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UTILIZATION OF NITROGEN BY THE ANIMAL ORGANISM

VI. FACTORS INFLUENCING NITROGEN RETENTION WHEN CERTAIN MIXTURES OF AMINO ACIDS ARE FED TO THE NORMAL ADULT ALBINO RAT

by

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INTRODUCTION

The ultimate goal of the nutritionist is the attainment, on a universal scale, of that state of nutrition which will contribute most effectively to the well-being of the individual throughout his entire span of life. Such a broad objective must be approached from many directions, by many procedures, and with insight and persistence, for its achievement depends upon the successful application of fundamental laws underlying both the sciences and the humanities.

It has become increasingly clear that the science of nutrition is interwoven intimately with all other sciences that impinge upon the life of man. In the development of its fundamental laws and principles, nutrition has borrowed heavily from the related sciences. As a result, workers in related fields are aware of the significance of the science of nutrition and are particularly well equipped to assist in the study of its varied aspects today.

The improvement of nutrition on a national scale is an undertaking of great magnitude, and nutritionists recognize that the goal itself needs to be defined in relation to the individual throughout the life cycle. Existing knowledge concerning the physical and biochemical characteristics associated with optimum nutriture of the growing and adult organism is not extensive. Even less is known concerning the nutritional demands during various phases of the reproductive cycle or in old age and the appropriate criteria for evaluation. Interrelationships between the successive phases of the life cycle are, however, apparent. Thus, the viability and the nutritional heritage of the infant are influenced decisively by the health of the mother, whose well-being is affected, in turn, by her dietary habits prior even to conception. Furthermore, accumulating evidence indicates that deprivation or overabundance of certain nutrients in early life may have far-reaching consequences. The nutritional status of an aged person, therefore, reflects not only current dietary practices but also a summation of the effects of many influencing factors during the preceding decades. Obviously, the task of defining, evaluating, and maintaining optimum nutriture in any single interval of the life cycle is exceedingly complex. It demands both intensive and extensive research coupled with careful integration of the information thus obtained.

In order to define optimum nutriture, workers in the field are attempting to develop reliable and convenient techniques for the evaluation of nutritional status and the nutritive value of foods. Thereafter, material resources must be mobilized effectively, and individuals and nations made so aware of the nature, desirability, and accessibility of excellent nutrition that they will desire to possess it. The existence of unsatisfactory food habits and of undernutrition within this nation, which has been so richly endowed with natural resources and is unsurpassed in its technological development, emphasizes the urgent necessity of applying knowledge that is already available. On the international plane, the Food and Agriculture Organization of the United Nations has undertaken collective action aimed at raising levels of nutrition and standards of living of the peoples of its 58 member nations.

Today, the delineation, qualitatively and quantitatively, of the nutritional requirements of man presents one of the most vital problems confronting nutritionists everywhere. Remarkable progress has been made toward elucidating the functional roles of all nutrients. For a time, the tremendous surge of interest that followed the discovery of the vitamins seemed to overshadow investigations related to carbohydrates, proteins, fats, and minerals. Now, however, research is going forward in respect not only to all of the individual nutrients but also to the manifold relationships among them.

The proteins represent a most important cornerstone in the basic structure describing the nutritional requirements. By virtue of the diversity, complexity, specificity, and biological significance of the proteins, researches designed to study the needs of the organism for nitrogen are particularly intriguing. Proteins are components of every living cell, being present in both the nucleus and the cytoplasm. They participate in the transport of oxygen and in the maintenance of normal osmotic pressure relationships and of acid-base balance. Moreover, they are necessary for the production of antibodies and for detoxification. Several important hormones and all enzymes that have been isolated exhibit the properties of proteins. Thus, the proteins and their constituent amino acids are of outstanding importance structurally and functionally, and their metabolic pathways interlock with those followed by carbohydrates, fats, vitamins, and minerals.

By means of recently developed techniques, insight has been gained into the amino acid composition of many types of proteins, into the intermediary metabolism of individual amino acids, and the interconversions that occur among them. Such information will contribute towards a fuller understanding of the nutritional roles of the amino acids and the specific requirements for each.

Indeed, the protein needs of man will be expressed ultimately in terms of the quantity of each amino acid that will permit optimum nutrition under the varying conditions of life. Already a tentative statement of the amino acid requirements of man for nitrogen equilibrium has been made.

Many questions of a fundamental nature must be answered, however, before a final precise statement can be made. It is known, for example, that adequate intakes of calories, of fat, of minerals, and of vitamins are essential for the retention of nitrogen. The specific relationships that are involved remain to be elucidated. Moreover, the maintenance of nitrogen equilibrium describes only the overall effect of a given diet and may not measure the need for amino acids required in the syntheses of body proteins vitally concerned in the establishment and regulation of a state of buoyant health.

The investigation herein reported represents an evaluation of the extent to which nitrogen equilibrium in the adult well nourished rat may be influenced by the kind and quantity of amino acids present in its dietary mixture. The study is directed specifically to the part played by the nonessential amino acids, the term, nonessential, being used in the sense defined by Rose and his associates in 1948. It is concerned further with the effect on nitrogen equilibrium of replacing individual nonessential acids with other nitrogenous protein components - some essential, others nonessential in character. Finally, the study undertakes to show that the method of administration of a test ration may have a profound influence on physiological response and thereby on interpretations of experimental findings. In general, it is believed that the present series of experiments provides a base that will permit a systematic approach to studies of the relative dietary importance of the individual amino acids, their specific functions in sustaining protein nutrition, and the relationships that exist between them and the other constituents of a well balanced diet.

REVIEW OF LITERATURE

This is, indeed, an era in which research dealing with the nutritive value of proteins and their constituent amino acids occupies a position of outstanding importance. Advances have been made in determining the effects of prolonged deprivation of nitrogen; in studying the functional roles of proteins in the maintenance of health and bodily integrity; in evaluating the nutritive properties of various proteins and mixtures of amino acids; in recognizing the varied factors that influence the utilization of nitrogen from endogenous or exogenous sources; in ascertaining the amino acid composition of proteins of biological importance; and in determining the nitrogenous requirements of different species. Such information contributes richly toward an understanding of the amino acid needs of experimental animals and of man.

The field is so broad, however, that it is impossible to discuss the importance of proteins in nutrition from all of these aspects in the present review. It, therefore, will be restricted to those topics that are especially relevant to the investigation herein reported, namely, (1) the metabolism of individual amino acids, (2) factors influencing the utilization of nitrogen from mixtures of amino acids, and (3) present knowledge concerning the amino acid requirements of the adult male rat.

METABOLISM OF INDIVIDUAL AMINO ACIDS

The classification of the amino acids into "essential" and "nonessential" acids was a logical outgrowth of animal feeding experiments in which it was demonstrated that whereas the omission of certain amino acids from the ration induced definite evidence of nutritive failure the removal of other amino acids neither retarded growth nor caused specific manifestations of deficiency. As defined by Rose (1938), an essential amino acid "is one which cannot be synthesized by the animal organism, out of the materials ordinarily available at a speed commensurate with the demands for normal growth". (p. 129)

An essential amino acid, however, needs to be defined in terms of physiological functions other than growth, for example, the maintenance of health in the adult and aging individual and the specialized requirements of the entire reproductive process. Moreover, since it has been demonstrated amply that the requirements of different species vary, amino acids essential in the support of these functions must be expressed in relation to a particular species. Any definition of an essential amino acid also needs amplification in respect to the state of nutriture of the organism in question.

Recent experimentation has indicated that the nature of other dietary components must be considered in evaluating the essentiality of individual amino acids. Thus, researches published after the present investigation was initiated have revealed that for optimal growth the essential amino acids in the ration must be supplemented with nitrogen from some nonessential dietary source (Rose, Smith, Womack, and Shane, 1949; Lardy and Feldott, 1950; Russell and Taylor, 1948). In addition, the vitamin content of the diet must be adequate (Rose, Oesterling, and Womack, 1948), and a positive energy balance (Rose, 1949) must be ensured. Perhaps even the relative proportions of fat and carbohydrate in the diet play an important role in determining the essentiality of specific amino acids (Hoover, 1950).

The author of this manuscript is interested particularly in the amino acids required for adult maintenance. Such needs are measured, in general, by the capacity of the organism to remain in nitrogen equilibrium when certain quantities of amino acids are fed. As already stated, this criterion reflects only the over-all effect of a given diet and may not depict accurately the amino acid requirements for synthesis of all functional proteins that are necessary for optimal nutriture. The quantities of specific amino acids that will maintain nitrogen balance represent, however, the first approximation of adult needs.

To date, information has accumulated in the literature concerning the amino acid requirements of the adult rat, the adult dog, and man.

For maintenance, the adult rat apparently needs nine amino acids, i.e., histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine (Wissler, Steffee, Frazier, Woolridge, and Benditt, 1948). Apparently arginine can be synthesized at a rate that fulfills the demands of the animal as measured by nitrogen balance. The same nine amino acids are necessary for regeneration of tissues by the adult protein-depleted rat (Frazier, Wissler, Steffee, Woolridge, and Cannon, 1947). These findings from Cannon's laboratory fail to support the earlier report by Burroughs, Burroughs, and Mitchell (1940) that the adult rat does not need a dietary source of leucine, lysine, histidine, phenylalanine, or arginine, presumably because the mature organism possesses "the residual capacity to synthesize cellular components, including the 'essential amino acids'" (Mitchell, 1947, p. 299). It seems probable that the variance in the results obtained in the two laboratories can be attributed to differences in experimental procedures, particularly since Wolf and Corley (1939) also concluded that nine amino acids were essential for the maintenance of nitrogen equilibrium by the adult rat.

Rose and Rice (1939) define the adult needs of dogs as being qualitatively the same as those of the growing rat except that arginine is not required.

Surprisingly, man can maintain nitrogen equilibrium when only eight amino acids, namely, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine are incorporated in the diet (Rose, 1949) when certain nonessential sources of nitrogen were available. Evidently neither arginine nor histidine are necessary components of the diet.

In the following pages, in an effort to understand more fully the qualities that make an amino acid "essential", available information regarding the role of each amino acid in nutritive and metabolic processes has been summarized. It must be kept in mind, however, that when several amino acids are involved in the synthesis of a particular tissue or the execution of a given function the omission of any single amino acid decreases the efficiency of the mixture. For this reason, many effects attributed to deprivation of individual amino acids later have been shown to be nonspecific. In the following discussion, only specific roles of the essential amino acids will be described.

The Essential Amino Acids

The aromatic amino acids

Tryptophane. A new era in the history of nutrition was ushered in when Willcock and Hopkins demonstrated in 1906 that a diet containing zein was incapable of maintaining life when fed to mice. However, supplementation of the ration with the single amino acid tryptophane, prolonged the survival of the animals. This discovery focussed attention upon the nutritional roles of *die Bausteine* and laid the foundation for the brilliant work of Osborne and Mendel, of Rose, and of many other investigators.

Despite its importance in the synthesis of tissues and in metabolism, tryptophane is present in relatively low concentrations in many proteins, especially muscular proteins. For this reason, Cannon (1948a) and his colleagues (Steffee, *et al.*, 1950) selected it as the basis for expressing the proportions of other amino acids required by the rat and for estimating the nutritive properties of proteins and mixtures of amino acids.

Tryptophane can be converted in the tissues to the vitamin, niacin. Thus, tryptophane as well as niacin is effective in the prevention and alleviation of pellagra in man (Vilter, Mueller, and Bean, 1949; Sarett and Goldsmith, 1950). That the amino acid was changed to niacin even when a large excess of dietary nicotinamide was available (Sarett, 1950) suggests that this conversion represents a normal rather than an alternative metabolic pathway. This interesting relationship between an amino acid and a vitamin has been reviewed ably by Krehl (1949).

Heidelberger and associates (1949a, 1949b) recently traced the steps involved in the transformation. By oxidation of the indole ring of tryptophane, kynurenine is formed. Kynurenine is then changed to niacin by way of 3-hydroxyanthranilic acid, carbon atom 3 of the indole ring becoming the carboxyl carbon of nicotinic acid. Kynurenine yields kynurenic acid also, but the latter compound is not an intermediate in the synthesis of niacin. In the absence of pyridoxine, the formation of kynurenine and kynurenic acid is disturbed and abnormal quantities of xanthurenic acid are excreted (Greenberg, Bohr, McGrath, and Rinehart, 1949). Apparently the animal organism possesses the capacity to rupture the indole ring under normal conditions, but not to synthesize it.

Spector and Adamstone (1950) have described a striking syndrome which they attributed to a deficiency of tryptophane. It was characterized by loss in weight, severe digestive disturbances, hyperexcitability, and a definite pattern of behavior. Histological changes in hepatic and muscular tissues were observed also by Adamstone and Spector (1950). The conditions depicted by these authors will be discussed fully in reporting the results obtained in Series I of the present investigation.

Williams and Elvehjem concluded recently (1949) that endogenous respiration and the activity of certain enzymes were reduced in the livers of tryptophane-deficient rats experiencing rapid losses in body weight, despite the fact that the dry weight and relative concentration of nitrogen in the liver were not affected.

Albanese and Buschke (1942) and Schaeffer and Geiger (1947) have reported that cataracts developed in rats when tryptophane was omitted from the diet. The lesion, however, probably is not specific (Sydenstricker, Hall, Bowles, and Schmidt, 1947).

Phenylalanine. That phenylalanine was essential for rat growth was demonstrated by Womack and Rose in 1934. The intermediary metabolism of phenylalanine and tyrosine has been reviewed recently by Lerner (1949). In rat liver slices the formation of ketone bodies represents a major metabolic pathway of both amino acids, two carbon atoms of the benzene ring and two carbon atoms of the side chain being utilized in this manner. The remaining four carbon atoms of the benzene ring form malic acid.

Certain interesting aspects of the biosynthesis of adrenalin from phenylalanine have been elucidated by Gurin and Delluva (1947). During the transformation, the side chain of the amino acid remains attached to the benzene ring after decarboxylation. Such stability contrasts with the rupture of the ring and the shift in position of the side chain that occur when ketone bodies are formed, as shown by Schepartz and Gurin (1949). Thus, in the synthesis of adrenalin, phenylalanine provides the basic structure, the necessary methyl group coming either directly or by way of an intermediate from other amino acids. Keller, Boissonnas, and du Vigneaud (1950) have demonstrated that the methyl group of epinephrine can be derived from dietary methionine *in vivo*.

The basic amino acids

Arginine. In respect to its classification as an essential amino acid for the growing rat, arginine occupies a mid-point position. Investigators in Rose's laboratory presented evidence early that the growing rat was capable of synthesizing arginine (Scull and Rose, 1930). Later, however, Borman and associates (1946) showed that this reaction did not occur at a rate that permitted maximum growth. Womack and Rose (1947) found that supplementation of a diet devoid of arginine, glutamic acid, proline, and hydroxyproline with either glutamic acid or proline, stimulated growth to some extent, although neither amino acid was as effective as arginine. Ramasarma, Henderson, and Elvehjem (1949) concluded that arginine improved the nutritive efficiency of a mixture of nine essential amino acids prepared in the proportions suggested by Rose when it was fed in conjunction with certain nonessential acids. It was very interesting, however, that under their experimental conditions, no increment in growth followed the inclusion of arginine when a very high proportion of glutamic acid replaced the mixture of nonessential acids.

Arginine is, therefore, an essential dietary constituent for the stimulation of maximum growth. Evidently the synthetic mechanisms of the cells cannot keep pace with the demands for rapid cell proliferation and for normal metabolic processes.

The omission of arginine from the diet did not induce a negative nitrogen balance in man (Rose, 1949), in the dog (Rose and Rice, 1939), in the female rat (Burroughs, Burroughs, and Mitchell, 1940), or in the adult male rat (Frazier et al., 1947; Wissler et al., 1948). On the other hand, the regeneration of serum proteins did not proceed normally in the hypoproteinemic dog (Madden, Carter, Kattus, Miller, and Whipple, 1943) unless arginine was present. Possibly such an acute state resembles growth in respect to the demands for rapid synthesis of tissue. Furthermore, the prolonged omission of arginine led to severe anatomical lesions of the testis of young rats (Holt, 1944) but not of adult rats (Frazier et al., 1947). In man, Holt, Albanese, Shettles, Kajdi, and Wangerin (1942) observed that nitrogen balance was maintained when arginine was omitted from the diet but that within 10 days spermatogenesis was greatly impaired. Moreover, the response to the incorporation of the missing amino acid in the diet was gradual. It is evident, therefore, that the capacity of certain species to maintain nitrogen balance in the absence of a dietary supply of arginine does not preclude the possibility of a serious break in metabolic processes. In this respect, Fischer's finding (1948) that arginine was second in importance in the metabolism of a pure culture of tissue cells in vitro may be very significant.

Arginine participates in the synthesis of urea. In addition, its guanidine group supplies the nitrogen atom for the amidine moiety of creatine (Bloch and Schoenheimer, 1941). Since creatine is irreversibly transformed to creatinine (Bloch and Schoenheimer, 1939), a small but steady loss of arginine occurs by this pathway. Shemin and Rittenberg (1945) showed that arginine was not reversibly deaminated and aminated to any considerable extent. Instead, the keto acid arising from its deamination was aminated to form ornithine which then yielded proline.

Histidine. The distinctive feature of histidine is the imidazole ring which is apparently very stable. When Schoenheimer, Rittenberg, and Keston (1939) fed isotopic ammonia or tyrosine to rats, they recovered N^{15} from only the α- amino group of histidine isolated from the tissues. The isotopic studies thus confirmed the earlier growth studies of Cox and Rose (1926) who demonstrated that histidine, which is essential for growth (Rose and Cox, 1924), could be replaced effectively by β-4-imidazole lactic acid but not by other derivatives which lacked an intact imidazole ring.

The hypotheses of certain investigators that histidine is a specific precursor of arginine and the purines (Ackroyd and Hopkins, 1916), of glutamic acid (Edlbacher and Neber, 1934), and of creatine (Steensholt, 1943) were not supported by the isotopic studies of Tesar and Rittenberg (1947). They found that nitrogen from the imidazole ring was not selectively utilized, but was redistributed in a manner similar to that of nitrogen derived from nonspecific precursors such as ammonium salts or other amino acids.

Although the omission of histidine from the diet of human subjects did not cause a negative nitrogen balance in the studies reported by Rose in 1949, the presence of an abnormal chromogen in the urine of histidine-deficient subjects (Albanese *et al.*, 1944) suggested that a metabolic disturbance is induced thereby. Sebrell (1949) has indicated also that histidine is especially important for blood regeneration as, indeed, the high concentration of the amino acid in hemoglobin would suggest.

Lysine. The essentiality of lysine for growth was demonstrated in 1914 by Osborne and Mendel who obtained satisfactory growth in rats by supplementing a diet that contained zein with both lysine and tryptophane. Lysine, in contrast to tryptophane, is present in high concentration in almost all the tissues of the rat (Dunn *et al.*, 1949).

Lysine is one of the essential amino acids that cannot accept nitrogen from the metabolic pool although it can contribute thereto (Weissman and Schoenheimer, 1941). Neither can it be attacked by amino acid oxidase unless the ϵ-amino group is acetylated first (Neuberger and Sanger, 1943, 1944a). The α-nitrogen-substituted derivatives cannot be substituted for lysine in the diet (Neuberger and Sanger, 1944b).

