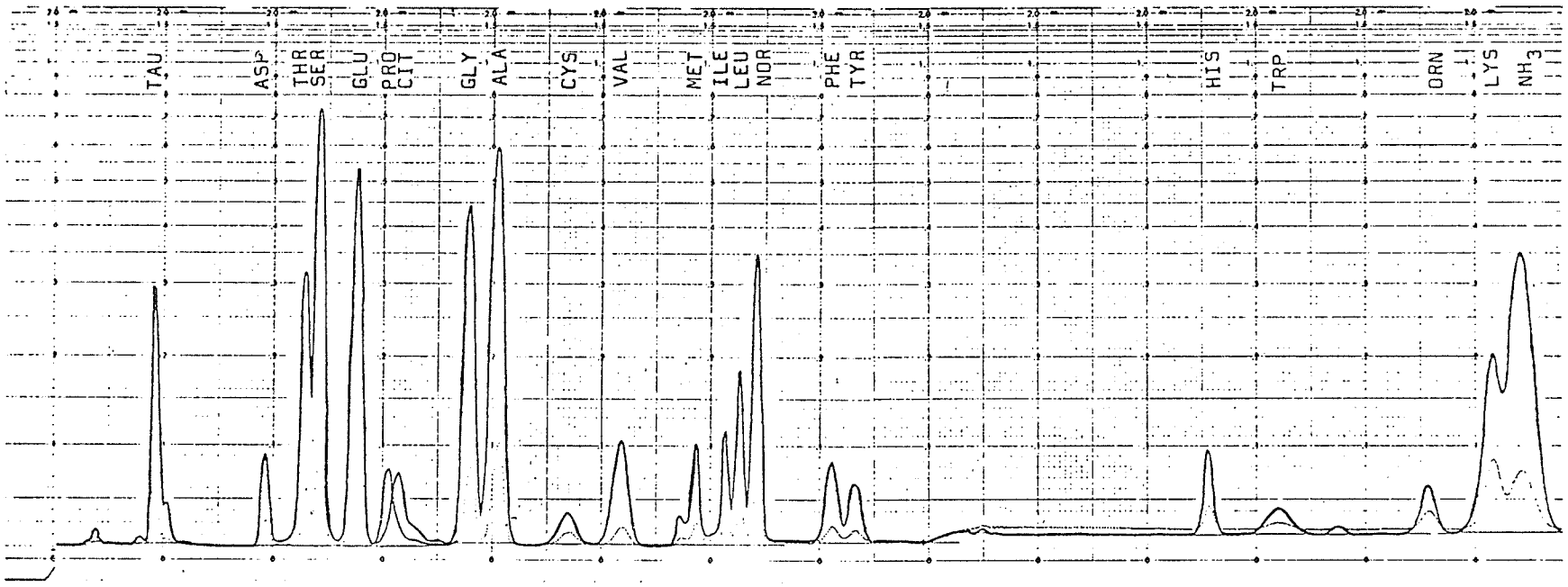
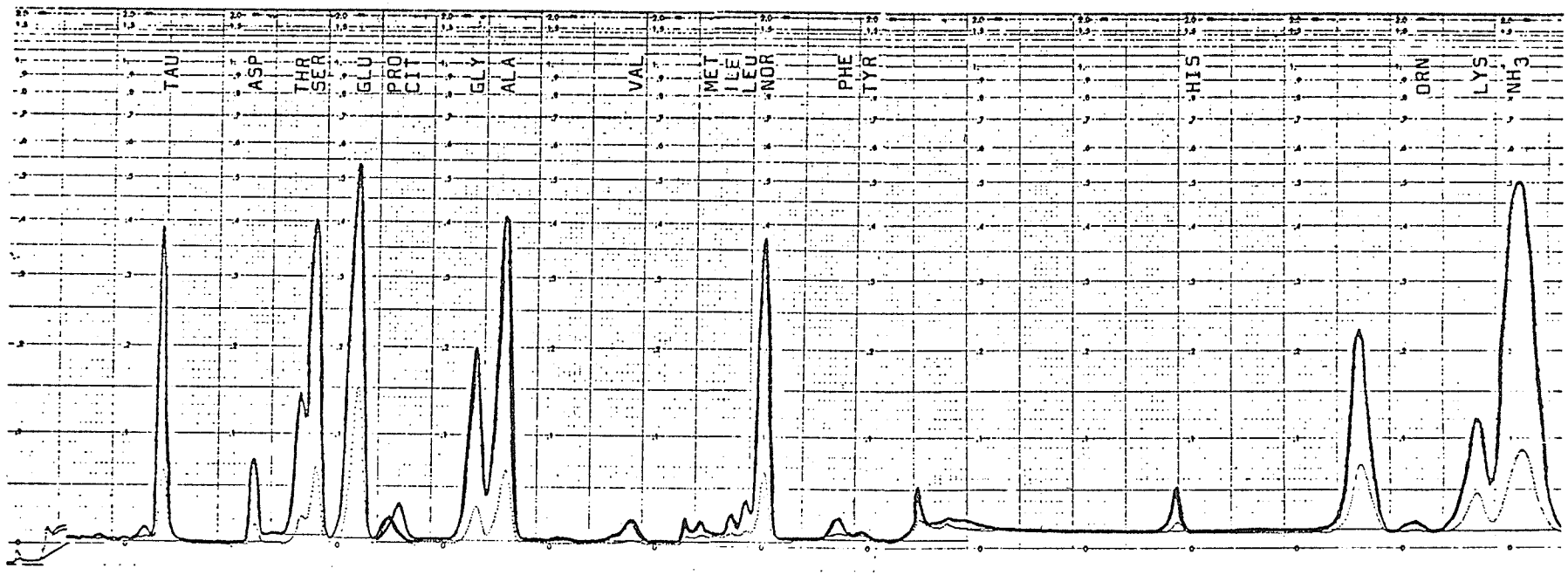


Appendix A, Fig. 1. Chromatogram of amino acid standard (0.1 μmole except 0.05 μmole cystine) eluted from 56 cm column with sodium citrate buffers (pH 3.17, 4.25 and 6.3)



Appendix A, Fig. 2. Chromatogram of serum free amino acids and non-amino acids eluted from 56 cm column with sodium citrate buffers (pH 3.17, 4.25 and 6.3)



Appendix A, Fig. 3. Chromatogram of free amino acids and non-amino acids of gastrocnemii eluted from 56 cm column with sodium citrate buffers (pH 3.17, 4.25 and 6.3)

Appendix B, Table I. Average whole body and tissue weights with standard errors for rats fed a casein (C-2) or wheat (W) diet 4, 8 or 12 weeks, experiment II

Weight of tissues	Control	Diets					
		C-2			W		
		4[1]	8[1]	12[1]	4[1]	8[1]	12[1]
Whole body	51.9±1.57	155.5±15.4	280.8±7.06	334.2±28.24	132.5±7.73	263.8 ±9.50	329.8±15.50
Liver	2.2±0.09	7.9± 0.93	13.6±0.53	12.6± 1.21	6.2±0.42	10.9 ±0.49	12.0± 0.76
Gastrocnemii[2]	0.4±0.03	1.5± 0.14	2.7±0.08	3.6± 0.23	1.1±0.06	2.60±0.10	3.4± 0.22
Thigh & leg[2]	--	10.4± 0.86	22.4±0.71	22.6± 1.48	7.9±0.57	19.6 ±0.59	21.5± 1.41

[1]Number of weeks on diet

[2]Muscles from both legs

Appendix B, Table II. Average weights and standard error of tissues for rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Weight of tissues	Control	Diets											
		C-1			C-2			C-3			SW		
		4[1]	8[1]	12[1]	4[1]	8[1]	12[1]	4[1]	8[1]	12[1]	4[1]	8[1]	12[1]
Whole body	65.8±3.55	93.6±6.59	153.4±6.78	227.2±56.0	173.9±13.0	201.1±13.1	361.4±66.4	219.4±8.03	361.1±21.33	455.6±33.38	237.5 ±8.10	367.1±13.0	460.4±14.4
Liver	2.9±0.24	6.2±0.42	8.2±0.64	7.4± 0.60	9.3± 1.03	12.4± 0.43	16.2± 0.09	11.5±0.64	15.5± 0.88	17.5± 0.65	11.42±0.64	14.3± 0.94	18.6± 1.21
Gastrocnemii[2]	0.6±0.01	0.9±0.10	1.6±0.07	1.8± 0.06	1.9± 0.09	3.0± 0.09	3.9± 0.11	2.4±0.17	4.0± 0.20	2.6± 0.08	2.6 ±0.08	4.3± 0.23	4.6± 0.31
Thigh & leg[2]	3.9±0.32	5.7±0.80	8.8±0.83	11.7± 0.74	10.8± 0.35	16.5± 1.00	25.4± 1.12	14.2±0.91	24.7± 2.73	29.3± 2.64	16.5 ±1.04	24.0± 0.67	30.9± 1.60

[1]Number of weeks on diet

[2]Muscles from both legs

[3]Only SW used in table headings

Appendix C, Table Ia. Summary of concentration means and standard error of ninhydrin-reacting substances from serum[x] of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances (mg/100 ml)	C-2			W		
	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	8.8±1.50	9.4±0.05	8.1±0.00	6.1±1.17	6.8±1.00	7.4±0.25
Ammonia	1.8±0.39	1.8±0.26	1.6±0.00	1.6±0.11	1.6±0.00	1.6±0.28
Arginine	1.5±0.08	1.6±0.33	3.2±0.04	2.0±0.78	3.8±0.00	3.8±0.57
Aspartic acid	2.2±0.35	1.4±0.02	1.5±0.01	2.0±0.34	1.2±0.01	1.1±0.12
Citrulline	1.5±0.09	1.3±0.10	1.3±0.00	1.6±0.27	1.6±0.04	1.5±0.00
Cystine	0.2±0.02	1.0±0.03	1.2±0.00	0.1±0.05	0.8±0.02	2.0±0.54
Glutamic acid	12.8±2.80	12.6±2.04	10.5±0.00	12.8±1.86	9.7±0.82	8.1±0.58
Glycine	1.2±0.12	1.3±0.08	1.6±0.00	2.8±0.51	2.4±0.18	3.3±0.33
Histidine	1.6±0.14	1.5±0.07	1.4±0.00	3.0±0.18	2.0±0.56	1.2±0.02
Isoleucine	1.8±0.30	1.6±0.11	1.6±0.00	1.4±0.30	1.1±0.06	1.2±0.07
Leucine	2.8±0.52	2.6±0.01	2.5±0.00	2.0±0.52	1.6±0.08	1.7±0.14
Lysine	12.3±0.23	9.2±0.46	10.1±0.00	3.0±0.18	2.4±0.02	2.8±1.64
Methionine	0.6±0.22	1.3±0.11	0.8±0.00	0.7±0.00	1.1±0.06	0.9±0.26
Ornithine	1.7±0.53	1.3±0.30	0.7±0.00	2.2±0.44	0.9±0.00	0.6±0.08
Phenylalanine	1.2±0.13	1.2±0.05	1.2±0.00	1.3±0.30	1.0±0.09	0.8±0.40
Proline	2.2±1.72	0.5±0.01	0.4±0.00	3.2±1.41	3.2±0.20	3.3±0.14
Serine	7.4±0.77	7.6±0.88	7.6±0.00	6.9±1.76	5.4±0.27	6.8±0.33
Taurine	1.0±0.12	1.9±0.32	2.3±0.02	4.9±0.74	3.2±0.88	3.2±0.18
Threonine	5.2±0.93	7.4±0.60	6.7±0.00	4.0±1.11	3.8±0.08	3.4±0.30
Tryptophan	0.9±0.14	1.4±0.61	1.5±0.29	0.6±0.05	1.4±0.00	2.2±0.51
Tyrosine	2.5±0.43	2.4±0.43	1.8±0.01	1.6±0.27	1.4±0.13	1.7±0.04
Valine	2.9±0.48	3.4±0.19	3.0±0.00	2.2±0.53	2.0±0.04	2.0±0.12

[x]With four rats per group, the tissues of every two rats were pooled

[z]Number of weeks on diet

Appendix C, Table Ib. Summary of concentration means and standard errors of ninhydrin-reacting substances from liver[x] of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances[y] (mg/100 g)	Zero-time controls	C-2			W		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	72.9±6.34	66.9± 8.44	56.0± 0.17	51.1±0.98	44.9±2.84	50.3±0.37	40.8±0.50
Ammonia	20.2±1.62	21.7± 0.36	17.0± 3.16	15.3±0.28	18.1±4.22	18.4±0.39	16.3±2.03
Aspartic acid	51.0±1.00	43.4± 4.44	62.5±17.00	55.6±4.40	33.5±2.18	50.5±0.66	47.4±1.40
Cystine	0.9±0.94	8.3± 1.99	0.8± 0.42	2.0±1.99	1.3±1.29	0.0±0.00	0.0±0.00
Glutamic acid	88.9±2.42	142.8±14.88	87.8± 3.92	100.6±1.04	110.0±0.17	62.5±6.68	68.8±0.05
Glycine	47.3±3.07	25.4± 5.13	16.9± 0.61	17.1±2.23	26.1±0.50	21.7±0.13	22.8±0.94
Histidine	15.1±1.12	12.0± 0.37	8.8± 0.55	8.8±0.84	10.6±1.30	10.1±0.56	8.4±0.47
Isoleucine	12.6±0.66	5.2± 0.04	3.6± 0.14	3.7±0.21	5.6±0.10	4.7±0.01	4.3±0.32
Leucine	27.1±1.93	13.0± 1.20	7.7± 0.41	8.3±0.41	12.4±0.49	10.1±0.16	9.5±1.20
Lysine	44.7±1.03	33.9± 4.63	18.1± 0.48	18.8±0.16	20.3±0.83	14.3±0.67	16.1±0.96
Methionine	14.5±0.98	6.8± 1.20	4.8± 0.05	4.8±0.12	7.4±0.05	6.0±0.37	5.6±0.50
Ornithine	29.1±2.25	12.1± 2.35	6.8± 0.21	6.8±0.60	12.6±1.53	12.1±0.76	8.5±1.29
Phenylalanine	14.1±0.91	6.4± 0.97	3.8± 0.16	4.2±0.31	8.2±1.94	5.2±0.13	4.7±0.72
Proline	12.2±2.54	5.1± 0.54	3.1± 0.43	4.6±0.26	8.5±0.58	4.1±0.65	4.6±1.59
Serine	32.1±1.52	37.5± 5.04	27.7± 2.04	24.2±3.98	29.4±5.84	30.3±3.27	20.9±2.30
Taurine	19.2±7.79	1.8± 0.24	2.6± 0.46	7.2±0.21	9.6±2.50	23.9±6.42	8.7±0.31
Threonine	20.6±1.38	17.5± 3.22	11.7± 1.92	9.9±0.93	13.0±2.31	10.6±0.33	7.6±1.01
Tryptophan	0.1±0.14	0.2± 0.00	0.5± 0.51	1.5±0.69	1.5±0.22	0.2±0.00	0.2±0.00
Tyrosine	13.8±0.80	8.2± 0.97	4.5± 0.17	4.6±0.10	7.4±0.29	5.5±0.19	5.0±0.75
Veline	19.4±1.16	7.4± 1.83	3.5± 0.09	4.9±0.70	6.6±0.11	5.3±0.39	4.3±0.20