An explanation of the failure of lysine to participate in reversible transamination reactions *in vivo* may be found in the recent studies of Borsook and associates (1948a, 1948b), who traced the steps involved in the degradation of lysine in guinea pig liver homogenate. They postulated that first the ϵ-amino group is removed to form α-aminoadipic acid. This acid then is oxidatively deaminated to α-ketoadipic acid, from which α-ketoglutaric acid arises by oxidative decarboxylation. The conversion of lysine to α-aminoadipic acid before it yields its α-amino nitrogen thus prevents its reamination to form lysine.

When fed to rats, α-aminoadipic acid, the product of the first step in the catabolism of lysine, not only failed to promote growth (Stevens and Ellman, 1950) but caused severe diarrhea (Geiger and Dunn, 1949). The only related compound thus far tested that seems capable of stimulating growth to any extent when added to a lysine-deficient diet is *homoarginine* (α-amino-ϵ-guanidino-n-caproic acid, Stevens and Bush, 1950).

In man, the absence of dietary lysine leads not only to a prompt and severely negative nitrogen balance (Rose, 1949), but also to a high urinary excretion of non-ketonic organic acids accompanied by nausea, dizziness, and hypersensitiveness to noise (Albanese, Holt, and Frankston, 1943).

That the normal pigmentation of the feathers of turkey poults depends upon the concentration of dietary lysine has been reported by German, Schweigert, Sherwood, and James (1949). To date, such a relationship has not been reported for other species, however.

The monoaminomonocarboxylic amino acids

In the nutrition of all mammalian species so far investigated, isoleucine, leucine, threonine, and valine play vital roles. Although they are structurally simple and similar, the organism is unable to synthesize them, at least to a significant extent. Prompt and profound nutritive failure invariably follows the omission of any member of the group from the diet. Their structural significance is indicated by the high concentration of each amino acid that is present in liver, muscle, and carcass of the rat (Dunn *et al.*, 1949). In addition, evidence is accumulating concerning their participation in metabolic processes, especially in relation to the lipids.

Isoleucine. The present literature carries little information concerning isoleucine, beyond evidence that it is essential for the growth of the young rat (Womack and Rose, 1936), and for the maintenance of nitrogen equilibrium in the adult rat (Frazier *et al.*, 1947; Wissler *et al.*, 1948), in the dog (Rose and Rice, 1939), and in man (Rose, 1949). Its concentration in hemoglobin is relatively low, as shown by Albanese (1945) and by Hegsted, McKibben, and Stare (1944).

Insight into the biosynthesis of isoleucine has been gained from the recent research of Adelberg and Tatum (1950), who utilized an isoleucine-requiring strain of Neurospora as the test organism. Formation of the branched chain involved the direct participation of acetate in a coupling with a four-carbon compound that was not formed directly from two-carbon fragments.

Isoleucine may be either glucogenic or ketogenic (Butts, Blunden, and Dunn, 1937) under different conditions.

Leucine. Dunn and colleagues (1949) found that leucine and lysine are the two essential amino acids found in the highest concentration in muscular tissue, liver, and carcass of the rat.

L-leucine enters swiftly into the metabolic pool as revealed by the entrance of the amino acid as such into various tissues and the transfer of its nitrogen atom to other amino acids (Schoenheimer, Ratner, and Rittenberg, 1939). Recently, Tanenbaum and Shemin (1950) discovered that leucine, like glutamic acid, was capable of transamination with ketoglutaric acid although at a slower rate.

In studying the ketogenic property of leucine, Coon and Gurin (1949) concluded that the α- and β-carbon atoms split off as a two-carbon intermediate, which then condensed with a similar unit to form acetoacetate. The isopropyl group also yielded a two-carbon intermediate which acted almost exclusively as an acetylating unit in the formation of acetoacetate (Coon, 1950).

A link between this amino acid and the synthesis of sterols was found by Zabin and Bloch (1950). They reported that isopropyl portion of isovalerate, an intermediate in the catabolism of leucine, was a more efficient source of carbon atoms for the synthesis of cholesterol than was acetate. Both compounds were, however, equally effective in the formation of acetyl groups and fatty acids.

Threonine. The discovery in 1935 by McCoy, Meyer, and Rose of the hydroxy-acid, threonine, and its synthesis in 1937 (West and Carter) laid the foundation for the brilliant investigations in Rose's laboratory of the amino acid requirements of rats and finally of man (Rose, 1949).

Threonine may be especially important for the synthesis of hemoglobin in the rat (Hall, Bowles, and Sydenstricker, 1949), and of serum proteins. After prolonged deficiency of this amino acid, an edematous fluid was present in the chest of young rats that was not observed when any of the other essential amino acids was missing.* A similar response to a threonine-deficient diet was reported by Bauer and Berg (1943) who studied the amino acid needs of growing mice.

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*From oral report by W. K. Hall at the meeting of the Federation of American Societies for Experimental Biology, at Detroit, 1949.

Isotopic studies have shown that threonine, like lysine, does not accept nitrogen from the metabolic pool (Meltzer and Sprinson, 1950). Animal feeding tests have indicated also that α-aminobutyric acid, which is formed by the deamination of threonine, does not support growth of rats fed a threonine-deficient diet (Armstrong and Binkley, 1949; Fromageot and Clauser, 1949). For this reason, partial deprivation of threonine might be more critical than reduction in the dietary supply of leucine, isoleucine, or valine. The interconversions which occur among the latter amino acids (Shemin, 1950), even though limited in extent, would permit the animal to adjust to a slight reduction in the quantity of leucine or isoleucine or valine.

Meltzer and Sprinson (1950) observed that in the rat approximately one-fourth of a given quantity of administered threonine was converted to acetate. A smaller fraction of it was transformed to glycine, which was used subsequently in the synthesis of serine.

Singal and coworkers reported in 1950 that when a diet deficient in threonine was fed, the turnover of phospholipids in the nucleoprotein fraction of the liver was depressed significantly. The condition was reversed by the administration of the missing amino acid.

Thus, threonine appears to function in important metabolic reactions as well as in the synthesis of essential tissues.

Valine. With the possible exception of tryptophane, valine is the only amino acid whose omission induces clear-cut deficiency symptoms. In growing rats, Rose and Eppstein (1939) observed a lack of coordination of the muscles, especially in the hind legs, a tendency to rotate in a circular manner, and a staggering gait. In addition to these gross symptoms, Ferraro and Roizin (1947) reported microscopic changes in the nerve cells of the spinal cord and degenerative changes in the muscles of adult rats.

Methionine

So complex are the metabolic roles of methionine that du Vigneaud (1948) wrote as follows of its methyl group alone:

> This vital grouping plays a role in nitrogen metabolism, in sulfur metabolism, in fat metabolism, and in carbohydrate metabolism. Thus, it can be seen that the process of transmethylation--or migration of methyl groups--truly transects all of these metabolic pathways. (p. 134)

Since 1948, reports emanating from many laboratories have revealed that methionine plays outstanding roles in metabolism, over and beyond its ability to supply the vital methyl group.

In view of the capacity of the animal organism to synthesize many complex chemical compounds, it is indeed surprising that under certain conditions a structure as simple as the methyl radical may become a dietary essential. That the organism may possess inadequate facilities to synthesize it at a rate compatible with the maintenance of health has been demonstrated. For example, Womack, Kemmerer, and Rose (1937) showed the importance of dietary methionine; du Vigneaud, Chandler, Moyer, and Keppel (1939) and Timmonds *et al.* (1943) the role of choline when administered simultaneously with homocystine.

In the biosynthesis of methionine, homocysteine apparently is not methylated directly. The close parallel between the presence of choline oxidase in animal tissues and the utilization of methyl groups suggested to Dubnoff (1949) that oxidation of the alcohol group of choline was necessary. Muntz proposed that choline must be transformed to betaine before the methyl group can be transferred (1950).

The high degree of activity of the thetins and the presence of specific transmethylases (Dubnoff and Borsook, 1948) suggest that these substances play a specific role in the biosynthesis of methionine. The structural criterion that was found by Maw and du Vigneaud (1948) and by Dubnoff and Borsook (1948) to be associated specifically with methyl transfer was the presence, in the molecule of the potential donor, of a methyl group or groups directly attached to an onium pole, i.e., a nitrogen or sulfur atom to which an additional methyl group is coordinated.

In contrast to earlier beliefs, data obtained in the current year reveal that the methyl group may be synthesized in vivo under certain conditions. Stekol and Weiss (1950) reported that rats at least 30 days old grew when they were fed amino acid-containing diets that were devoid of methyl groups, provided that vitamin B_{12} and homocystine or homocysteine were present. Growth of rats fed 0.8 per cent of homocystine or homocysteine and 15 mcg. of vitamin B_{12} daily equalled that obtained when 1.4 per cent of methionine was present. The rats did not survive if they were less than 30 days old when the experiment was initiated, and the response of both groups was inferior to that associated with the ingestion of 0.8 per cent of homocystine and 0.5 per cent choline.

The finding of C^{14} in the methyl groups of methionine and choline after methyl-labeled acetone was administered to rats suggested to Sakami (1950) that synthesis of labile methyl groups occurred via formate. Furthermore, Welch and Sakami (1950) demonstrated that methionine was synthesized by rat liver slices when C^{14}-formate, homocysteine, dimethylaminoethanol, folic acid and vitamin B_{12} were provided. These data indicate, therefore, that the conversion of labile methyl groups of methionine and other donors to formate (Mackenzie, 1950; Siekevitz and Greenberg, 1950) is indeed reversible.

That the methyl group is transferred from methionine to choline and creatine as an intact unit was demonstrated by Keller, Rachele, and du Vigneaud in 1949. Evidently the process is a cyclic one in which some steps, if not all, are irreversible (Dubnoff and Borsook, 1948), and in which specific methyl donors may exist for each methyl acceptor. Mackenzie and du Vigneaud (1950) noted also that the methyl groups of administered creatine and creatinine were not oxidized to any appreciable extent in the intact rat, whereas the methyl groups of methionine (Mackenzie and du Vigneaud, 1950) and of betaine and the thetins (Ferger and du Vigneaud, 1950) were oxidized readily.

By feeding L-methionine containing a high concentration of C^{14} in the methyl group, Mackenzie, Chandler, Keller, Rachele, Cross, and du Vigneaud (1949) studied the extent and rate of oxidation and the distribution of the methyl group. They concluded that the methyl group participates actively "in both degradative (oxidative) and synthetic (transmethylation) reactions" (p. 103). After 52 hours, 37 percent of the methyl carbon administered to a fasting rat was located in the methyl groups of creatine and choline, 44 per cent of the newly synthesized creatine having been formed from administered methionine. Also, the protein-bound methionine in the carcass represented approximately 40 per cent of the administered methyl carbon. The intense oxidation of the methyl group was accompanied by the appearance of C^{14} in all major tissues and organs, the concentration being particularly high in liver, kidneys, and adrenal glands.

The complete oxidation of methionine to carbon dioxide and water proceeds in two phases: a rapid initial period of assimilation followed by a period of equilibrium in which a low and relatively constant rate of oxidation continues (Mackenzie, Rachele, Cross, Chandler, and du Vigneaud, 1950). When the quantity of radiomethyl was doubled, the percentage of methyl groups oxidized during the period of assimilation was increased ninefold, whereas the rate of oxidation during the period of equilibrium was virtually unaltered.

Fromageot and Clauser (1949) suggested that methionine, like lysine and threonine, is irreversibly deaminated, since administration of α-aminobutyric acid, which is formed when either methionine or threonine is deaminated, failed to stimulate growth if methionine or threonine was omitted from the diet.

The demonstration by Keller, Boissonnas, and du Vigneaud (1950) that methionine participates in the synthesis of epinephrine may prove particularly significant, especially in elucidating the metabolic functions of the amino acid and its relationships with other amino acids.

Treadwell (1945) proposed that the total methionine requirement of growing rats could be divided almost equally into a need for growth and another for lipotropic activity. The metabolic relationships between methionine, choline, and cystine have been studied by Treadwell also (1948a, 1948b).

When a methionine-deficient diet was force-fed to growing rats, the expected negative balance did not occur. The only outward manifestations were bleeding of the feet and mouth, fatty livers, and very low liver xanthine oxidase activity (Williams <u>et al</u>., 1950).

In 1947, Brush, Willman, and Swanson demonstrated that when added alone to a protein-free diet offered to adult rats, methionine exerted a marked body-sparing action which exceeded that associated with feeding any other individual amino acid. In fact, methionine alone was as effective in reducing the excretion of nitrogen in the urine as was an equivalent quantity of nitrogen supplied in the form of egg proteins. It was more efficient than choline when both provided 4 mg. of nitrogen. Simultaneously, Allison, Anderson, and Seeley discovered that methionine reduced the excretion of nitrogen by normal adult dogs that were fed either protein-free diets or diets containing casein or eggwhite, the quantity of urea nitrogen being reduced notably. When the caloric intake was reduced to one-fourth of the normal intake, methionine was stikingly beneficial in depressing the urinary excretion of nitrogen (Stevenson, Swanson, Willman, and Brush, 1946; Hoover, 1950), especially when the diet was devoid of fat.

The Nonessential Amino Acids

The term "nonessential amino acid" was selected originally to designate those amino acids which the body can synthesize and thus to distinguish them from the "essential amino acids" which must be supplied pre-formed in the diet. The connotation is unfortunate, however, since many of the so-called nonessential amino acids perform specific, diverse and vital functions in the physiological economy. Under certain conditions, they may become a truly limiting factor in the diet.

The dicarboxylic amino acids

Both aspartic and glutamic acids play outstanding roles in transamination, glutamic acid being somewhat more active than aspartic acid. In fact, Shemin suggested in 1950 that glutamic acid formed by reductive amination is the key amino acid for nitrogen transfer to all other keto acids that may be formed via transamination. When labeled acetate was fed to rats, a major portion of the tagged carbon was located in the dicarboxylic amino acid fraction (Anfinsen _et al._, 1949; Greenberg and Winnick, 1949).

The central position of glutamic acid in the synthesis of urea and in the linking of the tricarboxylic acid and urea cycles was described by Ratner in 1949. In addition, Waelsch (1949) has postulated that the glutamic acid-glutamine system may aid in regulating the concentration of metabolites entering the tricarboxylic acid from various pathways, by ensuring adequate reserves of ketoglutaric acid. Glutamine itself represents a store of metabolically inert glutamic acid and ketoglutaric acid that is as easily mobilized by enzymatic action as glycogen is transformed to glucose.

The therapeutic effect of glutamic acid in epilepsy has been demonstrated by Waelsch (1949) and by Richter and Dawson (1949). Also, the administration of glutamic acid permitted the return of consciousness to patients in insulin coma at a lower level of blood sugar than did glucose (Mayer-Gross and Walker, 1949). The mechanisms involved in such relationships are unknown.

The ability of glutamic acid to replace arginine partially in the diet of growing rats has been discussed. Frost (1949) and Ramasarma, Henderson, and Elvehjem (1949) concluded that it could fulfill the functions of all or almost all other nonessential acids. Under the experimental conditions which they imposed, glutamic acid could fulfill the functions of all (Frost, 1949) or almost all (Ramasarma, Henderson, and Elvehjem, 1949) of the other nonessential acids.

Wu and Rittenberg (1949) concluded that aspartic acid was deaminated so rapidly that its amino group metabolically resembled ammonia instead of other natural amino acids in respect to the quantities of urinary ammonia and urea excreted by the rat.

The heterocyclic amino acids

The specific roles of proline and hydroxyproline have not been elucidated completely. Stetten and Schoenheimer (1944) demonstrated that proline and ornithine are interconvertible. Proline also may be converted irreversibly to hydroxyproline and thence to glutamic acid. These findings were confirmed by Taggart and Krakaur (1949), who concluded that the cyclophorase system of enzymes catalyzed the complete oxidation of proline to carbon dioxide, water, and ammonia by way of glutamic acid and the tricarboxylic acid cycle. Proline, like glutamic acid, can replace arginine at least partially for the growth of the rat

(Womack and Rose, 1947).

Hydroxyproline is present in relatively high concentration in collagen and gelatin, but Womack and Rose (1947) found that its addition to the diet did not enhance growth, even depressing it when certain concentrations were incorporated.

The monoaminomonocarboxylic amino acids

Alanine. Although no specific functions of alanine in the synthesis of tissues have been reported in the literature, its relationship to the metabolism of both carbohydrates and fats has been indicated. When Schofield and Lewis (1947) studied the glyconeogenetic properties of certain amino derivatives of propionic acid, they concluded that for the young rat, DL- and D-alanine and DL-serine were more effective than L-alanine, although all of them stimulated glycogen formation to some extent. The transformation of L-alanine to acetic acid by way of pyruvic acid was postulated by Anker in 1950.

Glycine and serine. The structurally simple amino acid, glycine, plays vital roles in the synthesis of tissues and in the regulation of metabolic processes that apparently cannot be performed by other amino acids. For example, it can be transformed to ethanolamine and thence to choline, probably by way of serine (Levene and Tarver, 1950), an amino acid which yields ethanolamine when it is decarboxylated (Stetten, 1942). Glycine is necessary for the synthesis of glutathione and glycocholic acid, both of which are important metabolites. Moreover, the isotopic studies of Bloch and Schoenheimer (1941) revealed that glycine is the only natural amino acid that can serve as a direct precursor of the sarcosine moiety of creatine.

The two carbon atoms of glycine perform specific functions. Both enter into the structure of hemoglobin, the α-carbon atom being utilized in the methene bridges (Wittenberg and Shemin, 1950) as well as in the four pyrrole rings. The carboxyl carbon atom participates in the synthesis of globin(Grinstein, Kamen, and Moore, 1949) but not of hemin (Radin, Rittenberg, and Shemin, 1950).

Close relationships between glycine and carbohydrate metabolism were indicated by the observations of Mayer-Gross and Walker (1949) and by Johlin (1949) that the effect of insulin was enhanced by glycine; also glycine was transformed to glycogen (Barnet and Wick, 1950), presumably by way of serine and pyruvic acid.

The interconversion of glycine and serine (Shemin, 1946; Sakami, 1949a) is both rapid and extensive. In fact, Goldsworthy, Winnick, and Greenberg (1949) concluded that they exist in "a rapid biochemical equilibrium" in the liver. Evidently serine was formed by the condensation of one intact molecule of glycine with the α-carbon atom of a second molecule of glycine (Siekevitz and Greenberg, 1949). Sakami postulated that the β-carbon atom of serine might arise from either the α-carbon atom of glycine (1949a) or the methyl group of choline (1949b). Shemin (1946) noted that serine was converted to glycine by the splitting off of its β-carbon atom.

The close relationship of glycine and serine to lipid metabolism emphasizes the existence of metabolic pathways that are involved in the synthesis and catabolism of both fats and proteins. Administered serine was incorporated as such in phosphatides (Stetten, 1942), and was present in high concentration in brain cephalin (Folch, 1949). Altman showed in 1949 that the α-carbon atom of glycine entered into the structure of fatty acids, especially saturated acids. Moreover, Sprinson (1949) found upon feeding to the rat glycine labeled only in the methylene group that acetic acid was formed which contained isotopic carbon in both positions. The synthesis of acetate may represent, therefore, one of the important roles of glycine, and one which conceivably might be depressed significantly if the dietary intake of nitrogen were inadequate.

On the other hand, Arnstein and Neuberger (1949) reported that acetate given to rats yielded glycine, the carboxyl carbon of acetate appearing exclusively in the carboxyl group of glycine. Medes (1950) has discussed mechanisms involving the tricarboxylic acid cycle, by means of which these interconversions may occur.

That both glycine (Shemin and Rittenberg, 1947) and serine (Elwyn and Sprinson, 1950) participate in purine metabolism was revealed by the presence of labeled nitrogen and carbon in specific positions of uric acid after these amino acids were administered.

The origin of the carbon chain of all of the nonessential amino acids except glycine and proline has been traced (Shemin, 1950). Anker proposed in 1948 that pyruvic acid is converted to glycine by way of serine. More recently, however, Shemin (1950) concluded that it is difficult to ascertain whether ketoglutaric acid or pyruvic acid is more closely associated with the immediate precursor of glycine or serine.

The sulfur-containing amino acids

Although methionine can replace cystine entirely for rat growth (Womack, Kemmerer, and Rose, 1937), Womack and Rose demonstrated in 1941 that cystine stimulated growth only in the presence of suboptimal quantities of the essential sulfur-containing amino acid. They found also that approximately one-sixth of the total requirement for methionine could be replaced by cystine.

That cystine *per se* is essential in cell metabolism was discovered by Fischer (1948). In evaluating the requirements of cultures of tissue cells *in vitro*, he ranked cystine as the most important single amino acid. Nevertheless, in the young rat excessive quantities of cystine may induce a distinct antilipotropic effect and finally death (Treadwell, 1948a). Hess recently reported (1949) that both cystine and cysteine were glycogenic whereas methionine was not.

In the biosynthesis of cystine, serine provides the carbon chain (Stetten, 1942; Binkley and du Vigneaud, 1942) and methionine the sulfur atom (Tarver and Schmidt, 1939; du Vigneaud, Kilmer, Rachele, and Cohn, 1944).

Tyrosine

Tyrosine can be synthesized readily *in vivo* from phenylalanine, which can replace it entirely in the diet of the growing rat (Rose and Womack, 1934). Indeed, Moss and Schoenheimer postulated in 1940 that the formation of tyrosine is an automatic and noninterruptable process. The participation of tyrosine *per se* in metabolism is indicated by its capacity to reduce the total requirement for phenylalanine. For example, Rose and Womack (1946) found that the quantity of phenylalanine needed for maximum growth was reduced to one-half when an adequate amount of tyrosine was included in the ration. Moreover, the first step in the synthesis of adrenaline involves the conversion of phenylalanine to tyrosine.

On the other hand, evidence that both amino acids play specific roles is available. Miller and Reiman (1943) noted that in the differentiation of rabbit ova tyrosine was a more effective stimulant to intracellular metabolism than was phenylalanine. Also, Ferger and du Vigneaud reported in 1949 that growth inhibition induced by thienylalanine could be reversed by phenylalanine but not by tyrosine. For this reason, Garst, Campaigne, and Day (1949) postulated that the antagonist competes with phenylalanine for an essential enzyme system distinct from that which converts phenylalanine into its para-hydroxy derivative.

Tyrosine, like phenylalanine, can be degraded to two intact four-carbon units, one of which is a ketone body and the other malic acid (Lerner, 1950). Weinhouse and Millington (1949) have confirmed earlier reports that p-hydroxyphenylpyruvic acid and homogentisic acid are intermediates in the formation of acetoacetic acid.

FACTORS INFLUENCING THE UTILIZATION OF NITROGEN WHEN MIXTURES OF AMINO ACIDS ARE FED

The utilization of nitrogen by the animal organism is a complex process that interlocks with the metabolism of many other nutrients. In the development of any experiment designed to investigate any aspect of protein utilization, many factors must be given cognizance, particularly in relation to the selection of test animals, and the formulation and administration of experimental rations. Discrepancies in the results obtained in different laboratories may be traced frequently to divergences in experimental procedures.

Species, Age, and Sex

Species

Although many biochemical processes have been found to be fundamentally similar in microorganisms, higher plants, and mammals, variations exist among species that make generalizations hazardous and necessitate the examination of experimental evidence secured from the particular species under consideration before final conclusions can be drawn. A few illustrations will suffice to emphasize the importance of this point.

For example, Sprinson and Rittenberg (1949b) have found that the original observations of extreme instability of cellular proteins, which gave rise to the concept of the dynamic state, are more typical of the rat than of the human being since the rates of protein synthesis per kilo of body weight were 1.0 and 0.2 gm. of nitrogen per day for the rat and the human being, respectively. Also, a larger fraction of the muscular proteins of man than of the rat were relatively inert metabolically.

Tatum (1949) has summarized reactions that are common to microorganisms and animals. Both groups exhibit the ability to convert tryptophane to niacin. On the other hand, while *Neurospora* can utilize α-aminoadipic acid as a precursor of lysine (Mitchell and Houlahan, 1948), the rat is unable to do so (Geiger and Dunn, 1949). Histidine is not needed for nitrogen equilibrium in man (Rose, 1949), but it is essential for the rat (Wissler *et al.*, 1948), and for the dog (Rose and Rice, 1939). Cox and associates (1947) discovered that the human requirement for sulfur-containing amino acids is lower than that of the rat and dog, presumably because of smaller demands for the synthesis of hair. Arginine, in contrast, is apparently dispensable for all mammalian species in adult life. In 1948, Almquist reviewed the amino acid requirements of avian species, which differ in several respects from those of the rat, dog, and man. Glycine, for example, is a dietary essential for the chick.

Differences in the ability of the mouse, the rat, the dog, and man to invert the unnatural isomers of certain amino acids have been summarized by Berg (1942) and by Albanese (1947).

Maddy and Elvehjem (1949) demonstrated that the mouse cannot utilize inorganic nitrogen efficiently for growth. On the other hand, the rat appeared to be able to do so (Rose, Smith, Womack, and Shane, 1949; Lardy and Feldott, 1950).

Even strains among a given species may differ in respect to the utilization of amino acids, as illustrated by the finding of Rutman, Dempster, and Tarver (1949) concerning methionine. The rate of uptake of this amino acid by liver tissue from one strain was four times as rapid as from another strain. The authors therefore emphasized the importance of avoiding genetic variation in the selection of experimental animals. Since strains may differ also in their requirements for vitamins (Light and Cracas, 1938), the possible effects of genetic variation upon experimental results are indeed intriguing.

Age

Arginine provides the best single illustration of the capacity of an adult animal to synthesize an amino acid at a rate that will fulfill its requirements in contrast to the inability of the growing animal to do so. Differences in the rate of uptake of amino acids between fetal and adult tissues are suggestive also (Winnick, 1950).

Robscheitt-Robbins and Whipple (1949a,b) noted that young and adult dogs tended to utilize certain test proteins differently.

Sex

Sometimes little consideration is given to the distribution of male and female rats among test groups employed in studies of protein metabolism. It seems reasonable that the requirement of the male rat for basic amino acids, especially arginine, should be greater at puberty than that of the female rat. Also, male rats are more susceptible than are female rats to a deficiency of biotin, which is related closely to several phases of protein utilization (Okey, Pencharz, and Lepkovsky, 1947).

Recently, Farber and colleagues discovered that fatty livers were induced more readily by the administration of ethionine to female rats of a given age and weight than to male rats (1950). Moreover, castrated mature rats developed fatty livers as quickly as female rats but became resistant if pellets of testosterone were implanted. Shipley, Chudzik, and György (1948) also noted that when a diet low in lipotropic factors was fed, adrenalectomy caused a significant reduction of fat in the livers of male but not of female rats. Advancing knowledge of the influence of specific hormones on the utilization of nitrogen will lead undoubtedly to an understanding of the responses of both sexes to a specific dietary treatment. Such knowledge will become increasingly necessary as attempts are made to elucidate requirements during the reproductive cycle.

Nutritional History

Data accumulated from many angles emphasize the influence of the diet consumed during the pre-experimental period upon the utilization of nitrogen from any given source. Such findings are pertinent to any consideration of nutritional needs in population groups, in the clinic, or in the laboratory.

The concept of a labile protein pool of considerable magnitude, originally advanced by Whipple (1942), has been reaffirmed by many investigators. The reserves comprise the cytoplasm of the liver, plasma albumin and gamma globulin, hemoglobin, and finally, structural tissues.

The influence of protein deprivation upon the nutriture of the organism and its capacity to utilize nitrogen has been studied thoroughly. The liver is unusually sensitive to changes in dietary protein. At reduced intakes, nitrogen is lost rapidly and extensively, the cytoplasm being especially labile (Campbell and Kosterlitz, 1948a). The withdrawal of pentosenucleic acid from the microsomatic fraction (Muntwyler, Seifter, and Harkness, 1950) may be especially significant in view of the conclusion by Borsook and colleagues (1949) that a synergism exists between microsomes and mitochondria in respect to the uptake of certain amino acids. When the dietary protein is lacking, the production of antibodies and the resistance to infection and intoxication is lowered (Benditt _et al._, 1949). Allison, Anderson, and White (1949) concluded that peptic digestion is depressed in protein undernutrition, since the depleted dog is less able than the normal dog to digest raw egg white. The concentration of certain enzymes in the liver also is decreased if protein is restricted in the diet (Williams and Elvehjem, 1949; Seifter _et al._, 1948). Necrotic changes retarded the regeneration of hepatic tissues by partially hepatectomized depleted rats (Gurd and Vars, 1949). During prolonged severed deficiency of protein, however, Wang and associates (1949) concluded from chemical and cytological data that the animal tends to conserve hepatic tissue. In addition to widespread alterations in the composition and structure of the liver when a protein-free diet is fed, edema and anemia develop also.

In view of such fundamental changes, a test animal that has been fed a diet devoid of protein might be expected to utilize nitrogen in a different manner than one that has ingested a diet adequate in respect to protein prior to the initiation of the experiment. Evidence from several laboratories has supported this hypothesis. Hoffman and McNeil (1949) and Allison (1948) found that the protein-deficient human subject and the dog, respectively, required smaller quantities of nitrogen to attain equilibrium than did those with greater reserves. Likewise, Mukhopadhay (1949, p. 59) demonstrated that partially depleted and well-nourished rats required 325 and 575 mg. of nitrogen, respectively, for nitrogen equilibrium when dehydrated defatted egg powder provided the protein of the diet. When estimated by means of the normal rat, the biological efficiency of egg proteins was 108, in contrast to 120 when depleted animals were used. Also, at all concentrations of dietary protein between 1.6 and 25.0 per cent, the undernourished animals utilized egg proteins more efficiently than did the well-nourished ones when nitrogen balance was used as the criterion. Moreover, the depleted animals increased their hepatic stores of nitrogen at a regular rate until 6.8 per cent of protein was supplied, after which a plateau was reached. In contrast, the well-nourished rats stored the same quantity of nitrogen in their livers whether 3.0 or 18.0 per cent of protein was offered.

The relative requirements of normal and depleted adult rats for individual essential amino acids have been expressed by Benditt _et_ _al_.(1950) and Steffee _et_ _al_. (1950). They will be discussed in a later section of this review.

Even the quality of a protein-containing diet in the pre-experimental period may alter the response of the test organism, since dietary proteins differ strikingly in their capacity to stimulate the synthesis of specific types of cellular protein. For example, Robscheitt-Robbins and Whipple (1949a, 1949b) demonstrated that egg proteins and lactalbumin favored the synthesis of plasma proteins whereas beef muscle augmented the production of hemoglobin markedly; casein was very effective and peanut flour ineffective in respect to the regeneration of both types of protein.

That the organism makes important hormonal adjustments in response to dietary modifications has been postulated by Samuels (1946), who concluded that adaptation to a high-protein diet or to utilization of endogenous nitrogen during fasting involved increased gluconeogenesis. When the protein intake was limited, the production of certain hormones by the pituitary gland was restricted and the demand for protein and calories was lowered thereby.

It is apparent, therefore, that the nutritional history of an animal may alter profoundly the manner and extent of utilization of protein, whether intact proteins or mixtures of amino acids are provided. The importance of standardization of the test organism cannot be overemphasized.

Total Quantity of Nitrogen Ingested

The influence upon the retention of nitrogen of the total quantity of dietary nitrogen consumed merits consideration. Two methods of expressing the nutritive value of a protein that are in current use, namely, nitrogen balance index (Allison, 1949) and biological efficiency (Swanson, 1948), are based on the existence of a linear relationship between nitrogen balance and absorbed nitrogen in the region of negative and low positive balance, the slope of the line being a function of the retention of nitrogen. Beyond a certain point, however, the line becomes curvilinear. The exact position of the break depends upon the nature of the dietary protein. That the shape of the curve also may vary is evident from data obtained in the Nutrition Laboratory of the Iowa State College. When certain proteins used in a collaborative assay* were fed, the curve flattened not far above the point of equilibrium, whereas an S-shaped curve characterized other proteins. It is believed that an upper limit exists for the utilization of each type of dietary protein. To provide more than that quantity not only may be inefficient but may impose a burden upon the mechanisms involved in the formation and excretion of nitrogenous waste compounds.

Since the nutritive property of a protein expresses, in reality, the combined effects of a variety of amino acids that are present in different concentrations, the total intake of nitrogen is particularly significant in relation to the utilization of mixtures of amino acids. In studying the needs of human beings, Rose (1949) gave careful consideration to this point, maintaining the daily supply of nitrogen within a range of 10.03 to 10.08 gm. per day throughout the test intervals. He stated that,

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*Sponsored by the Bureau of Biological Research, Rutgers University

> Thus, an abundance of nitrogen was always available to the cells for synthetic uses. This is an important point, for it does not follow that nitrogen balance could be maintained with the minimum levels of the eight essentials alone. Under the latter circumstances, the manufacture of the nonessentials by the tissues would be impossible. (p. 550)

Increments in the total quantity of protein present in a mixed diet led to marked elevations in the quantities of certain amino acids excreted in the urine by human subjects (Steele, Reynolds, and Baumann, 1950). Salmon (1950) has postulated that the proportion of dietary protein exerts a significant effect on the utilization of methionine by growing rats. In the chick, Grau (1948) and Almquist and Merritt (1950) discovered that the requirements for certain indispensable amino acids increased with the quantity of protein in the diet. Sprinson and Rittenberg (1949a) demonstrated that the rat employed an appreciable quantity of nitrogen that was supplied as ammonia when very low protein diets were provided, but excreted it nearly quantitatively when the diet was high in protein.

It is conceivable, therefore, that the plane of nitrogen metabolism may be so altered by the availability of nitrogen that at a certain point specific synthetic processes are set in motion that change the entire pattern of nitrogen utilization.

Presence of Nitrogen from Nonessential Amino Acids or from Inorganic Sources

The exact mechanisms involved in the biosynthesis of amino acids have not been elucidated (Shemin, 1950). It has become apparent, however, that a supply of labile nitrogen is indeed essential if excessive catabolism of body tissues is to be prevented.

Having based all previous estimates of the amino acid requirements of growing rats on data obtained in experiments in which ample quantities of the nonessential amino acids were provided, Rose, Oesterling, and Womack (1948) tested the effectiveness of a diet which contained only the essential amino acids. Growth was distinctly inferior to that of rats that were fed a mixture of nineteen amino acids, even though the essential amino acids provided the same total quantity of nitrogen. The authors concluded that

> ... the task of synthesizing ten amino acids simultaneously appears to present too great a burden upon the chemical resources of the cellsto permit the latter to keep pace with the needs of the organism for optimum growth. (p. 758)

Similarly, Frost (1949) noted that repletion of adult rats that were fed twice the minimum quantities of the essential amino acids recommended by Steffee and colleagues (1950) was less satisfactory than when the recommended mixture was supplemented with nonessential amino acids. On the other hand, Wissler and colleagues (1948) believed that the same acids that are nonessential for growth may be considered dispensable also for maintenance of appetite, weight, and nitrogen balance in the normal adult rat. It should be noted, however, that the quantities of all amino acids that were incorporated in their mixtures of nine or fifteen amino acids exceeded those offered by certain other investigators (See Table 1). Effects upon nitrogen balance of feeding limited quantities of essential amino acids alone to normal adult rats have not been reported in the literature. In depleted rats, Brush, Willman, and Swanson (1947) secured nitrogen equilibrium when only 52 mg. of nitrogen was provided daily per rat from a mixture of ten essential amino acids.

Of interest in this connection is the fact that a mixture of amino acids essential to the growth of a specific strain of microorganisms is ineffective for its culture unless supplemented with some source of nonessential nitrogen (Hegsted, 1944). Factors influencing the amino acid requirement of test organisms have been summarized by Snell (1945).

Rose, Smith, Womack, and Shane (1949) concluded that nitrogen required for the synthesis of nonessential amino acids in the growth process may be derived from several sources, including ammonium salts, certain amino acids, and urea. Glycine and urea were less efficacious than other sources, and diammonium citrate was particularly useful (Rose et al., 1949; Lardy and Feldott, 1950).

Ramasarma, Henderson, and Elvehjem (1949) found that certain individual nonessential amino acids may be important. They observed that when the essential amino acids were supplemented with glutamic acid alone, growth was enhanced to a smaller extent than when a mixture of nonessential acids was used. Tyrosine seemed to exert a slight stimulatory effect.

On the other hand, Frost (1949) concluded that glutamic acid could replace all of the other nonessential acids in the diet of the repleting rat. Amino acid mixtures which provided 20 to 25 per dent of the total nitrogen from sources other than the essential amino acids were efficient.

Under certain conditions, therefore, the nonessential amino acids indeed may become a limiting factor. The relative effectiveness of the individual acids has not been clarified, however.

Configuration of Amino Acids

Differences in the capacity of the animal organism to utilize the D- and L-isomers of certain amino acids illustrate the marked specificity which characterizes reactions of living matter. Only certain species possess the enzymes that are necessary for the inversion of D-amino acids to the L-forms thereof. Neuberger (1948) suggests that the extent to which D-amino acid becomes inverted depends upon "a) the rate of oxidation by the D-amino acid oxidase, b) the reamination to the L-amino acid, c) the relative rates of further irreversible oxidation of the keto-acid, and d) other oxidative reactions not involving the keto-acid stage."(p. 24). The D-amino acids may be broken down by reactions probably of an irreversible character that do not involve the keto-acid.

That mixtures of amino acids containing D-isomers are not utilized to the same degree as similar mixtures containing only the L-forms has been indicated recently by the work of several groups of investigators. For example, Graham and associates (1950) found that the DL-mixtures of leucine, tryptophane, and aspartic acid when added to a basal diet containing casein inhibited growth to a greater extent than did the natural forms.