[x]With four rats per group, the tissue of every two rats were pooled

[y]No arginine or citrulline were found in liver

[z]Number of weeks on diet

Appendix C, Table Ic. Summary of concentration means and standard errors of ninhydrin-reacting substances from gastrocnemii[x] of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances[y] (mg/100 g)	Zero-time controls[1]	C-2			W		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	32.2	47.8±3.05	36.6±2.88	33.0± 2.53	28.8±0.90	29.0±1.02	30.6±2.43
Ammonia		10.8±0.31	14.8±5.59	44.4± 4.84	35.1±6.02	39.0±6.69	18.3±2.56
Arginine	4.1	3.1±0.27	2.5±0.82	3.5± 0.27	3.3±0.18	1.8±0.12	3.1±0.37
Aspartic acid	8.3	7.4±0.33	6.1±0.35	4.9± 0.38	6.8±0.56	5.7±0.52	4.0±0.45
Citrulline	5.5	5.6±1.10	2.6±0.23	3.1± 0.24	6.3±0.31	4.1±0.44	3.7±0.33
Glutamic acid	44.2	83.8±8.48	45.5±2.94	37.0± 2.31	68.9±3.11	41.1±3.44	31.6±3.44
Glycine	46.9	15.1±3.8	13.0±3.28	12.2± 0.81	32.8±2.00	24.0±1.36	28.5±1.11
Histidine	9.7	5.6±0.35	4.3±0.28	4.2± 0.32	4.2±0.44	2.2±0.70	4.3±0.52
Isoleucine	3.1	2.0±0.29	2.6±0.17	2.4± 0.16	1.3±0.12	1.6±0.19	2.5±0.34
Leucine	5.7	3.5±0.33	3.7±0.26	3.7± 0.24	2.4±0.06	2.4±0.28	4.4±0.56
Lysine	20.4	21.3±3.18	9.8±0.37	10.6± 0.80	0.0±0.00	1.2±1.18	1.0±1.05
Methionine	3.2	2.6±0.16	3.2±0.15	2.9± 0.11	2.0±0.06	2.2±0.15	3.4±0.20
Ornithine	1.9	0.9±0.17	0.8±0.33	0.3± 0.14	1.8±0.37	1.4±0.42	0.8±0.34
Phenylalanine	2.9	1.6±0.21	2.7±0.74	2.8± 0.63	1.9±0.31	1.2±0.18	2.9±0.34
Proline	5.8	6.4±1.35	6.5±1.04	5.3± 0.66	6.8±0.52	6.4±0.89	3.9±0.89
Serine	41.4	34.3±2.47	26.3±1.55	33.3± 3.67	22.8±0.97	17.8±1.49	27.3±1.32
Taurine	64.8	35.2±1.78	45.3±6.15	88.1±13.96	118.0±9.16	119.3±5.73	150.1±6.83
Threonine	13.1	14.5±0.14	11.9±1.89	13.4± 1.14	9.4±0.67	6.2±1.08	9.0±1.50
Tryptophan		0.4±0.07	0.8±0.22	0.5± 0.15	0.2±0.08	0.3±0.07	0.5±0.13
Tyrosine	3.3	4.0±0.12	5.2±1.56	3.0± 0.13	2.5±0.24	2.0±0.13	3.7±0.35
Valine	3.5	3.1±0.17	2.6±0.13	2.7± 0.20	2.0±0.24	1.7±0.16	3.2±0.17

[1]No standard error of mean available as only a pooled sample from four rats was analyzed

[x]Samples from four rats per group were analyzed individually

[y]Cystine was not detected

[z]Number of weeks on diet

Appendix C, Table Id. Summary of concentration means of ninhydrin-reacting substances from thigh and leg muscle[x] of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances[y] (mg/100 g)	C-2			W		
	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	54.2	44.7	35.2	34.1	31.6	28.7
Ammonia	19.7	15.7	83.6	15.3	15.3	14.9
Arginine	4.2	1.7	2.2	4.4	1.8	3.4
Aspartic acid	10.9	11.7	6.9	11.6	10.0	5.8
Citrulline	7.8	4.5	3.6	6.2	4.9	4.0
Glutamic acid	99.1	66.0	51.1	71.1	42.7	40.3
Glycine	17.1	17.1	13.1	32.4	27.1	28.8
Histidine	8.0	5.6	5.5	4.4	4.7	3.6
Isoleucine	2.7	2.9	3.0	2.5	1.9	2.7
Leucine	5.8	5.4	5.6	5.5	4.0	5.2
Lysine	32.9	19.3	13.9	12.1	4.0	10.2
Methionine	1.4	3.4	3.5	3.4	2.5	3.3
Ornithine	4.3	0.8	0.7	2.2	1.5	1.0
Phenylalanine	3.4	3.5	3.3	3.6	3.2	3.6
Proline	7.9	7.9	3.2	7.4	4.8	3.4
Serine	30.8	30.0	24.3	25.0	20.3	21.0
Taurine	28.6	37.4	64.6	99.4	102.8	111.1
Threonine	16.6	15.3	11.9	9.5	7.2	7.4
Tryptophan	0.0	0.0	0.0	0.3	0.2	0.2
Tyrosine	6.0	6.0	4.6	4.2	4.0	4.2
Valine	5.0	4.3	4.0	3.8	3.3	3.3

[x]No standard error of the mean available as only a pooled sample from four rats was analyzed

[y]Cystine was not detected

[z]Number of weeks on diet

Appendix C, Table IIa. Summary of concentration means and standard errors of ninhydrin-reacting substances from serum of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[y] (mg/100 ml)	Zero-time controls	C-1			C-2			C-3			Mod. SW		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	4.0±0.43	9.2±1.77	10.6±0.68	12.5±0.04	9.3±0.66	8.3±0.24	7.8±0.36	6.1±0.42	5.4±0.00	6.7±0.60	6.0±0.42	5.7±0.14	5.7±0.21
Ammonia	1.6±0.21	2.1±0.04	1.9±0.01	2.4±0.03	1.8±0.13	1.8±0.07	1.7±0.01	1.9±0.24	1.7±0.14	1.7±0.11	1.6±0.28	1.6±0.14	1.8±0.08
Arginine	0.9±0.60	1.2±1.24	1.6±0.00	0.7±0.68	0.9±0.31	5.3±0.33	2.4±0.21	1.6±0.60	1.3±1.26	1.3±1.28	0.9±0.60	4.4±0.98	3.5±0.44
Aspartic acid	1.3±0.03	1.1±0.01	1.2±0.10	2.3±1.01	1.2±0.10	1.2±0.02	1.2±0.31	1.2±0.12	1.0±0.16	1.4±0.02	1.2±0.04	1.0±0.01	1.1±0.20
Citrulline	1.4±0.18	1.8±0.32	1.8±0.24	2.2±0.16	1.9±0.33	1.2±0.02	0.9±0.09	1.6±0.24	1.2±0.00	1.1±0.05	1.3±0.03	1.4±0.08	1.0±0.04
Cystine	0.0±0.00	0.9±0.15	0.3±0.02	0.5±0.07	1.0±0.47	1.2±0.07	1.5±0.08	0.9±0.08	0.6±0.29	1.9±1.23	0.0±0.00	0.0±0.00	0.0±0.00
Glutamic acid	8.0±0.74	11.9±0.52	10.8±0.44	14.5±4.31	12.0±0.41	10.8±0.63	9.2±0.28	10.0±0.44	8.8±1.00	7.9±0.48	8.5±0.45	7.6±0.63	7.4±0.13
Glycine	2.3±0.31	1.2±0.12	1.5±0.02	1.4±0.24	1.4±0.24	1.1±0.19	1.3±0.12	0.8±0.08	0.8±0.11	1.1±0.03	2.5±0.53	2.4±0.08	2.1±0.11
Histidine	0.9±0.07	2.2±0.17	2.1±0.26	2.2±0.45	1.5±0.14	1.4±0.10	1.2±0.10	1.3±0.00	1.1±0.04	1.3±0.08	1.4±0.03	1.1±0.01	1.2±0.01
Isoleucine	1.4±0.01	1.1±0.05	1.2±0.22	1.2±0.13	1.4±0.05	1.4±0.06	1.4±0.12	1.9±0.18	1.7±0.23	1.7±0.12	1.3±0.00	1.4±0.06	1.5±0.02
Leucine	2.0±0.11	1.7±0.10	1.8±0.34	1.9±0.36	2.0±0.10	2.3±0.12	2.2±0.10	3.1±0.14	2.9±0.51	3.0±0.20	2.0±0.08	2.1±0.02	2.4±0.04
Lysine	5.3±0.31	12.2±2.34	9.2±0.51	13.1±1.90	10.9±0.85	11.9±1.14	7.9±1.06	10.7±0.02	9.6±0.39	10.7±0.48	6.4±0.17	5.3±0.05	6.6±0.17
Methionine	0.7±0.02	0.6±0.12	0.7±0.08	0.6±0.12	0.9±0.02	1.0±0.13	1.0±0.06	1.0±0.00	1.1±0.04	0.8±0.00	0.9±0.02	1.1±0.07	0.9±0.14
Ornithine	1.7±0.28	1.7±0.84	2.1±0.76	2.0±0.18	2.1±0.16	0.6±0.00	0.8±0.00	1.5±0.11	1.0±0.41	0.7±0.02	2.1±0.26	1.2±0.31	1.1±0.14
Phenylalanine	1.0±0.04	0.9±0.04	0.9±0.19	1.0±0.09	0.9±0.01	1.1±0.05	1.1±0.10	1.2±0.01	1.3±0.19	1.2±0.00	1.0±0.06	1.0±0.00	1.2±0.00
Proline	2.0±0.36	4.2±0.45	3.6±0.38	3.5±0.39	7.4±0.21	4.2±0.64	3.8±0.36	4.8±0.30	4.2±0.37	4.3±0.62	3.3±0.15	3.6±0.36	3.2±0.34
Serine	3.7±0.04	8.3±0.57	9.2±1.56	9.5±0.04	7.3±0.10	6.5±0.64	6.5±0.22	4.4±0.11	4.2±0.05	5.3±0.54	4.7±0.28	4.3±0.36	4.3±0.16
Taurine	2.8±0.33	2.0±0.02	1.4±0.23	1.7±0.04	1.8±0.52	1.6±0.46	4.8±0.24	2.5±0.40	2.8±0.38	5.0±0.60	3.5±0.19	3.0±0.10	3.7±0.20
Threonine	1.9±0.02	2.3±0.24	4.1±0.25	4.0±0.63	5.7±0.88	6.3±0.94	5.6±0.56	7.2±0.32	5.8±0.07	5.5±0.02	4.3±0.04	3.9±0.06	3.3±0.32
Tryptophan	0.9±0.25	0.1±0.14	0.9±0.08	0.5±0.47	2.2±0.18	2.4±0.65	1.7±0.04	1.9±0.54	1.4±1.38	0.5±0.50	0.9±0.24	2.4±0.08	1.7±0.07
Tyrosine	1.1±0.10	1.0±0.01	1.4±0.28	1.0±0.14	3.2±0.42	2.7±0.03	2.2±0.18	2.4±0.13	2.7±0.16	2.1±0.02	1.8±0.23	1.6±0.03	1.5±0.12
Valine	1.9±0.11	2.0±0.07	2.4±0.31	2.2±0.00	3.0±0.05	2.9±0.00	3.1±0.04	4.1±0.39	3.8±0.58	4.0±0.55	2.4±0.11	2.6±0.00	2.7±0.05

[x] With four rats per group, the tissues of every two rats were pooled

[z] Number of weeks on diet

Appendix C, Table IIb. Summary of concentration means and standard errors of ninhydrin-reacting substances from liver[x] of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[y] (mg/100 g)	Zero-time controls	C-1			C-2			C-3			Mod. SW		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	52.5±2.16	56.4±3.18	70.3± 2.09	62.7± 0.32	65.2±1.51	62.7±5.33	53.9±3.47	57.6± 2.89	54.1± 0.56	40.8± 1.36	39.2± 0.27	33.0± 3.22	36.9±2.77
Ammonia	19.3±2.38	22.4±0.25	16.4± 0.74	17.0± 0.69	20.8±5.11	11.4±1.84	14.1±2.03	18.4± 1.30	14.2± 0.38	12.9± 2.08	9.7± 0.66	8.9± 0.57	9.5±0.90
Aspartic acid	43.7±5.64	55.5±6.02	45.5± 1.49	40.9± 8.94	47.6±1.75	48.7±0.33	43.6±7.68	51.0±10.34	40.7± 1.99	60.5±10.76	51.0± 2.22	53.8± 3.76	52.0±3.53
Cystine	0.0±0.00	10.9±4.49	4.2± 1.01	0.4± 0.19	4.0±1.37	11.1±0.21	2.4±1.94	0.0± 0.00	1.9± 0.52	0.0± 0.00	0.0± 0.00	0.0± 0.00	0.0±0.00
Glutamic acid	118.4±0.59	92.3±9.36	81.5±17.22	86.6±21.00	110.2±3.65	106.1±2.75	115.4±4.12	16.4±17.66	90.4± 0.19	91.8± 3.03	77.6± 0.44	59.9± 3.00	53.4±2.54
Glycine	34.7±1.37	19.1±2.52	21.4± 1.84	18.5± 2.12	24.6±1.06	15.6±0.39	18.3±0.19	21.3± 2.11	17.7± 1.12	13.0± 0.82	23.9± 2.20	19.2± 0.64	16.4±0.10
Histidine	11.8±0.14	9.1±0.10	9.3± 0.46	8.8± 0.93	10.5±0.56	8.4±0.29	9.4±0.20	13.8± 1.85	12.1± 1.19	9.5± 0.07	10.5± 0.51	8.3± 0.36	8.0±0.09
Isoleucine	7.6±0.14	3.7±0.41	2.7± 0.14	5.0± 0.93	6.7±0.50	4.3±0.02	4.8±0.53	5.9± 0.30	5.2± 1.51	3.1± 0.26	5.9± 0.36	4.4± 0.48	4.3±0.08
Leucine	18.8±0.03	8.2±0.14	7.2± 0.33	11.4± 2.81	15.9±0.93	10.0±0.25	10.0±0.07	14.0± 0.38	12.4± 1.96	8.2± 0.63	14.5± 0.78	10.0± 0.80	9.4±0.27
Lysine	35.8±0.54	24.0±0.89	25.8± 4.33	33.5± 8.42	33.5±8.42	15.9±2.97	16.8±6.90	28.6± 2.19	23.4± 2.36	19.6± 1.28	23.7± 3.33	16.2± 1.77	15.5±2.25
Methionine	10.1±0.06	5.1±0.25	4.5± 0.15	6.8± 1.39	8.1±0.55	6.0±0.28	7.6±2.58	7.2± 0.93	8.7± 0.41	1.3± 0.32	7.7± 0.27	5.8± 0.35	5.4±0.26
Ornithine	19.0±0.14	9.1±0.87	10.5± 0.54	12.5± 1.50	14.0±1.34	8.4±0.41	6.8±0.19	8.0± 0.80	7.2± 0.84	6.4± 1.23	12.7± 0.68	9.0± 0.81	7.6±0.30
Phenylalanine	9.1±0.31	4.1±0.28	4.2± 0.21	5.7± 1.41	7.9±0.46	5.5±0.41	5.3±0.04	7.1± 0.84	6.2± 0.27	4.2± 0.30	6.8± 0.33	5.0± 0.19	4.3±0.05
Proline	9.7±3.99	3.2±0.36	4.1± 0.62	5.0± 2.37	8.4±0.39	4.9±0.55	3.4±0.24	9.2± 1.72	5.6± 0.86	4.4± 0.44	5.1± 0.82	4.0± 0.51	3.7±1.21
Serine	24.5±0.66	34.9±3.97	38.4± 1.69	35.4± 3.00	40.6±1.10	29.0±1.16	19.7±0.62	19.6± 4.34	17.5± 1.70	15.5± 3.70	20.9± 0.89	14.4± 0.42	11.3±0.45
Taurine	31.3±8.23	2.3±0.10	2.0± 0.09	1.8± 0.15	2.1±0.22	2.1±0.04	5.6±0.62	39.5±15.93	60.6±10.32	65.2± 9.83	25.2±18.72	41.8±14.66	65.5±4.60
Threonine	13.5±0.22	8.0±0.34	11.1± 0.40	14.2± 2.43	16.4±0.18	12.2±1.86	10.8±0.64	11.3± 2.44	11.1± 0.55	6.8± 0.30	10.8± 0.99	5.8± 0.54	4.9±0.31
Tryptophan	0.2±0.00	0.5±0.49	0.6± 0.00	0.8± 0.76	1.8±0.34	0.7±0.00	0.0±0.00	0.0± 0.00	0.0± 0.00	0.5± 0.39	0.4± 0.05	0.1± 0.10	0.6±0.47
Tyrosine	9.5±0.11	4.5±0.56	4.5± 0.09	6.5± 2.03	9.3±0.62	5.6±0.18	6.1±0.19	8.9± 0.43	8.6± 0.97	5.6± 0.35	7.5± 0.26	5.3± 0.44	4.8±0.13
Valine	11.4±0.20	6.2±3.43	4.2± 0.14	5.0± 1.72	9.1±0.58	4.4±0.04	4.5±0.72	8.5± 0.32	7.9± 0.60	5.7± 0.56	6.8± 0.20	4.7± 0.20	4.1±0.16