Brand and Bosshardt (1948) reported that the growth response of mice was less satisfactory when certain DL-acids were used than when only the L-acids were fed. Frost (1949) observed that the appetite of depleted rats for amino acid solutions containing DL-valine, DL-threonine, and DL-isoleucine was less than for complete acid hydrolysates of proteins fortified with tryptophane. On the other hand, Van Pilsum and Berg (1950) concluded that the D-components of the DL-mixtures produced no appreciable deleterious effect per se when fed to rats. The unfavorable response of the animals to a mixture of amino acids made up only of DL-acids was ascribed to excess methionine, the L-form of which was more detrimental than its D-modification.

The reviews by Berg (1942), by Albanese (1947), and by Neuberger (1948) indicate that the rat is unable to utilize the D-forms of isoleucine, leucine, lysine, threonine, and valine. Histidine cannot be completely inverted but methionine, phenylalanine, and tryptophane seem to be fully available. However, in man, the recent findings of Baldwin and Berg (1949) and of Sarett and Goldsmith (1950) suggest that under certain conditions D-tryptophan is less effective for man than is the natural isomer.

The capacity of the organism to utilize the D-isomer may depend upon the presence of at least a small amount of the natural form, as illustrated by the findings of Anderson and Nasset (1950) with leucine.

Imbalance of Amino Acids

Ultimately a full understanding of the synthetic and degradative processes involved in protein metabolism will be achieved, together with a recognition of the forces that function in maintaining a balance between them. Then it will be possible to define amino acid requirements specifically under a given set of conditions. The concentrations of individual essential and non-essential amino acids that are required for growth and maintenance undoubtedly depend upon the amino acid composition of individual tissues and the respective rates of turnover in the processes of synthesis and regeneration. Under some conditions, the organism is unable, apparently, to metabolize large quantities of certain amino acids, an indication that a balance must exist among the nitrogenous components of the diet.

Cannon (1948a) expressed the "proportionality ratios" of essential amino acids in certain proteins in terms of their relationship to the concentration of tryptophane, the amino acid which is present in the lowest proportion in many proteins. Furthermore, he and his coworkers have attributed the ineffectiveness of several mixtures of amino acids to an imbalance among the essential amino acids (Steffee _et_ _al_., 1950).

That excessive quantities of certain essential amino acids may cause a retardation of growth was suggested by depression of growth observed in several laboratories when dietary nitrogen derived from both essential and non-essential acids was replaced by an equivalent quantity of nitrogen from the essential amino acids only (Rose, Oesterling, and Womack, 1948; Ramasarma, Henderson, and Elvehjem, 1949; Wretlind, 1949). Van Pilsum and Berg (1950) showed clearly that such effects could not be attributed to the presence of unnatural isomers. Moreover, Wretlind (1949) ascertained that the following percentages of individual acids in the diet would retard growth or cause death: DL-methionine 3; DL-tryptophane 4; DL-histidine 4 to 8; DL-threonine 6 to 7; DL-phenylalanine 12; DL-leucine and DL-valine 16; and DL-isoleucine 24.

Deleterious effects of excessive quantities of methionine upon nitrogen balance in dogs (Roth and Allison, 1949), upon recovery of weight in depleted rats (Steffee et al., 1950), and upon growth of rats (Van Pilsum and Berg, 1950) have been reported. The disturbance in nitrogen metabolism that followed the administration of excessive quantities of methionine could be counteracted by glycine or arginine (Roth and Allison, 1949), and a similar growth depression in the chick by increasing the supply of protein (Grau and Kamei, 1950). That glycine also may become toxic when fed in certain concentrations was shown by Handler, Kamin, and Harris (1949) in the dog, by Groschke, Anderson, and Briggs (1948) in the chick, and by Kelley and Totter (1950) in the rat.

Wyzan, Kade, and Shepherd (1950) concluded that the retention of nitrogen was enhanced by supplementing casein hydrolysates with methionine up to 40 mg. of nitrogen per kilogram as methionine. The addition of more than 50 per cent of the total amino acids as methionine apparently created an imbalance and seriously impaired utilization of nitrogen. The authors did not attribute the effects of the imbalance directly to the labile methyl group or sulfur atom, since excess choline chloride, cystine, and cysteine were fed without adverse effect.

Large amounts of phenylalanine, tyrosine, leucine, or glutamic acid apparently may be metabolized without difficulty (Wyzan et al, 1950; Ferger and du Vigneaud, 1949; Ramasarma et al., 1959). On the other hand, excessive quantities of hydroxyproline (Womack and Rose, 1947) and histidine (Martin, 1947) depressed the growth of rats. Treadwell (1948b) observed also that high concentrations of cystine exerted an antilipotropic effect and even caused death of growing rats.

Caloric Intake

The important bearing of the energy content of a ration upon the utilization of nitrogen has been demonstrated repeatedly. If available calories fail to meet the demands for energy, proteins are diverted from their normal roles in the maintenance and synthesis of tissues and of functional proteins to provide fuel. All species respond similarly. Beattie, Herbert, and Bell (1948) concluded that in the emaciated human subject, the absolute amount of nitrogen retained was related directly to caloric intake, provided that the nitrogen intake was above the critical level for nitrogen equilibrium, i.e., 0.17 mg./kg./da. In the growing rat and mouse, Bosshardt, Paul, O'Doherty, and Barnes (1946) noted that at a given level of protein intake, changes in caloric supply were reflected generally by alterations in apparent utilization of protein. For each protein source, an intake permitting maximal protein utilization could be found. At low caloric intakes, extra calories in the form of protein induced a much greater growth response in the mouse than did equivalent calories supplied as fat or carbohydrate (Bosshardt *et al.*, 1948). In the normal adult dog, Allison and Anderson (1945) concluded that excretion of nitrogen on a protein-free diet and the protein minimum for nitrogen equilibrium increased when the ingested calories fell below the optimal level.

Evidently a critical level of caloric intake exists, below which catabolism of tissues is accelerated markedly. In the depleted dog (Allison, Anderson, and Seeley, 1946) and the partially depleted rat (Stevenson, Swanson, Willman, and Brush, 1946; Willman, Brush, Clark, and Swanson, 1947), this point is apparently 50 per cent of the normal caloric intake. In the growing rat and mouse, it is 80 and 95 per cent, respectively, of the maximum intake obtained with *ad libitum* feeding (Bosshardt, Paul, O'Doherty, and Barnes, 1946). In the hypoproteinemic rat Benditt *et al.*, (1948) found that restriction of the calories below 1240 cal./sq.m./da limited utilization of protein for synthesis of tissue. Provision of more calories did not enhance nitrogen utilization, any increment in body weight being due almost entirely to the deposition of fat. Johnson, Deuel, Morehouse, and Mehl (1947) concluded that more than 1200 calories were necessary for the attainment of a minimum nitrogen excretion in man when low-protein diets were fed. Their findings were confirmed by Schwimmer and McGavack in 1948. Similarly, positive nitrogen balance in emaciated individuals was possible only when more than 35 cal./kg./day were fed (Beattie, Herbert, and Bell, 1948).

When diets containing amino acids were fed to human subjects, Rose (1949) found that the caloric requirement must be distinctly higher to ensure positive nitrogen balance than when casein was fed. Moreover, Anderson and Nasset (1948) noted that the specific dynamic action of a mixture of amino acids was increased definitely when the content of DL-isoleucine was reduced to one-third of normal. No change in specific dynamic action occurred as a result of decrements in methionine or valine, although the nitrogen of the mixture was used less efficiently than when adequate supplies of these amino acids were fed. Such observations highlight problems that merit consideration in planning experiments that are designed to test amino acid requirements and in interpreting results obtained therein.

In view of the critical importance of the caloric intake, a survey was made of the energy values of diets now in use by other investigators. All workers have provided 40 calories or more per rat per day. Benditt et al. (1950) supplied 1200 cal./sq. m., equivalent to 46 cal./300 gm. rat when studying amino acid requirements; Black, French, Cowan, and Swift (1949) offered 62 cal./390 gm. rat, equivalent to 48 cal./300 gm. rat; Forbes, Swift, Elliott, and James (1946) considered 45 calories ample for maintenance of adult rats, 65 calories being allowed for "supermaintenance". For mature male rats, Anderson and Nasset (1948) force-fed 38 calories. Also, Harte, Travers, and Sarich (1948) found that at five months of age, the voluntary caloric intake of rats stabilized at 12.5 to 13.0 cal./sq. cm., equivalent to 48.0 to 51.5 cal./300 gm. rat. Kuehl (1949) force-fed 60 cal./300 gm. rat/da.

Hegsted and Haffenreffer (1949) made the significant observation that, regardless of the nature and quantity of the protein offered to growing male rats, the voluntary mean caloric intake varied as the mean body weight raised to the 0.88 power. They postulated that "the food intake of the animals is controlled by some means (yet unknown) at a relatively constant per cent above the basal metabolism" (p. 146). They suggested, further, that when a protein of inferior quality is offered, an overabundance of calories may result in increased activity and a wastage of calories.

It is very clear that an adequate caloric intake constitutes an important factor in the formulation of diets to be utilized in evaluating the nutritional quality of a given mixture of amino acids. Moreover, the optimum quantity of calories for maximal utilization of nitrogen varies with the source of nitrogen.

Proportion of Fat in the Diet

As early as 1881 Voit undertook investigations of the relationship of dietary fat and carbohydrate to nitrogen utilization. That a full understanding of the nature of the relative sparing effects has not yet been attained is apparent from the review papers of Deuel (1948), of Swanson and Clark (1950), and of Medes (1950). Fat evidently exerts a role, over and above the provision of needed calories and essential fatty acids, that is directly related to the control of protein metabolism.

Allison, Anderson and Seeley (1946) and Bosshardt, Paul, O'Doherty, and Barnes (1948) concluded that fat and carbohydrate were equal in their protein-sparing effects when a low proportion of protein was offered. Nevertheless, Schwimmer and McGavack (1948) demonstrated that dietary fat assumed a special place in the diet of human subjects, particularly when the supply of calories was limited. The urinary nitrogen was lower when 30 per cent of fat was incorporated in a diet providing 900 calories and 6.0 gm. of nitrogen per day than when only 10 or 20 per cent was allowed. They associated these results with something intrinsic in the higher fat intake _per se_.

In the Nutrition Laboratory of the Iowa State College, Swanson and associates have observed repeatedly the striking influence of the proportion of dietary fat upon nitrogen metabolism of partially depleted rats deprived of dietary protein. When isocaloric quantities of high and low fat rations were offered _ad libitum_, the catabolism proceeded at approximately the same rate, but when the caloric value of the diets offered to both groups was restricted to approximately one-fourth of their voluntary intake, the destruction of tissue was twice as great in the absence of dietary fat as in the presence of 20 per cent of fat (Stevenson, Swanson, Willman, and Brush, 1946; Willman, Brush, Clark, and Swanson, 1947). Moreover, when 56 calories were administered by stomach tube daily, certain striking results were observed (Hoover, 1950). Between 30 and 40 per cent of the animals fed the low-fat ration died within four weeks. The increased excretion of nitrogen when calories were restricted was due largely to increases in urea and ammonia, the quantities of the latter in the urine being disproportionately large. Glucose tolerance was abnormal, and the concentration of riboflavin in hepatic tissue decreased.

In alleviating the conditions associated with dietary deprivation of fat, methionine was very effective (Stevenson et al., 1946; Willman et al., 1947; Hoover, 1950). When only 44 mg. of the amino acid were fed daily to rats receiving one-fourth of their normal caloric intake from a fat-free diet, an effect was induced that resembled the response to the inclusion of 20 per cent of fat in the diet. Marked decreases occurred in the total urinary excretion and in the respective quantities of urea and ammonia in the blood and urine. Also, tolerance to glucose and the concentration of riboflavin in the liver returned towards normal (Hoover, 1950).

Exploration of the mechanisms involved in the relationship of dietary fat to the utilization of nitrogen and in the particular roles of methionine offers a most intriguing approach to elucidation of the complex interlocking of metabolic pathways. The striking effect of minute quantities of methionine suggests a catalytic effect. In this connection, the interrelationship of methionine, choline, and vitamin B_{12} in various species is suggestive (Schaefer et al., 1950). A particularly interesting problem is whether or not the inclusion of dietary fats varying as to source, degree of hydrogenation, and method of preparation may alter the response of the test animal. There are many implications in the findings that may be significant in human nutrition.

Growth of young rats that were fed mixtures of amino acids was accelerated by reducing the proportion of fat in the diet (Borman et al., 1946; Van Pilsum and Berg, 1950).

Investigations of the influence of dietary fat upon various aspects of nutrition and upon the economy of food utilization are being pursued actively by Deuel (1947) and by Black (1949) and their associates. The interrelationships between fat and amino acids that were cited by Medes in 1950 suggest that the overall picture is very complex.

Time of Ingestion of Nutrients in Relation to Each Other

The importance of the times of ingestion of the amino acids in relation to each other has been reviewed by Swanson and Clark and by Geiger in 1950. The essential amino acids must be available simultaneously if they are to be utilized effectively for the synthesis of tissues (Geiger, 1947; Schaeffer and Geiger, 1947) or for the maintenance of nitrogen equilibrium (Cannon, Steffee, Frazier, Rowley, and Stepto, 1947). Thus, Geiger (1950) found that adult rats that received tryptophane as a delayed supplement lost weight as fast or faster than did control rats that received none; on the other hand, tryptophane was transformed efficiently to niacin when fed 12 hours after other amino acids. Geiger (1950) has emphasized the importance of the simultaneous availability of both essential and nonessential amino acids, if synthetic mechanisms are to proceed normally.

Munro (1949) has demonstrated recently that utilization of nitrogen is enhanced when carbohydrate is supplied with the protein moiety of the ration; fat apparently exerts no influence on the metabolism of protein.

Vitamin Supplementation

Animal feeding experiments have revealed that the nature and quantity of dietary protein or amino acids consumed may alter the requirements for specific vitamins. For example, a large proportion of casein elevates the demand for pyridoxine (Cerecedo and Foy, 1944); the concentration of protein influences the retention of riboflavin (Czaczkes and Guggenheim, 1946); tryptophane can replace niacin partially if not completely (Vilter, Mueller, and Bean, 1949); and the requirements for methionine, choline, vitamin B_{12} and folacin are closely interwoven (Schaefer et al., 1950).

On the other hand, evidence has accumulated that several vitamins are associated intimately with enzymatic processes involved in the metabolism of individual amino acids. In the oxidation of tyrosine and dihydroxyphenylalanine, a complex relationship exists between ascorbic acid, pteroylglutamic acid, and vitamin B_{12} (Basinski and Sealock, 1946; Clegg and Sealock, 1959; Rodney, Swendseid, and Swanson, 1949; Woodruff et al., 1949). Whether these vitamins function jointly or successively has not been elucidated. Moreover, vitamin B_{12}, folic acid, and choline are interrelated in the prevention of renal hemorrhage (Schaefer, Salmon, Strength, and Copeland, 1950) and of perosis. In fact, Stekol and Weiss (1950) demonstrated that rats would grow when fed a diet containing homocysteine and essential amino acids except methionine but devoid of donors of methyl groups, provided that vitamin B_{12} was present. Vitamin B_{12} in the ration enhanced the reduction of disulfide compounds (Dubnoff, 1950) and the synthesis of methionine (Oginsky, 1950). It did not, however, stimulate liver protein regeneration in partially hepatectomized rats (Vars, Karn, and Ferguson, 1950), nor did it improve nitrogen retention when diets containing soybean protein were used (Chow and Barrows, 1950), although growth was improved.

Keith and associates (1948) have proposed that folic acid may participate in regulating the activity of such enzymes as xanthine oxidase, L-amino acid oxidase, and D-amino acid oxidase. In folic acid deficiency, Elwyn and Sprinson (1950) observed that the rate of conversion of serine to glycine was reduced to about one-sixth of the normal value. It has been shown, too, that folic-acid treated rats fixed about ten times as much C^{14} from labeled formate into liver protein as did the deficient rats (Plaut, Bethel, and Lardy, 1950). The improvement was reflected clearly by higher concentrations of carbon in specific positions of aspartic acid, glutamic acid, and arginine, and serine.

Pyridoxine is particularly important in transamination (Umbreit, O'Kane, and Gunsalus, 1948), in decarboxylation (Umbreit and Gunsalus, 1945), in oxidation of D-amino acids (Armstrong, Feldott, and Lardy, 1950), and in the metabolism of tryptophane (Greenberg, Bohr, McGrath, and Rinehart, 1949).

The ability of tissues from biotin-deficient rats to fix C^{14} in aspartic acid, arginine, and certain nucleic acids was markedly depressed (MacLeod and Lardy, 1949), as was the synthesis of citrulline from ornithine. A lack of tocopherol strikingly lowered the glutamine content of skeletal muscles of dystrophic guinea pigs (Roderuck, 1949) and the aspartic-glutamic transminase activity of homogenates of skeletal muscle (Barber, Basinski, and Mattill, 1949).

Specific vitamins, therefore, play essential roles in oxidation, deamination, transamination, decarboxylation, transmethylation, and carbon dioxide fixation. Obviously, the composition of the vitamin mixture with which the basal diet is supplemented may influence the utilization of nitrogen derived from mixtures of amino acids.

Presence of Certain Unidentified Factors

The failure of certain mixtures of amino acids to induce growth equal to that resulting from a natural protein has led investigators to postulate that additional factors, including strepogenin, were necessary (Rose and Womack, 1946b;Woolley, 1946). This hypothesis has not been confirmed. Diets that were devoid of peptides permitted normal repletion in rats (Frost and Sandy, 1948), and satisfactory growth in rats (Ramasarma, Henderson, and Elvehjem, 1949), in dogs (Silber et al., 1949), and in mice (Maddy and Elvehjem, 1949). Furthermore, a clear-cut demonstration of the nutritional and metabolic equivalence of a protein and its corresponding amino acid mixture was presented by Brand and Bosshardt in 1948. They secured exactly the same growth response in mice whether they fed β-lactoglobulin or the L-amino acids contained therein.

The ineffectiveness of earlier mixtures of amino acids for growth may have been due, in part, to incomplete information concerning the exact amino acid composition of the protein, to such problems as the contamination of isoleucine with alloisoleucine, or to other factors involved in the formulation of the diet rather than to the absence of special peptide-like substances.

AMINO ACID REQUIREMENTS OF THE ADULT MALE RAT

Only within the past decade has it been possible to investigate the needs of the adult rat in respect to amino acids. Among the indices of protein nutrition that may be used are the ability of the nitrogenous portion of the diet to establish nitrogen equilibrium to maintain or restore body weight, and to maintain normal concentrations of plasma proteins and hemoglobin in the blood. The concentration of specific metabolites in the tissues, blood, or urine, alterations in the structure of the cells, and inability to metabolize certain test substances normally will supply additional information in respect to protein nutrition.

Bases used in the Formulation of Mixtures of Amino Acids

Two distinct approaches have been made in devising mixtures of amino acids for feeding to the adult rat: 1) the provision of the same quantity of nitrogen, arbitrarily selected, from each of the amino acids; and 2) simulation of the amino acid composition of specific proteins of high nutritive quality.