[x]With four rats per group, the tissues of every two rats were pooled

[y]No arginine or citrulline were found in liver

[z]Number of weeks on diet

Appendix C, Table IIc. Summary of concentration means and standard errors of ninhydrin-reacting substances from gastrocnemii[x] of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances (mg/100 g)	Zero-time controls	C-1			C-2			C-3			Mod. SW		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	28.8± 2.17	41.8±4.10	42.2±1.93	36.3±2.54	39.3±3.72	38.3±2.32	31.1± 2.11	38.8±3.51	28.8±0.44	26.1±0.34	25.8±0.87	22.4± 1.15	21.9±0.76
Ammonia	73.9±14.47	53.0±0.64	32.8±4.78	23.6±3.14	23.9±2.52	26.2±2.32	19.0± 2.44	17.7±0.65	17.4±0.95	16.9±1.22	17.8±1.60	16.6± 1.08	17.2±1.06
Arginine	5.2± 0.00	4.2±0.54	1.7±0.24	1.5±0.30	1.5±0.48	2.3±0.84	1.5± 0.25	2.0±0.44	1.9±0.30	1.9±0.23	2.3±0.42	2.3± 0.19	2.1±0.20
Aspartic acid	8.8± 1.19	5.5±0.44	4.9±0.22	5.0±0.48	8.4±0.37	7.0±0.20	6.4± 0.81	11.2±1.57	6.2±0.94	5.9±0.78	7.5±0.72	6.8± 0.83	6.5±1.12
Citrulline	6.3± 1.05	5.9±0.61	5.8±0.12	5.1±0.65	4.2±0.70	3.6±0.17	2.4± 0.33	4.2±0.23	2.3±0.15	2.3±0.22	3.7±0.40	2.5± 0.17	2.2±0.35
Glutamic acid	84.2± 6.06	67.2±4.90	46.0±1.90	30.1±1.99	90.4±6.12	52.4±1.90	31.6± 1.99	101.5±9.35	34.2±1.77	28.5±1.37	61.7±2.60	30.7± 2.10	27.2±2.34
Glycine	45.2± 3.27	14.6±1.47	15.8±1.72	18.9±2.59	18.4±1.24	14.5±1.48	11.3± 1.99	10.3±1.33	7.3±0.44	7.6±0.67	29.8±0.67	18.9± 0.85	15.9±1.64
Histidine	6.0± 0.36	6.3±0.39	7.0±0.38	5.3±0.41	5.4±0.56	4.6±0.26	3.5± 0.46	5.0±0.09	3.9±0.74	3.1±0.20	3.0±0.61	2.9± 0.14	2.8±0.35
Isoleucine	2.2± 0.36	2.0±0.21	1.6±0.17	1.7±0.28	2.0±0.19	1.8±0.19	2.2± 0.30	2.3±0.25	2.3±0.23	2.1±0.10	1.9±0.05	1.6± 0.23	1.6±0.07
Leucine	3.9± 0.46	4.0±0.32	2.8±0.23	2.8±0.47	3.5±0.19	2.8±0.18	3.4± 0.45	4.3±0.32	3.5±0.31	3.3±0.25	3.0±0.15	2.6± 0.53	2.7±0.24
Lysine	21.3± 3.10	22.9±2.59	22.3±1.97	6.6±1.81	20.7±7.49	11.6±5.38	6.4± 3.71	18.8±2.72	14.0±3.21	14.1±0.99	4.9±1.78	7.6± 0.90	5.2±2.28
Methionine	3.2± 0.37	2.8±0.24	2.4±0.15	2.3±0.25	2.5±0.38	2.0±0.34	1.7± 0.04	2.1±0.34	2.5±0.23	2.2±0.09	2.2±0.07	1.3± 0.56	1.9±0.10
Ornithine	2.8± 0.17	0.8±0.06	1.4±0.44	1.5±0.73	0.5±0.14	0.9±0.17	1.1± 0.52	0.9±0.19	1.5±0.99	0.8±0.08	1.5±0.34	0.9± 0.07	0.6±0.20
Phenylalanine	1.9± 0.22	2.5±0.24	1.6±0.16	2.5±0.62	2.1±0.42	1.6±0.06	1.9± 0.40	2.2±0.22	2.0±0.35	1.7±0.18	1.5±0.09	1.7± 0.35	1.5±0.20
Proline	6.3± 1.00	6.0±1.31	4.7±0.71	4.2±0.62	6.7±1.00	6.8±0.39	5.2± 0.97	11.8±1.70	5.1±0.34	5.2±0.52	4.6±0.36	3.5± 0.63	2.6±0.45
Serine	23.3± 0.66	40.7±1.28	42.8±3.57	36.1±3.01	28.0±1.61	30.2±0.61	19.7± 2.00	19.9±1.45	14.9±0.55	15.6±1.07	13.4±0.63	12.0± 1.05	10.5±0.31
Taurine	114.0±12.47	50.9±3.47	39.2±2.53	28.9±1.56	40.1±5.74	42.2±4.62	83.7±10.68	84.6±6.49	165.1±5.52	155.1±5.40	127.4±2.34	142.7±19.07	150.2±6.56
Threonine	8.6± 1.10	8.1±1.75	9.7±0.97	9.9±0.62	14.2±1.07	15.8±0.75	10.7± 1.66	20.6±2.32	9.4±0.46	8.7±0.23	8.8±0.57	5.9± 0.38	5.1±0.26
Tryptophan	0.6± 0.58	0.5±0.16	0.7±0.29	0.6±0.08	0.6±0.27	0.5±0.29	0.9± 0.08	0.8±0.16	0.5±0.18	1.0±0.05	0.9±0.08	0.7± 0.04	0.5±0.17
Tyrosine	2.4± 0.25	3.1±0.19	2.4±0.12	3.0±0.71	4.6±0.53	3.6±0.09	2.9± 0.35	3.6±0.28	3.4±0.07	2.8±0.17	2.5±0.06	2.4± 0.20	2.2±0.14
Valine	2.5± 0.18	2.9±0.09	2.4±0.26	2.5±0.26	3.2±0.25	3.0±0.36	3.2± 0.52	4.5±0.34	3.3±0.38	2.8±0.20	2.2±0.16	2.3± 0.28	1.9±0.19

[x]Samples from four rats per group were analyzed individually

[y]Cystine was not detected

[z]Number of weeks on diet

Appendix C, Table IId. Summary of concentration means of ninhydrin-reacting substances from thigh and leg muscle[x] of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[y] (mg/100 g)	Zero-time controls	C-1			C-2			C-3			Mod. SW		
		4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]	4[z]	8[z]	12[z]
Alanine	31.0	41.6	40.9	38.7	47.6	39.0	35.5	44.0	33.5	30.5	38.1	27.7	24.1
Ammonia	16.2	16.0	17.7	15.4	24.0	18.6	15.8	16.1	19.1	14.9	14.8	12.0	12.8
Arginine	6.6	4.5	2.8	2.4	3.5	3.1	2.3	3.6	4.4	3.9	5.1	5.1	2.7
Aspartic acid	15.0	7.7	7.0	7.1	12.6	9.8	6.5	15.6	11.2	10.1	7.6	5.1	5.7
Citrulline	7.1	6.4	6.3	5.6	6.6	4.0	2.9	4.4	2.4	2.4	3.8	1.9	3.2
Glutamic acid	86.2	71.2	59.4	52.8	95.0	61.7	48.8	92.2	43.6	42.6	75.8	32.0	32.6
Glycine	52.1	14.6	15.1	17.8	19.9	13.3	12.0	10.0	9.2	8.4	36.4	18.0	16.4
Histidine	8.2	6.1	7.5	5.9	6.8	5.0	4.1	6.9	6.1	4.1	7.6	3.5	3.1
Isoleucine	3.0	2.3	1.8	2.8	2.7	3.3	3.2	3.7	4.0	3.4	5.0	2.3	2.6
Leucine	5.2	4.7	3.9	4.6	5.6	5.9	5.3	6.5	7.2	6.4	8.7	4.2	4.6
Lysine	25.9	30.1	16.3	13.6	23.4	12.9	12.4	15.0	17.6	18.6	15.0	7.1	11.5
Methionine	3.1	3.1	2.8	3.1	3.5	3.1	3.8	3.9	3.9	4.1	6.3	2.8	3.1
Ornithine	4.5	2.0	1.3	1.1	2.0	0.6	1.1	1.0	0.8	0.6	2.1	0.8	0.7
Phenylalanine	3.2	3.0	2.7	3.2	3.9	3.7	3.2	4.0	4.4	3.8	4.9	2.7	2.7
Proline	6.1	7.5	5.2	7.0	7.3	5.3	8.1	13.0	6.1	7.4	4.6	3.8	3.7
Serine	22.4	41.9	34.4	40.9	33.4	25.4	21.4	23.1	17.6	16.8	19.4	11.3	13.2
Taurine	91.6	39.7	33.2	22.2	38.0	31.6	75.9	90.1	147.3	144.0	177.3	142.2	136.4
Threonine	7.7	6.1	8.8	12.2	15.4	13.8	10.2	18.7	11.3	9.4	14.0	6.6	6.0
Tryptophan	0.6	0.3	0.1	0.8	0.3	0.5	1.2	0.2	0.3	0.3	0.6	0.6	0.0
Tyrosine	3.9	3.7	3.8	3.6	6.5	5.5	4.6	5.2	6.1	5.0	6.4	3.7	3.5
Valine	3.8	3.2	3.2	3.6	6.4	4.1	4.2	6.0	6.3	5.5	4.7	2.3	3.4

[x]No standard error of the mean available as only a pooled sample from four rats was analyzed

[y]Cystine was not detected

[z]Number of weeks on diet

Appendix D, Table I. Comparison of ninhydrin-reacting substance concentrations in serum, liver and gastrocnemii of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances	Comparison of diets																	
	Serum[1]						Liver[2]						Gastrocnemii[3]					
	C-2>W			W>C-2			C-2>W			W>C-2			C-2>W			W>C-2		
	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																		
Arginine					*													
Histidine				*		*							*	*				
Isoleucine			*							*	*		*	*				
Leucine		**	*								*		*	*				
Lysine	**	**	*					*					**	**	**			
Methionine													**	**				
Phenylalanine											*			*				
Threonine		*											**	*	*			
Tryptophan										*								
Valine		*	*								*		**	**				
Non-essential amino acids:																		
Alanine									*				**	**				
Aspartic acid		*																
Cystine		*																
Glutamic acid									**									
Glycine					*	*					*					**	*	**
Proline					**					*								
Serine													**	**				
Tyrosine													**	*				*
Non-amino acids:																		
Ornithine											*							
Citrulline						*											*	
Ammonia															**	**	**	
Taurine				*		*										**	**	**

*Significant difference at $P<0.05$

**Significant difference at $P<0.01$

[1]No significant differences of methionine, phenylalanine, tryptophan, alanine, glutamic acid, serine, tyrosine, ornithine, ammonia.

[2]No detectable amount of arginine and citrulline; no significant differences of histidine, methionine, threonine, aspartic acid, cystine, serine, tyrosine, ammonia, taurine.

[3]No detectable amount of cystine; no significant differences of arginine, tryptophan, aspartic acid, glutamic acid, proline, ornithine.

Appendix D, Table IIa. Comparison of ninhydrin-reacting substance concentrations in serum of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances	Comparison of diets																																	
	C-1>C-2			C-1>C-3			C-1>Mod. SW			C-2>C-1			C-2>C-3			C-2>Mod. SW			C-3>C-1			C-3>C-2			C-3>Mod. SW			Mod. SW>C-1			Mod. SW>C-2			Mod. SW>C-3		
	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																																				
Arginine[b]																																				
Histidine	**	*		**	*		**	*																												
Isoleucine																			*			*			*										
Leucine																			*			*			*										
Lysine		*						*																												
Methionine[b]																																				
Phenylalanine																			**			**			*			*								
Threonine										**									**						*		*									
Tryptophan										*						*			*																	
Valine										*									**	*		*			**											
Non-essential amino acids:																																				
Alanine		*	**		**	**		**	**					**			**	*																		
Aspartic acid[b]																																				
Cystine											*						*																			
Glutamic acid				*			*						*			*																				
Glycine					**								*																							
Proline[b]																												**	**	*	**	**	*	**	**	*
Serine		**		**	*	**	**	*	**				**		*	**		**																			
Tyrosine										*	**	**				*		*	*	**	**						*									
Non-amino acids:																																				
Ornithine[b]																																				
Citrulline		**			**			**																												
Ammonia		**			**			**																												
Taurine																				*	**		*	**					*	*		*				

*Significant difference at P<0.05

**Significant difference at P<0.01

[b]No significant differences

Appendix D, Table IIb. Comparison of ninhydrin-reacting substance concentrations in liver of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[a]	Comparison of diets																							
	C-1>C-2			C-1>C-3			C-1>Mod. SW			C-2>C-1			C-2>C-3			C-2>Mod. SW			C-3>C-1			C-3>C-2			C-3>Mod. SW			Mod. SW>C-1			Mod. SW>C-2			Mod. SW>C-3		
	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																																				
Histidine																				°			°			°										
Isoleucine										°										°								°								
Leucine										°°			°			°			°°								°°			°					
Lysine[b]																																				
Methionine											°					°				°°			°°			°°			°							
Phenylalanine										°	°								°	°						°		°								
Threonine								°	°	°						°	°	°								°										
Tryptophan					°			°						°			°																			
Valine																				°°			°°			°°										
Non-essential amino acids:																																				
Alanine					°	°°		°	°°	°°				°		°°	°°	°°							°	°°	°°									
Aspartic acid[b]																																				
Cystine					°			°°			°°			°°			°°																			
Glutamic acid[b]																																				
Glycine[b]																																				
Proline										°																										
Serine	°°	°°		°	°°	°°		°	°°	°				°	°°		°	°°		°						°										
Tyrosine										°°									°°	°			°			°										
Non-amino acids:																																				
Ornithine										°			°																							
Ammonia	°						°																											°		
Taurine																				°°			°			°			°	°°		°	°°			

°Significant difference at P<0,05

°°Significant difference at P<0,01

[a]No detectable amounts of arginine or citrulline

[b]No significant differences

Appendix D, Table IIc. Comparison of ninhydrin-reacting substance concentrations in gastrocnemii of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[a]	Comparison of diets																														
	C-1>C-2			C-1>C-3			C-1>Mod. SW			C-2>C-1			C-2>C-3			C-2>Mod. SW			C-3>C-1			C-3>C-2			C-3>Mod. SW			Mod. SW>C-1			Mod. SW>C-2			Mod. SW>C-3		
	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																																				
Arginine	**			**			*																													
Histidine		**	**		**	**	**	**	**							**	*								**										
Isoleucine[b]																																				
Leucine							*																													
Lysine		*					*	*								*									*										
Methionine[b]																									*											
Phenylalanine[b]																																				
Threonine								**	**	*	**			**		*	**	**	**			*			**	**	**									
Tryptophan												*						*			*						*									
Valine																*			**			**			**											
Non-essential amino acids:																																				
Alanine					**	**	*	**	**					**		*	**	**	•						*	*										
Aspartic acid																			**						*											
Glutamic acid					**			**		*	*			**		**	**		**						**											
Glycine			*	*	**	**				*			**	**														**			**	*		**	**	*
Proline											*						**		**						**											
Serine	**	**	**	**	**	**	**	**	**				**	**		**	**	**							**											
Tyrosine										**	**		*			**	**			**					*	**										
Non-amino acids:																																				
Ornithine																															**					
Citrulline	*	**	**	*	**	**	*	**	**					**			**																			
Ammonia	**			**	*		**	*					*	*		*	*																			
Taurine												**							**	**	**	**	**	**				**	**	**	**	**	**	**			