Wolf and Corley (1939) first succeeded in maintaining nitrogen balance in adult rats when amino acids were added to a protein-free diet. They believed that since the requirements for maintenance were included in the need for growth, the same amino acids should be provided. Possibly a smaller number would suffice. No data were then available, however, concerning the amino acid composition of the tissues that must be maintained or their relative rates of turnover. Consequently, Wolf and Corley arbitrarily supplied in their basal mixture 4 mg.

of nitrogen daily from each of ten amino acids that are essential for growth, except threonine, 13 mg. of which was included. Similarly, Burrough, Burroughs, and Mitchell (1940) provided 80 mg. of nitrogen daily from mixtures comprising 20 amino acids or less. In 1947, Brush, Willman, and Swanson secured nitrogen balance in protein-depleted adult male rats when they fed a mixture in which the utilizable forms of each of the ten essential amino acids contributed 4 mg. of nitrogen.

More recently, natural proteins have been selected as a pattern. Thus, in Cannon's laboratory a mixture of sixteen amino acids simulating casein was developed and used by Frazier (1947), Wissler (1948), Benditt (1950), and Steffee (1950) and their colleagues to study the minimum requirements of the normal and depleted rat. The excellent nutritive properties of egg proteins led Anderson and Nasset (1948) to formulate, with egg proteins as the reference standard, a composite of ten essential amino acids supplemented with glutamic acid. Simultaneously, workers in Swanson's laboratory selected lactalbumin as a model (Kuehl, 1949), because its biological efficiency was slightly higher than that of egg proteins, and distinctly superior to that of casein, _i.e._, 106 _vs_. 74, as determined by Metz in 1947. Furthermore, lactalbumin consists largely if not entirely of

B-lactoglobulin (McMeekin and Polis, 1950), whereas neither casein nor egg proteins are homomolecular.

The composition of the mixtures of amino acids that have been utilized for studies involving the adult male rat is presented in Table 1. The values indicated therein have been corrected for hydrochloric acid and water of crystallization, but not for the presence of the D-isomer, since the investigators differed in their interpretation of the availability of the unnatural isomers. For example, Brush doubled the quantity of isoleucine and threonine, whereas Wissler and Kuehl increased the amount of valine also. On the other hand, Anderson and Nasset increased the concentrations of methionine, phenylalanine, and tryptophane as well as isoleucine, threonine, and valine.

Certain striking differences in respect to the total concentration of nitrogen and the distribution of the individual amino acids are apparent from the data in Table 1. The workers in Cannon's laboratory, for example, fed four or five times as great a quantity of the amino acid mixture daily as did the other investigators. Anderson and Nasset included considerably larger proportions of methionine and phenylalanine than did Kuehl, but smaller quantities of isoleucine, leucine, lysine, and threonine. All of the mixtures described above have

Table 1. Composition of mixtures of amino acids fed by different investigators to the adult male rat (mg. of amino acid /rat/day)

		Investigator				
		Brush (1946)	Wissler(1948)		Anderson & Nasset*	Kuehl (1949)
Amino acid	Form		A	B		
Arginine	L	12	63	99	27	16
Histidine	L	15	52	60	9	9
Isoleucine	DL	75	200	314	40	59
Leucine	L	37	190	294	27	47
Lysine	L	20	106	167	22	37
Methionine	DL	44	54	85	22	13
Phenylalanine	DL	47	80	126	34	26
Threonine	DL	68	120	189	28	49
Tryptophane	DL	--	28	43	9	--
Tryptophane	L	29	--	--	--	11
Valine	DL	67	216	338	41	37
Total		414	1109	1715	259	304
Alanine	DL	--	86	--	--	--
Alanine	L	--	--	--	--	13
Aspartic acid	DL	--	97	--	--	--
Aspartic acid	L	--	--	--	--	49
Cystine	L	--	6	--	--	14
Glutamic acid	L	--	366	--	99	68
Glycine	-	--	8	--	--	11
Proline	L	--	--	--	--	20
Serine	L	--	--	--	--	13
Tyrosine	L	--	99	--	--	24
Total			662		99	212

*Estimated from data presented in J. Nutr. 36, 706 (1948) contributed, however, towards a fuller understanding of the capacity of the animal to utilize nitrogen from amino acids and of the requirements for each.

Present Knowledge concerning Amino Acid Requirements

The classic nitrogen balance technique developed by Thomas (1909) and modified by Mitchell (1924)was employed in all early evaluations of the nutritive quality of proteins or amino acids. This procedure involved the comparison of nitrogen balances in two successive periods, an interval in which the diet was devoid of protein, and a subsequent interval in which the test protein was administered. All data therefore reflected the response of a partially depleted animal. Furthermore, under the conditions of World War II, information concerning the utilization of nitrogen by severely depleted human beings was needed urgently.

The question inevitably arose, however, as to whether the well-nourished organism utilized nitrogen more or less efficiently than did the depleted one, and whether the amino acid needs of the two types of organisms were similar. Investigations of amino acid requirements of the adult male rat have developed, consequently, in two phases, the first being related to the depleted animal and the second to the well-nourished rat.

Of the protein-depleted rat

In 1947, Brush, Willman, and Swanson demonstrated that a mixture of the ten essential amino acids in which each contributed 4 mg. of nitrogen daily (Table 1) was as effective in sparing body tissue as an equivalent quantity of nitrogen from egg proteins. Such an assortment induced a slightly positive balance in rats weighing approximately 230 gm. The authors discovered that the sum of the quantities of nitrogen spared by individual amino acids, when fed alone, approximated that resulting from administration of the ten acids simultaneously. Methionine proved unusually effective. Although the authors did not attempt to establish the quantitative needs of partially depleted animals for each of the amino acids, they postulated that the daily methionine requirement of a rat weighing 260 gm. was 11 mg. or less.

Simultaneously, Frazier and associates showed that a composite of sixteen amino acids simulating casein, or of the ten essential amino acids providing the same total quantity of nitrogen as the first assortment, permitted satisfactory weight recovery in rats that were previously depleted for three months. The omission of any amino acid except arginine induced a prompt decline in appetite followed by a loss in weight. They concluded that arginine was not necessary for normal appetite, recovery of weight, or spermatogenesis. Moreover, only nine amino acids were indispensable for the regeneration of serum proteins and erythrocytes by the adult hypoproteinemic rat (Benditt et al., 1947). The finding that lysine was essential confirmed the report of Neuberger and Webster (1945). Mitchell (1947) concluded, however, that the adult rat could synthesize a sufficient quantity of lysine to meet the needs for nitrogen equilibrium. The latter idea seems untenable in view of the high concentration of lysine in the carcass of the rat (Dunn et al., 1949) and the irreversible deamination of lysine (Borsook et al., 1948). That histidine was essential for the adult rat was shown by Albanese and Frankston (1945), as well as by Benditt and associates (1947).

In 1950, Steffee and associates estimated the quantitative requirements of the repleting male rat for individual amino acids by ascertaining the quantity of each that would induce a predetermined gain in weight. Since Diet A in Table 1 was used, fifteen other amino acids were present in relatively high concentrations when the need for any given acid was being ascertained. Finally, a mixture of nine amino acids was prepared that contained the minimum quantities of each of the nine essential amino acids that permitted weight recovery and the amounts of the nonessential acids that were provided in previous trials. Satisfactory recovery of weight was induced that was paralleled by gains in carcass protein.

In interpreting their data, the authors subdivided the total daily requirements for a given amino acid into two distinct parts; 1) a maintenance requirement related to the continuing metabolism and calculated from the needs of the well-nourished rat and 2) the quantity of amino acid utilized for the construction of tissues over and beyond the need for maintenance. Steffee and colleagues concluded that the minimum and optimum quantities were very similar for the repleting animal, as indicated by the rapid stabilization of the curve.

The data from Steffee's paper are reproduced in Table 2 and are supplemented with an estimate calculated by the author in terms of the rat weighing 300 gm.

Anderson and Nasset (1948, 1950) have made significant contributions concerning the effect of altering the concentrations of certain amino acids in a mixture based on the composition of egg proteins (Table 1). Reduction of the quantity of DL-leucine to one-third of that in the original mixture decreased the nitrogen balance index, with a concomitant increase in the maintenance requirement

Table 2. Minimum daily requirements of the adult male rat for the utilizable forms of the essential amino acids

	The well-nourished rat				The depleted rat		
	As reported*		Calculated/ 300 gm. rat**		As reported***		Per 300 gm.
Amino acid	For nitrogen balance	For maintenance of weight	For nitrogen balance	For maintenance of weight	For maintenance	For repletion	For repletion
	mg./100 sq. cm.	mg./100 sq. cm.	mg.	mg.	mg./kg.	mg./kg.	mg.
Histidine	2.1	2.2	8.0	8.5	35	85	28
Isoleucine	12.9	13.7	49.5	52.6	180	165	55
Leucine	6.5	8.0	24.9	30.7	110	305	102
Lysine	3.7	4.5	14.2	17.3	60	270	90
Methionine	5.3	7.3	20.3	28.0	90	130	43
Phenylalanine	3.1	6.0	11.9	23.0	50	205	68
Threonine	5.1	5.3	19.6	20.3	85	160	53
Tryptophane	1.8	2.2	6.9	8.5	30	50	17
Valine	7.0	10.0	26.9	38.4	120	170	57

*Benditt *et al.*, J. Nutr. *40*, 341 (1950)

**Body weight of 300 gm. is equivalent to body surface of 384 sq.cm. by Lee's formula $(12.54 \times W^{0.6})$

***Steffee *et al.*, J. Nutr. *40*, 491 (1950) of nitrogen from 162 to 212 mg N/day/kg$^{3/4}$. A similar reduction in DL-methionine lowered the nitrogen balance index to one-half, and increased the maintenance requirement to 323 mg N/day/kg$^{3/4}$, whereas the lowering of DL-valine to one-third of the amount in the basal mixture did not alter the utilization of nitrogen significantly. Moreover, an increase in specific dynamic action followed a low intake of isoleucine but not of methionine or valine. When the quantity of L-leucine was limited to one-sixth of that present in the complete mixture, utilization of nitrogen from the composite was depressed significantly (1950). Nasset and Anderson (1950) estimated that the adult rat requires 2.1 mg of nitrogen from L-valine and 4.2 mg from DL-valine per day per kilogram$^{3/4}$.

Of the normal adult rat

Wissler and colleagues reported in 1948 that well-nourished adult rats maintained appetite, weight, and nitrogen balance when they were fed a diet containing the ten essential amino acids, as shown in Table 1, Diet B. The omission of any single amino acid, except arginine, resulted in an unmistakable decline in weight and in nitrogen balance. The food intakes of rats deprived of arginine were variable, and nitrogen balances sometimes negative. When the intake of nitrogen was maintained by force-feeding, however, removal of arginine did not depress nitrogen balance.

In 1950, Benditt extended the investigation to an estimation of the minimum quantitative requirements of the normal adult rat for essential amino acids. As a basic mixture, he used Diet A in Table 1, which contributed approximately 150 mg. and 70 mg. of nitrogen from the essential and nonessential acids, respectively. All of the mixtures that were fed contained between 203 and 223 mg. of nitrogen per day. The estimated quantities for the maintenance of nitrogen balance and of body weight are presented in Table 2 in terms of body surface as reported and of body weight as calculated by the author of this manuscript. It is evident that the demands for maintenance of weight are greater than those for nitrogen balance. The requirement for phenylalanine, for example, was almost doubled, although the needs for histidine and threonine were similar in both cases. Benditt believed that the value for lysine probably represented the minimum, but that certain other values would require modification under different experimental conditions.

Striking results were obtained by Kuehl (1949) when she fed the diet described in Table 1. The complete mixture containing both essential and nonessential amino acids permitted nitrogen equilibrium and a normal response when the animals were fed by stomach tube twice daily. When the ten essential amino acids were fed alone in the same quantities, however, the animals lost over 200 mg. of nitrogen in 7 days, and developed a condition that has been designated the Kuehl syndrome. It was characterized by hyperexcitability, severe digestive disturbances, and a peculiar behavior pattern that was never observed in animals that were fed either the lactalbumin control ration or the same mixture of essential amino acids supplemented with the nonessential acids.

Such observations emphasized the necessity of exploring the factors that influence the retention of nitrogen when mixtures of amino acids are fed to the normal adult male rat.

EXPERIMENTAL PROCEDURE

PLAN OF THE EXPERIMENT

For some years, studies have been in progress in the Nutrition Laboratory of the Home Economics Department of the Iowa Agricultural Experiment Station which are directed toward the expression, in terms of specific amino acids, of the nitrogen requirement of the adult rat that is well-nourished in respect to protein. The investigation reported herein comprises one unit of the series.

Although values representing supposedly minimum requirements of the rat for the essential amino acids have been reported by Benditt et al. (1950) and Steffee et al. (1950), workers in the Iowa laboratory believe that many points must be clarified before the need for nitrogen is established quantitatively on a firm basis. The latter investigators have obtained data in experiments dealing with the metabolism of nitrogen and the utilization of intact proteins and mixtures of amino acids that emphasize the complex nature of the protein requirement.

Nitrogen retention offers an admirable tool for the first approximation of the dietary requirements of the animal for this element, whether it be provided as the intact protein or as constituent amino acids. It has been shown that nitrogen equilibrium reflects dietary manipulation readily. At points that approach equilibrium on a curve showing the relation between intake and utilization, a linear relationship exists (Swanson, 1948; Mukhopadhay, 1949; Allison, 1950).

It is also clear that nitrogen equilibrium is not a measure of the full needs of the animal (Swanson, 1948; Mukhopadhay, 1949; Glabais, 1946). Increased concentrations of nitrogen in the liver and plasma, of hemoglobin in the blood, of immune proteins, and of other essential metabolites occur when the food supplies nitrogen in a quantity well over the amount needed to support equilibrium.

At the present stage of progress, however, it was not deemed advisable to attempt a quantitative estimation of the amino acids needed for the establishment of optimal nutrition. Many problems relating to the interplay of dietary constituents and of the specific amino acids are unsettled. It seemed well, therefore, to set up the present study in terms of minimal requirements easily measured by the balance test.

A preliminary investigation in which the author participated indicated that feeding assortments of amino acids modeled after those in a protein of high quality offered a very interesting approach to the study of amino acid requirements (Kuehl, 1949). In this research, lactalbumin served as the reference protein. It was chosen for this purpose because it had the highest biological efficiency of any protein tested in this laboratory (Swanson, 1948; Metz, 1947). Furthermore, one might assume that in a protein of as high nutritive value as lactalbumin the various amino acids are present in proportions that lead to efficient utilization.

The present experiment, therefore, represents a critical evaluation and enlargement of the study conducted by Kuehl in 1949. Retention of nitrogen by the adequately nourished adult albino rat has been measured when a mixture of amino acids equivalent to those present in a ration containing 4 per cent of lactalbumin, or modifications thereof, are fed in the pure form as the sole source of dietary nitrogen. In addition, data pertaining to the retention of nitrogen under the experimental conditions imposed were supported by estimation of the quantity of nitrogen present in the liver, hepatic nitrogen being a sensitive indicator of the state of protein nutrition (Harrison and Long, 1945; Campbell and Kosterlitz, 1948; Clark, 1945).

The research has been developed in seven distinct parts. The objectives of the various series are stated below:

Series I. To replicate the "Kuehl syndrome"

Series II. To evaluate the force-feeding technique and to establish procedures for the present experiment in respect to

A. Manner of feeding vitamins
B. Influence of number of daily feedings
C. *Ad libitum vs.* forced feeding
D. Best experimental interval for metabolic studies
E. Efficiency of delivery of day's quota of food by stomach tube

Series III. To study nitrogen utilization when a mixture of the ten essential amino acids present in a ration containing 4.27 per cent of lactalbumin is supplemented with graded quantities of a mixture of nonessential acids

Series IV. To determine whether the ten essential amino acids can support nitrogen equilibrium in the absence of the nonessential acids if they furnish a quantity of nitrogen approximating the total nitrogen present in a 4.27 per cent lactalbumin diet

Series V. To determine whether the low nutritive value of a mixture of the ten essential amino acids simulating those present in lactalbumin reflects low concentrations of methionine and/or phenylalanine

Series VI. To determine whether specific nonessential amino acids play particular roles in the maintenance of nitrogen equilibrium

Series VII. To determine whether the addition of a single nonessential amino acid to the essential components is as effective in establishing nitrogen equilibrium as a mixture of nonessentials

EXPERIMENTAL GROUPS

The animals were divided among the various groups within each series in accordance with the plan outlined in Table 3. Certain groups appear in more than one series because they were used as control groups therein. The number of animals fed a given ration, however, is reported only once.

Table 3. Distribution of experimental animals

Series	Group	Description of diet	Number assigned to diet	No. of rats
I	A	The standard low-nitrogen diet	1	--
	B	Diet 1 with dextrin quantitatively replaced by 2.5 per cent of a mixture of the ten essential amino acids, the quantities of the specific amino acids used being those reported in the literature to be present in a diet containing 4 per cent of the Harris preparation of lactalbumin, as used by Kuehl (1949); force-fed twice daily	2a	6
II	A	Diet 2a, force-fed in two daily portions, the vitamins being offered separately	2a	6
	B	Diet 2a, force-fed in two daily portions, the vitamins being homogenized with the diet	2a	6
	C	Diet 2a, force-fed in three daily portions, the vitamins being homogenized with the diet	2a	6
	D	Diet 2a, offered ad libitum	2a	6
	E	Diet 1, with dextrin quantitatively replaced by 4.9 per cent of a Nutritional Biochemicals preparation of lactalbumin; diet offered ad libitum	3a	6

(Continued on next page)

Table 3 (Cont'd)

Series	Group	Description of diet	Number assigned to diet	No. of rats
III	A	The standard low-nitrogen ration	1	5
	B	Diet 1, with dextrin quantitatively replaced by 4.27 per cent of a Harris preparation of lactalbumin analyzed as to its amino acid content	3	6
	C	Diet 1, with dextrin quantitatively replaced by 2.6 per cent of a mixture of the ten essential amino acids, quantities of the specific amino acids used being those shown by microbiological assay to be present in Diet 3; this diet designated as the "basal essential amino acid diet"	2	6
	D	Diet 2, supplemented with 10 mg. of nitrogen per rat per day from nonessential acids, amino acids in the mixture being present in the proportions found in the Harris preparation of lactalbumin	4	6
	E	Diet 2, supplemented with 20 mg. of nitrogen from nonessential acids	5	6
	F	Diet 2, supplemented with 30 mg. of nitrogen from nonessential acids	6	5
	G	Diet 2, supplemented with 40 mg. of nitrogen from nonessential acids	7	12

(Continued on next page)

Table 3 (Cont'd)

Series	Group	Description of diet	Number assigned to diet	No. of rats
	H	Diet 2, supplemented with 50 mg. of nitrogen from nonessential acids	8	5
	I	Diet 2, supplemented with 60 mg. of nitrogen from nonessential acids	9	5
	J	Diet 7, with L-alanine, L-aspartic acid and L-serine replacing DL-forms of these acids	10	5
IV	A	Twice the quantity of the ten essential amino acids provided by the basal diet 2	11	5
	B	Diet 2	--	-
	C	Diet 7	--	-
	D	Diet 8	--	-
V	A	Methionine of diet 7 increased, cystine being replaced isonitrogenously with methionine	12	6
	B	Phenylalanine of diet 7 in creased, tyrosine being replaced isonitrogenously with phenylalanine	13	6
	C	Methionine and phenylalanine of diet 7 increased, cystine and tyrosine being replaced isonitrogenously with methionine and phenylalanine, respectively	14	5

(Continued on next page)

Table 3 (Cont'd)

Series	Group	Description of diet	Number assigned to diet	No. of rats
	D	Diet 7	--	-
VI	A	Alanine, glycine and serine omitted from diet 7, the remaining nonessential amino acids being adjusted proportionately	15	5
	B	Aspartic and glutamic acids omitted from diet 7, the remaining nonessential amino acids being proportionately adjusted	16	5
	C	Cystine omitted from diet 7, the remaining nonessential amino acids being adjusted proportionately	17	5
	D	Proline omitted from diet 7, the remaining nonessential amino acids being adjusted proportionately	18	5
	E	Tyrosine omitted from diet 7, the remaining nonessential amino acids being adjusted proportionately	19	5
	F	Diet 7	--	-
VII	A	Nonessential amino acids of diet 6 replaced isonitrogenously with glutamic acid	20	6
	B	Cystine and tyrosine of diet 7 replaced isonitrogenously with methionine and phenylalanine,	21	5

(Continued on next page)

Table 3 (Cont'd)

Series	Group	Description of diet	Number assigned to diet	No. of rats
		respectively, and all other nonessential acids replaced with glutamic acid		
	C	Diet 6		
	D	Diet 7		
	E	Diet 14	--	--

EXPERIMENTAL ANIMALS

Adult male albino rats reared in the Nutrition Laboratory were used. They represented animals of the 100th generation of an inbred Wistar stock, strain A. After weaning at four weeks, the young rats were fed the Steenbock XVII ration (see Appendix II) until the experiment was initiated. This is the first investigation dealing with protein metabolism in which the rats were fed the Steenbock XVII diet. Since June 1949, this ration has replaced the Steenbock V, a diet formerly used as the stock ration for male rats grown in the laboratory.

The animals ranged in age from seven to nine months.

They were all of approximately the same weight at six months of age and weighed from 300 to 420 gm. when assigned to the various experimental series. They were considered normal in all respects except a few of the older animals which showed a tendency toward lung infection.

The rats were distributed among the various groups as uniformly as possible in respect to weight, age, and physical characteristics. In the final selection of the animals, both the pre-experimental weight, which represented the average of three daily weighings immediately prior to the experiment, and the initial weight, i.e., the weight recorded on the first day of the experiment after the animals had been starved for ten hours, were considered.

FORMULATION AND PREPARATION OF EXPERIMENTAL DIETS

The Basic Diet

All of the diets used in the present experiment represented modifications of the low-nitrogen ration containing 20 per cent of fat which has been used in this laboratory for the past seven years in experiments pertaining to nitrogen utilization. This ration is considered adequate in all respects except for protein. Either lactalbumin or a composite of amino acids was substituted for an equal weight of dextrin in the various diets prepared for this investigation.

The composition of the basic portion of the basal diet is indicated in Table 4. The same lot of all ingredients, except butterfat, was used throughout the experiment. Butterfat was prepared in 4 pound lots as needed. The salt mixture and Ruffex were passed through a fine sieve; otherwise, small lumps occasionally blocked the needle or catheter during the feeding process.

The dry ingredients were thoroughly blended and transferred to the bowl of the Hobart mixer and the fats melted to a semi-fluid state before they were added. The dietary components were then mixed at medium speed for thirty minutes, any fat adhering to the paddle or bowl being scraped into the mixture at intervals. Representative samples were removed for determination of nitrogen and moisture, and the diets were refrigerated immediately.

The basic diet was supplemented with a mixture of vitamins believed to be adequate for the maintenance of the adult rat. It contained all of the vitamins now available in pure form except folic acid and vitamin B_{12}. The rice bran polish factor II, which was also included, supplied approximately .17 mcg. of folic acid per day# and

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#Analyzed by courtesy of Dr. Gladys Everson, Foods and Nutrition Dept.

Table 4. Composition of the basal portion of the low-nitrogen diet

Ingredients	Per cent
Dextrin[1]	73
Osborne and Mendel salts[2]	4
Sodium chloride[3]	1
Ruffex[4]	2
Butterfat[5]	10
Lard[6]	10
	100

[1] Fisher Scientific Co.

[2] Osborne, T. B., and Mendel, L. B., J. Biol. Chem. <u>37</u>, 557-601 (1919)

[3] Baker Chemical Co.

[4] Eimer and Amend, New York; also termed Fisher Celluration

[5] Butter was purchased on the local market; butterfat was prepared by standard procedures in the Nutrition Laboratory and centrifuged for 15 minutes at speed 15 in the IEC centrifuge

[6] Swiftning, from Swift & Co., made from animal and vegetable fats and containing propyl gallate and citric acid as preservatives

served as a source of unidentified factors. It may be deficient in vitamin B_{12} but recent research indicated that, under the experimental conditions employed, the presence of vitamin B_{12} does not enhance either nitrogen retention (Chow and Barrows, 1950) or regeneration of hepatic tissue (Vars, et al., 1950). It is apparent, however, that the requirements of the growing rat for vitamin B_{12}, folic acid, choline and methionine are closely related (Schaefer, et al., 1950).

For convenience, all vitamins except tocopherol and cod liver oil were dispersed with dextrin so that 500 mg. of the resulting mixture carried the daily allowance of each vitamin. The composition of the mixture is shown in Table 5. The quantities of the specific vitamins required for 1000 such doses were mixed in one lot. A sufficient number of these lots were prepared to meet the estimated needs for the entire experiment and carefully blended. Precautions were taken to prevent undue exposure to light and heat, either of which might reduce the potency of the mixture. The mixtures were refrigerated except during immediate use.

In preparing the mixture, choline and the rice bran polish were quickly weighed on the Torsion balance; biotin was quantitatively transferred from the ampoule with 10 ml. of 50 per cent alcohol. These materials were mixed

Table 5. Composition of the vitamin supplement

Group	Vitamin	Allowance per rat per day	Gm. per 1000 doses
A	Choline chloride	5.0 mg.	5.000
	Biotin	1.0 mcg.	0.001
	Rice Bran Polish Factor II	100.0 mg.	100.000
B	Thiamine	40.0 mcg.	0.040
	Riboflavin	60.0 mcg.	0.060
	Pyridoxine	40.0 mcg.	0.040
	Inositol	10.0 mg.	10.000
	Para-aminobenzoic acid	10.0 mg.	10.000
	Calcium pantothenate	0.1 mg.	1.000
	Nicotinic acid	0.5 mg.	0.500
	Ascorbic acid	1.0 mg.	1.000
C	Alpha tocopherol	0.75 mg.	---
	Cod liver oil	50.0 mg.	---

with 75 gm. of dextrin to a smooth golden brown paste. Then an additional portion of 275 gm. of dextrin was added and the whole thoroughly blended. The paste was transferred to a large plate with 10 ml. of 50 per cent alcohol. The product was dried before a large fan operated at high speed until it no longer had a glossy appearance, approximately 1-1/2 hours being required. The other water-soluble vitamins were weighed on an analytical balance and tabled with 25 gm. of dextrin. They were then added to the partially dry mixture of choline, rice bran polish, biotin, and dextrin. All ingredients were rubbed together until homogeneous, pressed through a medium sieve, and allowed to dry completely before a fan set at low speed. Sufficient dextrin to make the final weight of the mixture 500 gm. was then added. If necessary, the dry mixture was rubbed lightly in a mortar to yield a smooth powder. The entire operation was carried through as swiftly as possible, i.e., in four hours or less.

Alpha tocopherol was diluted with Wesson oil so that 50 mg. of the solution provided 0.75 mg. of tocopherol per rat per day. The daily dosesof the vitamin E solution and the cod liver oil (50 mg.) were delivered by calibrated droppers when the vitamins were offered apart from the basal diet. Otherwise, they were homogenized with the diet in appropriate amounts.

The Lactalbumin-Containing Diets

A preparation of lactalbumin of high quality was used as the reference protein, it serving as the base against which the rations containing amino acids were formulated. A portion of the preparation used by Kuehl (1949) originally secured from Harris Laboratories, Division of Bristol-Myers of New York, and subsequently refrigerated, was used in the present experiment. By analysis the diet contained 583 mg. of nitrogen per 100 gm., and thus contributed 76 mg. of nitrogen per 13 gm., the amount selected as the daily quota for each rat (see later section).

The sample of lactalbumin contained 12.725 per cent of nitrogen, 1.04 per cent of ash, and 8.18 per cent of moisture. The nitrogen content on the ash-free, water-free basis was therefore 14.01 per cent, from which was derived the factor 7.14 for conversion of nitrogen to protein. On this basis, the control ration used in Series III supplied 4.16 per cent of protein.

Diets Containing Amino Acids

As with the lactalbumin-containing ration, the amino acids replaced an equal weight of dextrin in the basic nitrogen-low ration. Every precaution was taken to ensure quantitative transfer and thorough blending of amino acid mixtures with other ingredients. For example, the amino acid composite and the appropriate quantity of Na_2CO_3 were transferred to cellophane and tabled; the Osborne and Mendel salts were placed in the beaker that had contained the amino acids to remove the small fraction of ground amino acids that adhered to the beaker despite dareful brushing; the salts were tabled with the amino acids, then the Ruffex. Finally, dextrin was added portion by portion and the mixture tabled carefully before it was placed in the Hobart mixer and blended in the same manner as was the lactalbumin diet.

Formulation and Preparation of Amino Acid Mixtures

Nature of the amino acids used

All amino acids were purchased from Merck and Co., except tryptophane which was procured from General Biochemicals Co., and L-alanine, L-aspartic acid, and L-serine which were available from Bios Laboratories.

At the present time, the natural isomers of several amino acids are not readily obtainable. Although the rat is able to invert certain D-forms either wholly or partially, the enzymes necessary for the inversion of others are apparently lacking. Twice the required amounts of isoleucine, threonine, and valine were included in the amino acid mixture but no correction was made for methionine, phenylalanine, alanine, or aspartic acid, since both forms of these amino acids are believed to be almost completely utilized. DL-serine was omitted and isonitrogenously replaced with glycine because of the nephrotoxic effects that have been associated with the DL-form. Since glycine and serine are interconvertible (Goldsworthy *et al.*, 1949), this procedure was deemed satisfactory.

The necessary corrections were made for the hydrochloric acid and water present in the preparations of arginine, histidine, and lysine that were fed. The quantity was increased as indicated by the relationship of the molecular weight of the amino acid as fed to that of the free amino acid. For example, the molecular weight of lysine hydrochloride hydrate is 200.7 and that of lysine is 146.2. To supply 100 mg. of lysine, therefore, 137 mg. of lysine.HCl.H_2O were incorporated.

For the neutralization of the hydrochlorides, sodium carbonate was used. The data in Table 6 proved useful during the course of the experiment.

Table 6. Neutralization of amino acid hydrochlorides

Amino acid	Molecular weight of amino acid.HCl	Per cent HCl as fed	Mg. Na_2CO_3 to neutralize HCl in 100 mg. of amino acid.HCl
Arginine.HCl	210.7	17.32	25.15
Histidine.HCl.H_2O	209.7	17.41	25.28
Lysine.HCl.H_2O	200.7	18.19	26.41

The concentration of nitrogen in the amino acids was determined (Appendix I, Table A). All were within a satisfactory range except L-alanine and L-serine. They contained, respectively, 13.8 and 10.8 per cent of nitrogen instead of the theoretical values, 15.7 and 13.3 per cent. The quantity of each was increased to yield the required proportion of nitrogen in the composite.

Amino acid mixtures used in diets fed in Series I and II

This mixture contained only the ten amino acids classically listed as essential. In these basic experiments, it was deemed advisable to include arginine in the list. The mixture was identical with that used by Kuehl (1949) and differed from those fed in all subsequent series. It was formulated so as to be equivalent in kind and quantity to the amino acids present in a ration containing 4.06 per cent of lactalbumin. Estimations were made from analyses published in the literature.

The tables of amino acid composition prepared by Block and Bolling (1945) were used except in the case of leucine and isoleucine. Values for these acids were provided by Harris[#]. The data from both sources were calculated to 16 per cent nitrogen. The control ration contained 4.06 per cent of ash-free, water-free lactalbumin, of which 14.01 per cent was nitrogen. The value, 0.875, therefore represents the factor by which the Block and Bolling figures must be multiplied to secure the approximate amino acid composition of the preparation (Block and Bolling, p. 299). For example, since Block and Bolling state that lactalbumin contains 3.5 per cent of arginine calculated

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[#] Personal communication from J. W. Brooks, of Harris Laboratories, to Purchasing Dept., the Iowa State College, April, 1946 to 16 per cent nitrogen, the quantity of arginine to be incorporated in the test ration used in the present experiment would be 4.06 x 0.035 x 0.875 x 1000 or 124.2 mg. per 100 gm. of diet.

The composition of the mixture used in Diet 2a is presented in Table 7.

One group of animals in the series weighed more than the others, _i.e._, 350 gm. _vs_. 310 gm. Although data secured in this laboratory and from the literature indicated that a daily caloric intake of approximately 60 calories as provided by 13 gm. of ration was adequate for the larger rats, it seemed advisable to increase the concentration of amino acids in the daily allotment of their food. In so doing, the proportions of the individual amino acids were held constant but the quantity of each was increased in direct relation to body weight. This last mixture contained 169 mg. arginine.HCl, 107 mg. histidine.HCl.H_2O, 514 mg. isoleucine, 405 mg. leucine, 440 mg. lysine, 112 mg. methionine, 225 mg. phenylalanine, 426 mg. threonine, 93 mg. tryptophane, and 321 mg. valine. Both composites of essential amino acids contained by analysis 12.6 per cent nitrogen.

Amino acid mixtures used in diets fed in Series III

Whenever the experimental plan called for the use of

Table 7. Composition of the mixture of ten essential amino acids indi in a control ration containing 4.06 per cent of lactalbumin

Amino acid	Utilized by the rat	Fed	Formula of amino acid as fed	Mol. wt. of amino acid as fed	Mol. wt. of amino acid $HCl.H_2O$	Per cent N in amino acid	Per cent N in amino acid as fed	Per cent of acid reporte present in a purified lac bumin prepar	
								Block & Bolling*	Ha
Arginine	L	L	$C_6H_{14}N_4O_2.HCl$	174.2	210.7	32.2	26.6	3.5	-
Histidine	D,L	L	$C_6H_9N_3O_2.HCl\ H_2O$	155.2	209.6	27.0	20.0	2.4	-
Isoleucine	L	DL	$C_6H_{13}O_2N$	131.2	—	10.7	—	—	
Leucine	L	L	$C_6H_{13}O_2N$	131.2	—	10.7	—	—	1
Lysine	L	L	$C_6H_{14}O_2N_2.HCl.H_2O$	146.2	200.7	19.2	13.9	8.0	-
Methionine	D,L	DL	$C_5H_{11}O_2NS$	149.2	—	9.4	—	2.8	-
Phenylalanine	D.L	DL	$C_9H_{11}O_2N$	165.2	—	8.5	—	5.6	-
Threonine	L	DL	$C_4H_9O_3N$	119.1	—	11.8	—	5.3	-
Tryptophane	D,L	L	$C_{11}H_{12}O_2N_2$	204.2	—	13.7	—	2.3	-
Valine	L	DL	$C_5H_{11}O_2N$	117.2	—	11.9	—	4.0	-
Total:									

ls indicated by data in the literature to be present
lbumin (used in Series I and II)

nt of amino eported t in a ed lactal- preparation		Per cent of amino acid in control ration	Mg. amino acid					Mg. utilizable amino acid per rat/day	Mg. total N/100 gm. diet	Mg. utilizable N/100 gm. diet	Mg. total N/rat/day in 13 gm. diet	Mg. utilizable N/rat/day in 13 gm. diet
& ng*	Harris*		Per 100 gm. diet	Adjusted for		As fed/ 100 gm. diet	As fed/ 13 gm. diet					
				HCl or HCl.H_2O	DL-form							
	-	3.06	124.2	150	—	150	19.5	16.1	39.9	39.9	5.2	5.2
	-	1.75	71.0	95	—	95	12.3	9.2	19.0	19.0	2.5	2.5
	6.4	5.60	227.4	—	455	455	59.1	29.6	48.7	24.3	6.3	3.1
	10.1	8.84	358.9	—	—	359	46.7	46.7	38.4	38.4	5.0	5.0
	-	7.00	284.2	390	—	390	50.7	36.9	54.2	54.2	7.1	7.1
	-	2.45	99.5	—	—	99	12.9	12.9	9.3	9.3	1.2	1.2
	-	4.90	198.9	—	—	199	25.9	25.9	16.9	16.9	2.2	2.2
	-	4.64	188.4	—	377	377	49.0	24.5	44.5	22.2	5.8	2.9
	-	2.01	81.6	—	—	82	10.7	10.7	11.2	11.2	1.5	1.5
	-	3.50	142.1	—	284	284	36.9	18.5	33.8	16.9	4.4	2.2
		43.75	1776.2			2490	323.7	231.0	315.9	252.3	41.2	32.9 ✓

only the ten essential amino acids in studies following Series II, the mixture was formulated on the basis of (1) the concentration of nitrogen in the control ration containing 4.27 per cent of lactalbumin and (2) the concentration of the amino acids in the lactalbumin as determined by microbiological assay.

The analyses of the specific concentrations of 15 amino acids in this sample of lactalbumin were conducted by Dr. M. N. Camien through the courtesy of Dr. M. S. Dunn, Professor of Chemistry, University of California, whose generosity is gratefully acknowledged. He expressed the amino acid values as percentages of the amino acids in the samples as received without correction for ash or moisture (see Appendix II, Personal Communication to Dr. P. Swanson from Dr. M. S. Dunn). Because values were not available from this source for threonine, alanine, and serine, data provided by Block and Bolling (1944) and by Schmidt (1944) were applied.