*Significant difference at P<0.05

**Significant difference at P<0.01

[a]No detectable amounts of cystine

[b]No significant difference

Appendix D, Table IIIa. Concentration changes of ninhydrin-reacting substances in serum of rats fed a casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances	Increases						Decreases					
	4-8 wk		4-12 wk		8-12 wk		4-8 wk		4-12 wk		8-12 wk	
	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W
Essential amino acids:												
Arginine			*		*							
Histidine[b]												
Isoleucine[b]												
Leucine[b]												
Lysine							**		**			
Methionine[b]												
Phenylalanine[b]												
Threonine[b]												
Tryptophan[b]												
Valine[b]												
Non-essential amino acids:												
Alanine[b]												
Aspartic acid[b]												
Cystine	**		**		**							
Glutamic acid[b]												
Glycine[b]												
Proline[b]												
Serine[b]												
Tyrosine[b]												
Non-amino acids:												
Ornithine							*		*			
Citrulline[b]												
Ammonia[b]												
Taurine	*		*									

*Significant difference at P<0.05
**Significant difference at P<0.01
[b]No significant difference

Appendix D, Table IIIb. Concentration changes of ninhydrin-reacting substances in liver of rats fed casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances[a]	Increases												Decreases											
	0-4 wk		0-8 wk		0-12 wk		4-8 wk		4-12 wk		8-12 wk		0-4 wk		0-8 wk		0-12 wk		4-8 wk		4-12 wk		8-12 wk	
	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W
Essential amino acids:																								
Histidine													*	*	*	*	*	*	*		*			
Isoleucine													**	**	**	**	**	**	*		*			
Leucine													**	**	**	**	**	**	*		*			
Lysine													*	**	**	**	**	**	*	**	*	*		
Methionine													**	**	**	**	**	**						
Phenylalanine													**	*	**	*	**	*						
Threonine														*		*		*						
Tryptophan	**																		**		**			
Valine													**	**	**	**	**	**			*			
Non-essential amino acids:																								
Alanine														*		*		*						
Aspartic acid								**						**										
Cystine[b]																								
Glutamic acid	**	*														**		*	**	**	**	**		
Glycine													**	**	**	**	**	**						
Proline													**		**		**							
Serine[b]																								
Tyrosine													**	**	**	**	**	**	*		*	*		
Non-amino acids:																								
Ornithine													**	**	**	**	**	**						
Ammonia[b]																								
Taurine[b]																								

*Significant difference at P<0.05

**Significant difference at P<0.01

[a]No detectable amount of arginine or citrulline

[b]No significant difference

Appendix D, Table IIIc. Concentration changes of ninhydrin-reacting substances in gastrocnemii of rats fed casein (C-2) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances[a]	Increases												Decreases											
	0-4 wk		0-8 wk		0-12 wk		4-8 wk		4-12 wk		8-12 wk		0-4 wk		0-8 wk		0-12 wk		4-8 wk		4-12 wk		8-12 wk	
	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W	C-2	W
Essential amino acids:																								
Arginine											**			*		**		*		**				
Histidine											*		**	**	**	**	**	**	**	*	**			
Isoleucine										**	*		**	**		**		*						
Leucine										**	**		**	**	**	**	**	*						
Lysine	*													**	*	**	*	**	**			**		
Methionine							*		*	**	**		*	**	*	**	*							*
Phenylalanine										*	**			*		**								
Threonine														*		**		*		*				
Tryptophan[b]																								
Valine															**		**							*
Non-essential amino acids:																								
Alanine	**																		**		**			
Aspartic acid														*	**	**	**	**	*		**	**	*	*
Glutamic acid	**	**																*	**	**	**	**		*
Glycine											*		**	**	**	**	**	**		**		*		
Proline[b]																								
Serine										*	**			**	**	**	*	**	*	*	**			
Tyrosine										**	**			*		**								
Non-amino acids:																								
Ornithine													**		**		**				*			
Citrulline															**		*		**	*	*	*		
Ammonia									*		*													
Taurine									**		**		*											

*Significant difference at P<0.05

**Significant difference at P<0.01

[a]No detectable amounts of cystine

[b]No significant differences

Appendix D, Table IVa. Concentration changes of ninhydrin-reacting substances in serum of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW) for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances	Increases																								Decreases																			
	0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk				0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk			
	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW
Essential amino acids[†]																																															
Arginine[b]																																															
Histidine			•	••			•	•				••												•												••				•						•
Isoleucine[b]																																														
Leucine[b]																																														
Lysine			••	•			••				••	•																												•				•		
Methionine			••				••				••				••																											••				••		
Phenylalanine[b]																																														
Threonine		•	•	•	•	•	•	•	•	•	•	•	°				•																							•				•	•		
Tryptophan								•				•			•				•																												
Valine		••		••		••		••		••		••										•																									
Non-essential amino acids[†]																																															
Alanine		••	•	•	•	••		•	•	••	•	•					•																														
Aspartic acid[b]																																															
Cystine	••	•				•				•	•																													••				•		
Glutamic acid		•				•																																			•				•		
Glycine																									•																						
Proline[b]																																															
Serine	•	••			•	••			•	••																																					
Tyrosine		•	••			•	••			•	••																																		•			•
Non-amino acids[†]																																															
Ornithine																										•																						
Citrulline[b]																																															
Ammonia	•								•																																						
Taurine											•								•				•	•			•				•																

*Significant difference at P<0.05
**Significant difference at P<0.01
[b]No significant differences
[†]Only SW used in table headings

Appendix D, Table IVb. Concentration changes of ninhydrin-reacting substances in liver of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW)[1] diet for 4, 8 or 12 weeks, experiment III

Ninhydrin-reacting substances[a]	Increases																				Decreases																			
	0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk				0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk			
	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW
Essential amino acids:																																															
Histidine																									•	••			◦	••		••	◦	••		••		•		••				••				
Isoleucine																					◦				••			•	••	••		••	•	••		••		•		•		•							
Leucine																									•	◦	•	••	•	••	◦	••	•	••	••	••		••		•		••	•	••					
Lysine																											•	•			•	••			•	••				◦			•					
Methionine																									•		•	••	•			••	•	••	••	••		••				••		••			••	
Phenylalanine																									•		•	••	•	••	•	••		••	••	••		••		•		••		••			•	
Threonine																												•				••				••						••					
Tryptophan		••				••																																•						••				
Valine																										◦	◦	••		••	••	••		••	••	••		•	◦	◦		•	◦	◦				•	
Non-essential amino acids:																																															
Alanine					•				•				•															•				•												•			•	
Aspartic acid[b]																																															
Cystine						•	•								•	•																													•	•	
Glutamic acid																												••				••															
Glycine																									•	••	••	••		••	••	••	◦	••	••	••		••				••	◦	•					
Proline[b]																																															
Serine		••				•																																••						••				
Tyrosine																														••				••	•			••				••	◦					
Non-amino acids:																																															
Ornithine																									••	••	••	••	••	••	••	••	••	••	••	••		••				••		••					
Ammonia																												•				•				•												
Taurine																									••	◦			••	•			••	◦														

*Significant difference at P<0.05

**Significant difference at P<0.01

[a]No detectable amount of arginine or citrulline

[b]No significant difference

Appendix D, Table IVc. Concentration changes of ninhydrin-reacting substances in gastrocnemii of rats fed diets containing casein (C-1, C-2, C-3) or wheat (mod. SW) for 4, 8 or 12 weeks, experiment III