The lactalbumin-containing ration (Diet 3) prepared for the present experiment had by analysis 583 mg. of nitrogen per 100 gm., of which 40 mg. was contributed by ingredients other than lactalbumin. Since the lactalbumin preparation as fed possessed 12.725 per cent of nitrogen, 543 mg. represents the quantity of nitrogen present in 4.27 gm. of the lactalbumin preparation. In formulating

Table 8. Composition of a mixture of ten essential amino acids incorporated in the basal t basis of a microbiological assay* of the lactalbumin preparation used in a contro cent of the lactalbumin preparation

Amino acid	Per cent amino acid in lactalbumin	Mg. amino acid in 4.27 gm. lactalbumin**	Mg. amino acid/100 gm. diet			Mg. amino acid as fed/rat/day in 13 gm. diet	Mg. utilisable amino acid/rat/day	Mg. total N/100 gm. diet
			Adjusted for HCl or $HCl.H_2O$	Adjusted for DL-form	As fed			
Arginine	2.56	109.1	132	—	132	17.2	14.2	35.1
Histidine	1.65	70.3	95	—	95	12.3	9.1	19.0
Isoleucine	5.00	213.0	—	426	426	55.4	27.7	45.6
Leucine	9.71	413.6	—	—	414	53.8	53.8	44.3
Lysine	7.57	322.5	442	—	442	57.5	41.9	61.9
Methionine	1.86	79.2	—	—	79	10.3	10.3	7.4
Phenylalanine	3.29	140.2	—	—	140	18.2	18.2	11.9
Threonine***	4.64	197.7	—	394	394	51.2	25.7	46.6
Tryptophane	1.66	70.7	—		71	9.2	9.2	9.7
Valine	5.13	218.5	—	437	437	56.8	28.4	52.0
Total	43.07	1834.8			2630	341.9	238.5	333.5

*Analyzed through the courtesy of Dr. M. S. Dunn, University of California

**As fed

***Value taken from Schmidt (1944)

ten essential amino acids incorporated in the basal test ration and formulated on the
ssay* of the lactalbumin preparation used in a control diet which contained 4.27 per
aration

g. amino acid/100 gm. diet			Mg. amino acid as fed/rat/day in 13 gm. diet	Mg. utilisable amino acid/rat/day	Mg. total N/100 gm. diet	Mg. utilisable N/100 gm. diet	Mg. total N/rat/day	Mg. utilisable N/rat/day
Adjusted for		As fed						
l or l.H_2O	DL-form							
132	—	132	17.2	14.2	35.1	35.1	4.6	4.6
95	—	95	12.3	9.1	19.0	19.0	2.5	2.5
—	426	426	55.4	27.7	45.6	22.8	6.0	3.0
—	—	414	53.8	53.8	44.3	44.3	5.8	5.8
442	—	442	57.5	41.9	61.9	61.9	8.0	8.0
—	—	79	10.3	10.3	7.4	7.4	1.0	1.0
—	—	140	18.2	18.2	11.9	11.9	1.5	1.5
—	394	394	51.2	25.7	46.6	23.3	6.0	3.0
—		71	9.2	9.2	9.7	9.7	1.3	1.3
—	437	437	56.8	28.4	52.0	26.0	6.8	3.4
		2630	341.9	238.5	333.5	261.4	43.5	34.1

of Dr. M. S. Dunn, University of California

)

the amino acid mixture that simulated the quantities of the essential amino acids present in the control ration (Table 8), calculations were, therefore, based on 4.27 gm. of lactalbumin. The diet containing this mixture was designated as Diet 2, the "basal essential amino acid diet".

In Series III, graded quantities of the nonessential amino acids were added to the basal essential amino acid diet to provide 10, 20, 30, 40, 50, and 60 mg. of nitrogen per rat per day (Diets 2 and 4 to 10). The proportions used in the development of these mixtures of nonessential acids were also based on the amino acid composition of lactalbumin as indicated by microbiological assay.

The lactalbumin-containing control ration provided 192.1 mg. of nitrogen from the eight nonessential amino acids occurring in the 4.27 gm. of lactalbumin preparation used. The quantity of nitrogen required in any desired mixture then was calculated with the appropriate proportion. From this value the amount of amino acid that would supply the necessary quantity of nitrogen was ascertained. The calculations involved in the formulation of the mixtures are shown in the example presented in Table 9.

The composition of the six mixtures of nonessential acids that supplemented the amino acids in the basal essential amino acid diet is stated in Table 10. With

Table 9. Composition of a mixture of nonessential amino acids which supplied 10 mg. of nitrogen in the proportions found in lactalbumin*

Amino acid	Per cent amino acid in lactalbumin	Mg. amino acid in 4.27 gm. lactalbumin	Per cent N in amino acid	Mg. N in 4.27 gm. lactalbumin	Mg. N from each amino acid in a mixture providing 10 mg. N	Mg. amino acid in a mixture providing 10 mg. N
Alanine**	2.40	102.2	15.7	16.04	.835	5.32
Aspartic acid	8.50	362.1	10.5	38.02	1.979	18.85
Cystine	1.82	77.5	11.6	8.99	.468	4.03
Glutamic acid	14.60	622.0	9.5	59.09	3.077	32.39
Glycine	1.63	69.4	18.6	12.91	.672	3.61
Proline	4.35	185.3	12.2	22.61	1.177	9.65
Serine**	4.29	182.7	13.3	24.30	1.266	9.52
Tyrosine	3.08	131.2	7.7	10.10	.526	6.83
				192.06	10.000	90.20

*Analyzed through the courtesy of Dr. M. S. Dunn, University of California, at Los Angeles

**Values taken from Schmidt (1944)

Table 10. Composition of mixtures of nonessential amino acids formulated for Series III so
quantities of nitrogen (used in Diets 2 and 4 to 9)

Amino acid	Mg. amino acid/100 gm. diet to supply						Mg. amino acid/rat/day in 13 gm. diet to supply						
	10 mg. N	20 mg. N	30 mg. N	40 mg. N	50 mg. N	60 mg. N	10 mg. N	20 mg. N	30 mg. N	40 mg. N	50 mg. N	60 mg. N	10 mg. N
Alanine	41	82	123	164	205	246	5.3	10.6	16.0	21.3	26.6	31.9	0.83
Aspartic acid	145	290	435	580	725	870	18.8	37.7	56.6	75.4	94.3	113.1	1.98
Cystine	31	62	93	124	155	186	4.0	8.1	12.1	16.1	20.2	24.2	0.47
Glutamic acid	249	498	747	997	1246	1495	32.4	64.8	97.2	129.6	161.9	194.3	3.08
Glycine	80	160	240	320	401	481	10.4	20.8	32.2	41.7	52.1	62.5	1.94
Proline	74	148	223	297	371	445	9.7	19.3	28.9	38.6	48.2	57.9	1.18
Tyrosine	52	105	158	210	263	315	6.8	13.7	20.5	27.3	34.2	41.0	0.52
Total	672	1345	2019	2692	3366	4038	87.4	175.0	263.5	350.0	437.5	524.9	10.00

nonessential amino acids formulated for Series III so as to provide graded
d in Diets 2 and 4 to 9)

iet	Mg. amino acid/rat/day in 13 gm. diet to supply						Mg. N in 13 gm. diet to supply/rat/day					
60 mg. N	10 mg. N	20 mg. N	30 mg. N	40 mg. N	50 mg. N	60 mg. N	10 mg. N	20 mg. N	30 mg. N	40 mg. N	50 mg. N	60 mg. N
246	5.3	10.6	16.0	21.3	26.6	31.9	0.83	1.67	2.50	3.34	4.17	5.01
870	18.8	37.7	56.6	75.4	94.3	113.1	1.98	3.96	5.94	7.92	9.90	11.87
186	4.0	8.1	12.1	16.1	20.2	24.2	0.47	.94	1.40	1.87	2.34	2.81
1495	32.4	64.8	97.2	129.6	161.9	194.3	3.08	6.15	9.23	12.31	15.39	18.46
481	10.4	20.8	32.2	41.7	52.1	62.5	1.94	3.88	5.82	7.75	9.69	11.63
445	9.7	19.3	28.9	38.6	48.2	57.9	1.18	2.35	3.53	4.71	5.88	7.06
315	6.8	13.7	20.5	27.3	34.2	41.0	0.52	1.05	1.58	2.10	2.63	3.16
4038	87.4	175.0	263.5	350.0	437.5	524.9	10.00	20.00	30.00	40.00	50.00	60.00

one exception, glycine was substituted for serine since the L-form of the latter amino acid is very expensive. In Diet 10, L-alanine, L-aspartic acid, and L-serine replaced the DL-forms of these acids used in formulating Diet 7. The diet therefore contained 111.2 mg. of glycine and 292.9 mg. of serine per 100 gm. Each rat received daily 14.4 mg. of glycine and 38.1 mg. of serine which supplied 2.7 and 5.1 mg., respectively, of nitrogen.

Amino acid mixture used in the diet fed in Series IV

The quantity of each of the essential amino acids in the basal essential amino acid diet (Diet 2) described in Table 8 was doubled. No nonessential amino acids were included. The resulting mixture is indicated in Table 11.

Amino acid mixtures used in diets fed in Series V, VI, and VII

The diets used in Series V represented modifications of Diet 7 which supplied 40 mg. of nitrogen from the nonessential amino acids in addition to the basal essential amino acid diet. In Diet 12, the quantity of methionine was increased as necessary to replace isonitrogenously the amount of cystine present in Diet 7. In Diet 13, the quantity of phenylalanine was increased to replace tyrosine isonitrogenously. In Diet 14, both methionine and phenylalanine were increased, cystine and tyrosine being

Table 11. Composition of the mixture of essential amino acids used in Diet 11 and fed in Series IV

Amino acid	Mg. amino acid as fed per 100 gm. diet	Mg. utilizable amino acid per rat per day (*i.e.*, in 13 gm. diet)	Mg. total N per rat per day	Mg. utilizable N per rat per day
Arginine.HCl	264	28.4	9.2	9.2
Histidine.HCl.H_2O	190	18.2	5.0	5.0
Isoleucine	852	55.4	12.0	6.0
Leucine	828	107.6	11.6	11.6
Lysine.HCl.H_2O	884	83.8	16.0	16.0
Methionine	158	20.6	2.0	2.0
Phenylalanine	280	36.4	3.0	3.0
Threonine	788	51.4	12.0	6.0
Tryptophane	142	18.4	2.6	2.6
Valine	874	56.8	13.6	6.8
Total	5260	477.0	87.0	68.2

replaced isonitrogenously by methionine and phenylalanine, respectively.

In Series VI, the basal essential amino acid diet was used without alteration, and all diets contained 40 mg. of nitrogen from the nonessential amino acids. However, from each mixture, a single nonessential acid or a group of acids was omitted, the remaining nonessential acids being adjusted proportionately to keep the total supply of nitrogen constant. Thus, in formulating Diet 15, three monoaminomonocarboxylic acids, alanine, glycine, and serine were deleted. Diet 16 lacked the dicarboxylic acids. Diets 17, 18, and 19 were devoid of cystine, proline, and tyrosine, respectively.

In Series VII, only two diets were fed. Diet 20 contained the basal essential amino acid mixture supplemented with 30 mg. of glutamic acid per day. Diet 21 resembled Diet 14 in that methionine and phenylalanine were increased to replace isonitrogenously cystine and tyrosine, respectively; glutamic acid was then added to provide the same quantity of nitrogen present in all other nonessential acids in Diet 7.

The basic calculations involved in the formulation of the various mixtures have been illustrated in previous sections. The composition of the mixtures is presented in Tables 12 and 13.

Table 12. Composition of the mixtures of amino acids used in Ser

Amino acid	Mg. amino acid as fed/ 100 gm. of Diet No.*					Mg. utilizable amino acid/rat/day (*i.e.*, per 13 gm. of Diet No.)					Mg.
	12	13	14	20	21	12	13	14	20	21	12
Arginine.HCl	132	132	132	132	132	14.2	14.2	14.2	14.2	14.2	4.6
Histidine.HCl.H_2O	95	95	95	95	95	9.1	9.1	9.1	9.1	9.1	2.5
Isoleucine	426	426	426	426	426	27.7	27.7	27.7	27.7	27.7	6.0
Leucine	414	414	414	414	414	53.8	53.8	53.8	53.8	53.8	5.8
Lysine.HCl.H_2O	442	442	442	442	442	41.9	41.9	41.9	41.9	41.9	8.0
Methionine	232	79	232	79	232	30.2	10.3	30.2	10.3	30.2	2.9
Phenylalanine	140	350	350	140	350	18.2	45.5	45.5	18.2	45.5	1.5
Threonine	394	394	394	394	394	25.7	25.7	25.7	25.7	25.7	6.0
Tryptophane	71	71	71	71	71	9.2	9.2	9.2	9.2	9.2	1.3
Valine	437	437	437	437	437	28.4	28.4	28.4	28.4	28.4	6.8
Total	2783	2840	2993	2630	2993	258.4	265.8	285.7	238.5	285.7	45.4
Alanine	164	164	164	—	—	21.3	21.3	21.3	—	—	3.3
Aspartic acid	580	580	580	—	—	75.4	75.4	75.4	—	—	7.9
Cystine	—	124	—	—	—	—	16.1	—	—	—	—
Glutamic acid	997	997	997	2429	2917	129.6	129.6	129.6	315.8	379.2	12.3
Glycine	320	320	320	—	—	41.7	41.7	41.7	—	—	7.7
Proline	297	297	297	—	—	38.6	38.6	38.6	—	—	4.7
Tyrosine	210	—	—	—	—	27.3	—	—	—	—	2.1
Total	2568	2482	2358	2429	2917	333.9	322.7	306.6	315.8	379.2	38.0

*Diets 12, 13 and 14 were fed in Series V, and Diets 20 and 21 in Series VII

res of amino acids used in Series V and VII*

le amino acid/rat/day .3 gm. of Diet No.)			Mg. total N/rat/day in Diet No.					Mg. utilizable N/rat/day in Diet No.				
14	20	21	12	13	14	20	21	12	13	14	20	21
14.2	14.2	14.2	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
9.1	9.1	9.1	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
27.7	27.7	27.7	6.0	6.0	6.0	6.0	6.0	3.0	3.0	3.0	3.0	3.0
53.8	53.8	53.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
41.9	41.9	41.9	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
30.2	10.3	30.2	2.9	1.0	2.9	1.0	2.9	2.9	1.0	2.9	1.0	2.9
45.5	18.2	45.5	1.5	3.6	3.6	1.5	3.6	1.5	3.6	3.6	1.5	3.6
25.7	25.7	25.7	6.0	6.0	6.0	6.0	6.0	3.0	3.0	3.0	3.0	3.0
9.2	9.2	9.2	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
28.4	28.4	28.4	6.8	6.8	6.8	6.8	6.8	3.4	3.4	3.4	3.4	3.4
285.7	238.5	285.7	45.4	45.6	47.5	43.5	47.5	36.0	36.6	38.1	34.1	38.1
21.3	—	—	3.3	3.3	3.3	—	—	3.3	3.3	3.3	—	—
75.4	—	—	7.9	7.9	7.9	—	—	7.9	7.9	7.9	—	—
—	—	—	—	1.9	—	—	—	—	1.9	—	—	—
129.6	315.8	379.2	12.3	12.3	12.3	30.0	36.0	12.3	12.3	12.3	30.0	36.0
41.7	—	—	7.7	7.7	7.7	—	—	7.7	7.7	7.7	—	—
38.6	—	—	4.7	4.7	4.7	—	—	4.7	4.7	4.7	—	—
—	—	—	2.1	—	—	—	—	2.1	—	—	—	—
306.6	315.8	379.2	38.0	37.8	35.9	30.0	36.0	38.0	37.8	35.9	30.0	36.0

nd 21 in Series VII

Table 13. Composition of the mixtures of amino acids used in Serie

Amino acid	Mg. amino acid as fed/ 100 gm. of Diet no.					Mg. utilizable amino acid/rat/ day (i.e., in 13 gm. Diet no.					Mg. ut
	15	16	17	18	19	15	16	17	18	19	15
Arginine.HCl	132	132	132	132	132	14.2	14.2	14.2	14.2	14.2	4.6
Histidine.HCl.H_2O	95	95	95	95	95	9.1	9.1	9.1	9.1	9.1	2.5
Isoleucine	426	426	426	426	426	27.7	27.7	27.7	27.7	27.7	3.0
Leucine	414	414	414	414	414	53.8	53.8	53.8	53.8	53.8	5.8
Lysine.HCl.H_2O	442	442	442	442	442	41.9	41.9	41.9	41.9	41.9	8.0
Methionine	79	79	79	79	79	10.3	10.3	10.3	10.3	10.3	1.0
Phenylalanine	140	140	140	140	140	18.2	18.2	18.2	18.2	18.2	1.5
Threonine	394	394	394	394	394	25.7	25.7	25.7	25.7	25.7	3.0
Tryptophane	71	71	71	71	71	9.2	9.2	9.2	9.2	9.2	1.3
Valine	437	437	437	437	437	28.4	28.4	28.4	28.4	28.4	3.4
Total	2630	2630	2630	2630	2630	238.5	238.5	238.5	238.5	238.5	34.1
Alanine	—	331	172	185	173	—	43.0	22.4	24.1	22.5	—
Aspartic acid	802	—	6–8	657	612	104.3	—	79.0	85.4	79.6	11.0
Cystine	172	251	—	141	131	22.3	32.6	—	18.3	17.0	2.6
Glutamic acid	1379	—	1045	1130	1052	179.3	—	135.9	146.8	136.7	17.0
Glycine	—	648	336	363	338	—	84.3	43.7	47.2	44.0	—
Proline	411	600	311	—	313	53.4	78.0	40.5	—	40.7	6.5
Tyrosine	291	425	220	238	—	37.8	55.3	28.7	30.9	—	2.9
Total	3055	2256	2692	2714	2619	397.1	293.2	350.2	352.7	340.5	40.0

*The total quantity of nitrogen provided per rat per day by the ten essential amino aci
the D-forms of isoleucine, threonine, and valine contributed 3.0, 3.0, and 3.4 mg. of nitro

le 13. Composition of the mixtures of amino acids used in Series VI

ino acid as fed/ . of Diet no.				Mg. utilizable amino acid/rat/ day (i.e., in 13 gm. Diet no.					Mg. utilizable N*/rat/day in Diet no.				
	17	18	19	15	16	17	18	19	15	16	17	18	19
2	132	132	132	14.2	14.2	14.2	14.2	14.2	4.6	4.6	4.6	4.6	4.6
5	95	95	95	9.1	9.1	9.1	9.1	9.1	2.5	2.5	2.5	2.5	2.5
6	426	426	426	27.7	27.7	27.7	27.7	27.7	3.0	3.0	3.0	3.0	3.0
4	414	414	414	53.8	53.8	53.8	53.8	53.8	5.8	5.8	5.8	5.8	5.8
2	442	442	442	41.9	41.9	41.9	41.9	41.9	8.0	8.0	8.0	8.0	8.0
9	79	79	79	10.3	10.3	10.3	10.3	10.3	1.0	1.0	1.0	1.0	1.0
0	140	140	140	18.2	18.2	18.2	18.2	18.2	1.5	1.5	1.5	1.5	1.5
4	394	394	394	25.7	25.7	25.7	25.7	25.7	3.0	3.0	3.0	3.0	3.0
1	71	71	71	9.2	9.2	9.2	9.2	9.2	1.3	1.3	1.3	1.3	1.3
7	437	437	437	28.4	28.4	28.4	28.4	28.4	3.4	3.4	3.4	3.4	3.4
0	2630	2630	2630	238.5	238.5	238.5	238.5	238.5	34.1	34.1	34.1	34.1	34.1
1	172	185	173	—	43.0	22.4	24.1	22.5	—	6.8	3.5	3.8	3.5
-	6-8	657	612	104.3	—	79.0	85.4	79.6	11.0	—	8.3	9.0	8.3
1	—	141	131	22.3	32.6	—	18.3	17.0	2.6	3.8	—	2.1	2.0
-	1045	1130	1052	179.3	—	135.9	146.8	136.7	17.0	—	12.9	13.9	13.0
8	336	363	338	—	84.3	43.7	47.2	44.0	—	15.7	8.1	8.8	8.2
0	311	—	313	53.4	78.0	40.5	—	40.7	6.5	9.5	5.0	—	5.0
5	220	238	—	37.8	55.3	28.7	30.9	—	2.9	4.2	2.2	2.4	—
6	2692	2714	2619	397.1	293.2	350.2	352.7	340.5	40.0	40.0	40.0	40.0	40.0

nitrogen provided per rat per day by the ten essential amino acids was 43.5 mg. since
hreonine, and valine contributed 3.0, 3.0, and 3.4 mg. of nitrogen, respectively

Preparation of amino acid mixtures

A sufficient quantity of each composite of amino acids was prepared for the entire feeding interval. Despite careful control, a relatively large quantity of diet is wasted by the force-feeding technique. In calculating the quantity of each amino acid required, provision was made for the determination of nitrogen in the composite, and for adjustment of the amount of amino acid mixture to be incorporated in the diet in case the nitrogen found in the composite failed to correspond with the theoretical value. The amount prepared also covered the needs for analysis of the diet in the dry and homogenized states.