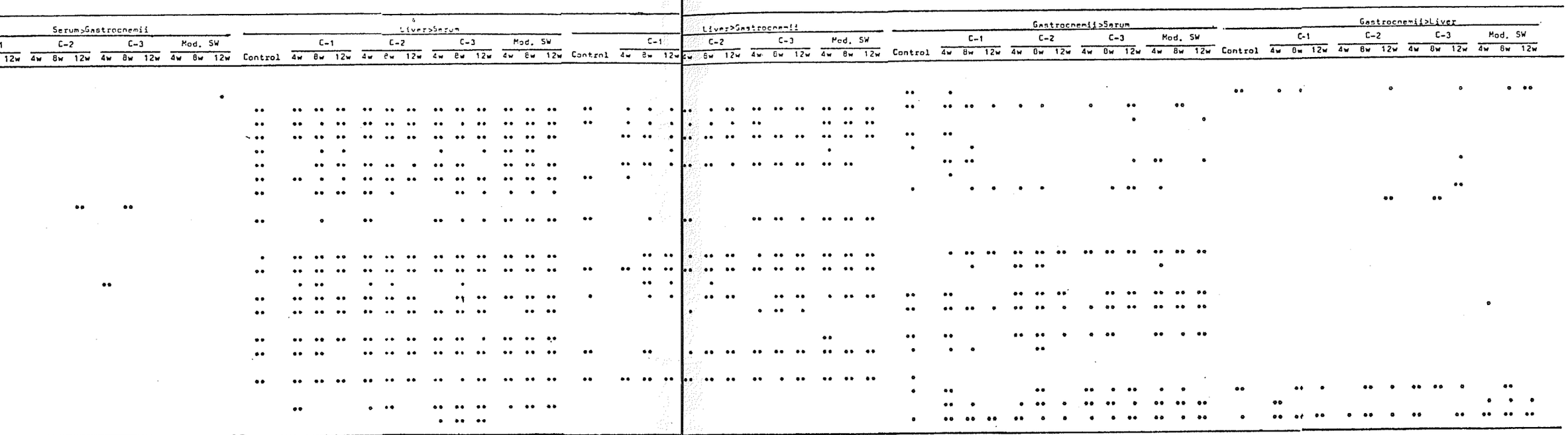
Ninhydrin-reacting substances[a]	Increases																				Decreases																			
	0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk				0-4 wk				0-8 wk				0-12 wk				4-8 wk				4-12 wk				8-12 wk			
	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW	C-1	C-2	C-3	SW
Essential amino acids:																																															
Arginine																									••	••	••		••	••	••	••	••	••	••	••					••							
Histidine																														••		••		••		••		•		••		•		••				
Isoleucine[b]																																															
Leucine[b]																																															
Lysine																											••					••				••							••				
Methionine																										•				•		•			•	•											
Phenylalanine[b]																																															
Threonine		••	••			••																										••				••		••				••	••		•	
Tryptophan[b]																																															
Valine			••																																	••						••				
Non-essential amino acids:																																															
Alanine	•	•	••		•	•			•																							•				••						••				
Aspartic acid																									••				••				••										•				
Glutamic acid																									•			••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••
Glycine																									••	••	••	••	••	••	••	••	••	••	••	••					••				•		••			
Proline			••																													•				••						••				
Serine	••				••	••			••																		•	•				•	•									••	•	•		••		
Tyrosine		••	••			••	••																										•									••	•				
Non-amino acids:																																															
Ornithine																									•	••		••	•	••		••	•	••		••								•				
Citrulline																										•	•	••		•	••	••		••	••	••								•				
Ammonia																										••	••	••	••	••	••	••	••	••	••	••					°							
Taurine								••				••				••				••				••	••	••	••		••			••		••		••		•				°							

*Significant difference at P<0,05
**Significant difference at P<0,01
[a]No detectable amounts of cystine
[b]No significant difference
[1]Only SW used in table headings

Appendix D, Table V. Comparison of ninhydrin-reacting substance concentrations in serum, liver and gastrocnemii of rats fed a casein (C-1, C-2, C-3) or wheat (W) diet for 4, 8 or 12 weeks, experiment II

Ninhydrin-reacting substances	Serum>Liver						Serum>Gastrocnemii						Liver>Serum						Liver>Gastrocnemii						Gastrocnemii>Serum						Gastrocnemii>Liver					
	C-2			W			C-2			W			C-2			W			C-2			W			C-2			W			C-2			W		
	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																																				
Arginine	*		**	*	**	**					**														*				*		**		**	*	**	**
Histidine													**	**	**	**	**	**	**	**	**	*	**		**	**	*			*						
Isoleucine													**	**	**	**	**	**	**	*	*	**	**	*												
Leucine													**	**	**	**	**	**	**	**	**	**	**	*												
Lysine										**	**									**	**	**	**	**												
Methionine													**	**	**	**	**	**	**	**	**	**	**	*		**	**		**	**	**					
Phenylalanine													**			**	**		**			**	**	*												
Threonine													**			*	*								*											
Tryptophan	*				**		*					*	**	**					**	**																
Valine								*					*			**	**	**	*			**	**	*					*							
Non-essential amino acids:																																				
Alanine													**	**	**	**	**	**		**	*	**	**		**	**	**	**	**	**						
Aspartic acid													**	**	**	**	**	**	**	**	*	**	**	**					**							
Cystine					**						**	**	**						**																	
Glutamic acid													**	**	**	**	**	**	*	**	**	**		**	*	**	**	**	**	*	**					
Glycine													*		**	**	**	**									**	**	**	**						
Proline															*	*																				
Serine													**	**	*	**	**	**					*		**	**	*	*	*	**						
Tyrosine													**		**	**	**	*	**		**	**	**				**									
Non-amino acids:																																				
Ornithine													**	**	**	**	**	**	**	**	**	**	**	**												
Citrulline		*	*	*		*																				*	*	**	*	*		**	**	**	*	**
Ammonia													**					*	**						**		*						*			
Taurine																									**	**		**	**	**	**	*	*	**	**	**

-reacting substance concentrations in serum, liver and gastrocnemii of rats fed a casein (C-1, C-2, C-3) or wheat (mod. SW) diet for 4, 8



Appendix D, Table VI. Comparison of ninhydrin-reacting substance concentrations in serum, liver and gastrocnemii of rats fed a casein (C-
or 12 weeks, experiment III

Ninhydrin-reacting substances	Serum>Liver												Serum>Gastrocnemii											
	Control	C-1			C-2			C-3			Mod. SW			Control	C-1			C-2			C-3			Mod. SW	
		4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w		4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																										
Arginine			•	•		••			••			••	••													
Histidine																										
Isoleucine																										
Leucine																										
Lysine																										
Methionine																										
Phenylalanine																										
Threonine																										
Tryptophan					•	••	••	••	••	•																
Valine																										
Non-essential amino acids:																										
Alanine																										
Aspartic acid																										
Cystine					••		••																			
Glutamic acid																										
Glycine												••														
Proline																										
Serine																										
Tyrosine																										
Non-amino acids:																										
Ornithine																										
Citrulline		••			••			•	•		••				••			•	••		••	••	••			
Ammonia																										
Taurine																										

Ninhydrin-reacting substances	Liver>Serum												Liver>Gastrocnemii											
	Control	C-1			C-2			C-3			Mod. SW			Control	C-1			C-2			C-3			Mod. SW	
		4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w		4w	8w	12w	4w	8w	12w	4w	8w	12w	4w	8w	12w
Essential amino acids:																										
Arginine	••	••	••	••	••	••	••	••	••	••	••	••	••	••	•	•	•	••	•	••	••	••	••	••	••	••
Histidine	••	••	•	••	••	••	••	••	•	••	••	••	••	••							••	••	••			
Isoleucine	••	••	••	••	••	••	••	••	••	••	••	••	••		••	••	•	••	•	••	••	••	••	••	••	••
Leucine	••		•	•				•		•	••	••												•		
Lysine	••		••	••	••	••	•	••	••		••	••	••		••	••	•	••	••	••	••	••				
Methionine	••	••	•	•	••	••	••	••	••	••	••	••	••	••	•											
Phenylalanine	••		••	••	••	•			••	•	•	•	•											•	•	•
Threonine																										
Tryptophan	••		•		••			••	•	•	••	••	••	••		•		••	••	•	••	••	••			
Valine																										
Non-essential amino acids:	•	••	••	••	••	••	••	••	••	••	••	••	••			••	••	•	••	••	•	••	••	••	••	••
Alanine	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••	••
Aspartic acid		•	••		•	•		•								••	•		•							
Cystine	••	••	••	••	••	••	••	••	••		••	••	••	•		•	•	••	••		••	••	•	••	••	••	••
Glutamic acid	••	••	••	••	••	••	••	••	••	••		••	••		•			••	••	•	••	•					
Glycine																										
Proline	••	••	••	••	••	••	••	••	••	•	••	••	••											••	••	••
Serine	••	••	••		••	••	••	••	••	••	••	••	••		•	••	••	••	••	••	••	••	••	•	•	•
Tyrosine																										
Non-amino acids:	••	••	••	••	••	••	••	••	•	••	••	••	••		••	••	••	••	•	••	••	••	••	•		
Ornithine																								•	••	
Citrulline		••			•	••		••	••	••	••	••	••											••	•	•
Ammonia								••	••	••														•	••	••
Taurine																										