The amino acids were weighed on an analytical balance when humidity was relatively low, transferred to a mortar approximately 6 inches in diameter, and ground to a fine powder. They were carefully tabled together and the mixtures analyzed immediately.

Estimation of the Daily Allowance of Food

The force-feeding technique eliminates the errors inherent in experiments wherein the animals may refuse to eat a sufficient quantity of ration for their body needs. Nevertheless, certain problems are raised by the arbitrary selection of a specific weight of food to represent the daily quota. For example, physiological or metabolic disorders may arise if an excessive amount is administered, particularly in the rat which cannot regurgitate; on the other hand, the dietary demands of the organism may be underestimated. Body weight and stomach capacity are important factors controlling the quantity of food eaten or that can be administered. Probably the quantity which can be efficiently metabolized varies also with the nature and completeness of the test ration. The use of a high proportion of dextrin in a diet that is force-fed adds a further complication because it affects the viscosity of the slurry which in turn introduces difficulties in the measurement and administration of the daily quota of food.

Kuehl (1949) more or less arbitrarily selected 13 gm. as the quota of ration to be provided daily. The striking behavior of her animals suggested that excess food might be a predisposing factor. In order to rule out this possibility, the food intakes of groups of rats that had been used for other experiments in the laboratory were studied. In one experiment, the average daily food intakes of 11 adult male rats weighing approximately 325 gm. that had been transferred directly from the stock colony and offered a ration containing 14 per cent protein from lactalbumin were 12.3, 12.6, 12.9, and 13.0 gm., in four successive periods of four days each.* Individual variations were observed, but rarely exceeded 1.0 gm. Similar results were obtained when a diet containing 18 per cent protein from defatted dehydrated whole eggs was provided, the daily intakes being 12.1, 12.8, 12.7, and 12.5 gm. in periods corresponding to those in the preceding experiment.

To obtain further information under the conditions of the present experiment concerning the voluntary food intake of undepleted adult male rats an adequate synthetic diet containing lactalbumin** was fed to 12 healthy rats. The ration contained 618 mg. of nitrogen per 100 gm., a proportion estimated to permit only slightly positive nitrogen balance. Data relative to food consumption and body weight during 25 days are presented in Table 14.

With a single exception, body weight was maintained throughout the test. It is apparent that the animals tended to reduce their food intake as the experiment progressed. The smaller animals consistently ate about one gm. less per day than the larger rats. That the quantity was adequate was indicated by the attainment of nitrogen equilibrium by 60 per cent of the animals.

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*Unpublished data, Files of Nutrition Laboratory, The Iowa State College

**From Nutritional Biochemicals, Ltd.

After studying the above data, 13 gm. was selected as the daily allowance of all rations that were force-fed, since the rats available for study weighed about 350 gm.

Table 14. Average daily food intake of adult rats offered an adequate diet containing approximately 4 per cent of lactalbumin*

Rat	Body wt.	Days of experiment					Av. for 25 days
		1-3	4-7	8-11	18-18	19-25	
1	336	13.7	12.4	8.6	12.5	11.5	11.7
2	340	13.1	12.7	11.6	10.7	10.9	11.8
3	338	14.2	13.2	13.9	10.3	9.8	12.3
4	338	14.3	14.0	15.4	12.9	12.1	13.8
5	349	14.8	14.5	14.9	13.3	12.1	13.9
6	348	14.4	14.3	14.3	12.8	11.8	13.5
Av.	341	14.1	13.5	13.1	12.1	11.4	12.8
7	304	11.8	11.5	10.5	9.8	10.0	10.7
8	290	13.6	11.0	11.2	9.7	9.7	11.0
9	293	13.4	11.8	12.0	10.2	10.1	11.5
10	286	13.3	12.5	11.6	11.0	12.2	12.1
11	286	14.2	14.0	12.7	11.6	11.5	12.8
12	290	14.0	13.5	12.8	11.1	10.9	12.5
Av.	290	13.4	12.4	11.4	10.6	10.7	11.7

*From Nutritional Biochemicals, Ltd., as fed in Series II

Homogenization of the Diets

Preliminary trials indicated that a satisfactory consistency for force-feeding was achieved when 250 gm. of the basal diet, 9.6 gm. of the dry vitamin mixture, 0.96

gm. of cod liver oil, and 0.96 gm. of a solution of alpha tocopherol in Wesson oil were homogenized. The oils were delivered by means of a calibrated dropper. The resulting quantity of diet (465 to 470 ml.) was sufficient to feed five rats for three days. The appropriate volume of distilled water was warmed to a point between 38 and 40 degrees C. and transferred to a Stevens blendor. The dietary ingredients were added and homogenized for two minutes. The homogenate was placed in two half-pint jars instead of a single large container to avoid repeated warming of the diet before feeding. Homogenized diets were refrigerated immediately and continually, except at feeding intervals. By calculation, 100 gm. of the basal diet and the appropriate vitamin supplements contained 465 calories. Analysis of similar diets by bomb calorimeter yielded an average value of 464 calories per 100 gm. Each rat was given approximately 60 calories daily, the caloric density of the diet being 2.5 cal. per ml.

Administration of the Diets

The force-feeding technique described by Reinecke *et al*. (1939) was used. The diets were allowed to reach room temperature before they were administered.

A veterinary syringe (No. I.D. 105) with a capcity of 20 ml. was fitted with a blunt needle carrying a No. 8 French catheter. The necessary quantity of diet was drawn into the syringe; the needle and catheter were attached and filled, and the syringe adjusted to the proper mark. The syringe was supported on a box approximately as high as the rat cage and close to it. By resting the left wrist on the edge of the open cage, the worker was able to support the animal within the cage during feeding so that only the head was above the cage, thereby reducing the possibility of losing metabolic materials. The front feet of the animal were immobilized with the thumb and second finger of the left hand, and the first finger was placed firmly on the head. The mandibles were separated by gentle pressure, and with the right hand the catheter, moistened in cool water, was inserted to a point approximately 4-1/4 inches from the tip.

The number, volume, and times of feeding were established on the basis of data obtained in Series I and II.

CRITERIA SELECTED FOR EVALUATION OF NITROGEN UTILIZATION

Nitrogen Balance

Plan of the balance experiment

The balance test was divided into two periods: (1) the preliminary period in which the animals became adjusted to the experimental ration and the force-feeding technique, and (2) the balance period. The most desirable interval for the study of metabolism was determined in Series II.

On the day preceding the initiation of the experiment, the stock ration was removed from the cage at 10 P.M., approximately ten hours before the experimental diet was administered. On the day preceding a balance period, the total quantity of food was reduced from 24 ml. to 20 ml., and the last feeding given at 4 P.M. Food jars were removed at 10 P.M. from animals that were fed *ad libitum*. On the first day of the balance period, the animals were transferred to wide-meshed metabolism cages between 7:30 and 8:00 A.M. On the last day of the balance test, the feeding schedule was similar to that on the day preceding the balance period, and at the conclusion of the balance period, animals were removed from the metabolism cages at the same hour that they had been placed on balance.

Procedure for the collection of urine and feces

The urine was collected on acid-treated filter papers*, one being allowed for each day of the balance test and two additional papers being added per period to ensure complete absorption of urine. One filter paper was removed daily, and placed in a 400-ml. flask containing 200 ml. of 20 per cent hydrochloric acid.

Feces were removed daily, brushed free from hair, and placed in an Erlenmeyer flask containing 50 ml. of 20 per cent hydrochloric acid. To mark the beginning and end of the balance interval, 100 mg. of ferric oxide were added per 100 gm. of dry diet or an equivalent quantity of homogenized diet. On the first day on which feces were collected, only the red feces were considered to belong to the metabolism period and at the conclusion of the experiment, all feces were collected until the first red ones appeared.

On the day on which rats were removed from the metabolism cages, the cages were quantitatively washed with hot distilled water, and the washings added to the flask

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*500 9-inch filter papers (Schleicher and Schull, No. 597) were left overnight in a solution of 900 ml. of 95 per cent alcohol and 100 ml. of glacial acetic acid and dried.

containing filter papers and urine in hydrochloric acid. The urine was removed from the papers with suction, and the resulting solution diluted to a volume of 2000 ml.

Feces were autoclaved for 2 hours at 15 pounds pressure, pressed through a fine sieve, and diluted to a final volume of 250 ml.

The determination of nitrogen

The concentration of nitrogen present in the diets, livers, and excreta was determined by the Kjeldahl-Gunning procedure. The ammonia formed during distillation was collected in approximately 0.1N hydrochloric acid which contained a mixed indicator of methylene blue and methyl red. Some experimental details are shown in Table 15.

The ability of the author to recover nitrogen from a standard solution has been demonstrated previously (Clark, 1945). Recovery of nitrogen after dispersion of a standard solution of $(NH_4)_2SO_4$ over the bottom of the metabolism cage was satisfactory (Table B, Appendix I). Similar evidence of the reliability of the method as applied by S. P. Yang, who has conducted the major portion of the nitrogen determinations, is on file in the Foods and Nutrition Laboratory.

Table 15. Determination of nitrogen in various materials

Nature of sample	Total volume	Size of sample	H_2SO_4	K_2SO_4	HgO	Length of digestion period
	ml.		ml.	gm.	gm.	hr.
Diets*	--	2-4 gm.	35	15	.7	2
Vitamins*	--	1 gm.	35	15	.7	2
Amino acids	--	120-300 mg.	20	10	.7	1-1/4
Urine	2000	50 ml.	20	10	.7	1-1/4
Feces	250	25 ml.	20	15	.7	1-1/2
Hepatic tissue	250	10 ml.	20	15	.7	1-1/2

*The addition of 50 ml. of water before the sulfuric acid facilitated digestion of these samples

Analyses of Hepatic Tissue

The weight of the liver and the concentrations of nitrogen and water were determined. The organ was removed 24 hours after the completion of the balance test, a procedure necessitated by the time required for the appearance of the red feces. During this interval, the same feeding schedule was followed as on the last day of the balance period.

The animals were sacrificed at 8 A.M., one rat being taken from each group in turn. They were anesthetized with a solution of sodium pentobarbital injected intrapleurally. After an incision was made along the linea alba, the abdominal aorta was severed to permit speedy withdrawal of blood from the liver. The kidney, adrenals, and liver were removed. The adrenal glands and liver were carefully and quickly trimmed. A cross-section of the largest lobe and the tip of the longest lobe of the liver were placed in a weighed glass-stoppered weighing bottle for the determination of water and fat. The remaining portion of the liver was placed in a weighed Erlenmeyer flask, weighed, and covered with 50 ml. of a 20 per cent solution of hydrochloric acid. All procedures were carried out as rapidly as possible.

For later analytical purposes, samples of hepatic tissue were dried at 105 degrees C in an air oven until they reached constant weight, the time required being approximately one week. Samples used for the determination of nitrogen were autoclaved at 15 pounds pressure for 2 hours, pressed through a fine sieve, and diluted to a final volume of 250 ml.

RESULTS AND DISCUSSION

SERIES I

The Kuehl Syndrome

The chief external manifestations associated with the omission of amino acids from the diet are loss of weight in the adult animal and retardation of growth in the young. No specific syndrome has been attributed to the absence from the diet of any single amino acid except valine (Rose and Eppstein, 1939) and tryptophane (Spector and Adamstone, 1950). For this reason, the significance of the syndrome induced when Kuehl (1949) fed the essential amino acids as the sole source of nitrogen in an otherwise adequate diet assumed interesting proportions, and the ability to reproduce the disorder became important.

In Series I of the present investigation, the experimental conditions imposed by Kuehl were replicated as closely as possible, and the response of the test animals was studied in respect to external manifestations, nitrogen balances, and necropsy findings.

Adult well-nourished male rats weighing approximately 320 gm. were used. Again the test ration (Diet 2a,

Table 7) supplied quantities of the ten essential amino acids equivalent to those present in the control ration prepared by Kuehl containing 4.06 per cent of water-free, ash-free lactalbumin. It was force-fed. The daily quota of homogenized diet, 24 ml., was prepared from 13 gm. of solid diet, and was administered in two equal portions. It provided approximately 60 calories per rat per day.

That a mixture of amino acids containing only the ten essential amino acids was incapable of supporting protein nutrition under conditions of force-feeding has been demonstrated again. Indeed, the responses of the animals were even more dramatic than those observed in 1949.

As in Kuehl's experiment, the nitrogen balances of all rats that were force-fed the mixture of ten essential amino acids were distinctly negative. On the average, the rats lost 238 mg. of nitrogen per 300 gm. of body weight in the 7-day experimental period (See Table 16). Negative balances reported by Kuehl averaged 213 mg. per 7 days.

A greater proportion of the rats died than in Kuehl's study. From the group of 16 animals, eight died (Table 17), and another rat was almost moribund at the conclusion of the experiment. Many of these animals showed a precipitous loss in weight just prior to death. The critical

Table 16. Nitrogen balances per 7 days of rats in Series II, force-fed their daily quotas of Diet 2a in two portions when the vitamin supplement was either fed separately or homogenized with the basal diet

Method of feeding vitamins	Rat no.	Body wt.	N in food	Total N excreted	N balance	N balance per 300 gm. rat
		gm.	mg.	mg.	mg.	mg.
Separate from basal diet	49837	285	322	578	-256	-269
	49868	313	322	662	-340	-326
	49987	297	322	589	-267	-270
	50042	298	322	492	-170	-171
	Average	298	322	580	-258	-259
Homogenized with basal diet	50177	344	349	614	-265	-231
	50201	319	349	554	-205	-193
	50209	327	349	572	-223	-205
	Average	330	349	580	-231	-210

Table 17. Body weights of rats in Series I and II at successive intervals of the experiment when Diet 2a containing only the ten essential amino acids was force-fed in two portions daily

Group	Rat no.	Pre-experimental weight	Weight on different days					
			1st	7th	11th	14th	18th	25th
		gm.	gm.	gm.	gm.	gm.	gm.	gm.
A***	49837	325	304	301	286	283	284	283
	49868	321	316	314	314	310	312	310
	49987	328	320	315	296	296	294	292
	49994	316	307	298	300	292*	---	---
	50033	325	316	304	293*	---	---	---
	50042	308	298	296	303	296	301	298
	Average	320	310	311	301	298	299	295
B****	49806**	315	304	303	288	274	271	---
	49963**	323	309	306	---	---	---	---
	49989	314	292	286*	---	---	---	---
	49995	326	317	314	302*	---	---	---
	50034	331	321	321	302*	---	---	---
	50041	320	308	306	286	272	272	252
	Average	321	308	308	296	273	271	252
C****	50130	332	320	308	302	288*	---	
	50177	375	366	360	352	346	343	
	50185	353	325	320	316*	---	---	
	50201	358	338	336	327	320	320	
	50209	368	350	348	330	330	330	
	50216	353	342	328	324	312*	---	
	Average	356	340	333	325	319	331	

*Died

**Accidentally killed by introducing the catheter into the trachea when they struggled

***Vitamins fed separately

****Vitamins homogenized with basal diet period apparently lay between the eleventh and fourteenth days of the experiment.

Of the surviving animals, all but two lost weight. The average loss in the first 18 days of the test was 20 gm., a response nearly identical to the one recorded by Kuehl.

Almost all of the rats had diarrhea during the first week of force-feeding. This symptom then gradually disappeared, although a few rats eliminated loose masses of fecal material until death or until the termination of the experiment. Abdominal distension was observed in some rats but not in all.

The general behavior pattern of the animals fed only the ten essential amino acids closely resembled that depicted by Kuehl in 1949. In fact, it was so similar that the photographs in Plate 1 typify rats in Series I although they were taken during Kuehl's test. Their unkempt appearance in contrast with that of the normal rat fed the control diet containing 4.06 per cent of lactalbumin is apparent, also.

From the time that the experiment was initiated, the rats were difficult to feed. They avoided being picked up, resisted the introduction of the catheter between their jaws, and scratched vigorously with their hind feet to free themselves. The violent struggling of two animals led to the accidental introduction of the catheter into the trachea, and to death one-half hour later (Table 17).

The membranes lining the mouth seemed sensitive; they became red and inflamed within a few days. Within a week, excessive salivation, usually accompanied by a foul odor, was observed in most of the animals. The mouths of such rats frequently had a foamy appearance. Later, a denuding of the chin occurred.

The hyperexcitability described by Kuehl was pronounced in these animals. They frequently scratched the sides and floor of the cage violently, particularly just after they were fed. They rubbed their faces vigorously, as shown in Plate 1, and shook their paws rapidly. The latter motion resembled the reaction that is produced when one transfers the limbs from very cold to hot water. It sometimes preceded, sometimes followed the cage-scratching activity. The paws became red, a condition probably due, in part at least, to friction with the cage. Peripheral flow of blood, however, may be stimulated by the administration of amino acids, Collentine (1948) having reported a sensation of warmth and tingling in the extremities of a patient who had been given glycine by infusion. The behavior of one animal supported the latter idea. It often placed its forepaws in the water as though cooling them.

Convulsions occurred in only one rat, and developed after food had been delivered inadvertently into the trachea. In general, the animals tested in Series I tended to become comatose instead of convulsive.

A very characteristic posture was observed during periods of rest and quiet. After as short a time as four days of force-feeding, the rats began resting their heads on the holders of the water fountains. Within ten days, almost all of them placed their heads or bodies there. Sometimes they reclined against the side of the cage in a slanting position as though elevation and extension of the body provided some relief. The rats usually assumed this pose in the interval immediately subsequent to feeding. Sometimes they remained in the same position for an hour or more.

The animals in the present experiment did not crawl about the floor of the cage in a circular manner as consistently as did those observed by Kuehl. More often they lay limp, as though dead, or staggered dizzily across the cage. The actions of certain animals two or three hours after feeding suggested water intoxication and/or thiamine deficiency. Instability, rotary movement, and occasionally a slight stiffness of the tail were noted. Approximately four hours after feeding, the animals became more active. Those animals that developed a coma-like condition were

Upper left.	Rat fed the control ration containing 4.06 per cent of lactalbumin
Upper right.	Rat fed a ration containing only the ten essential amino acids
Center.	Posture assumed when shaking paws
Bottom.	Characteristic actions: rubbing face and scratching cage

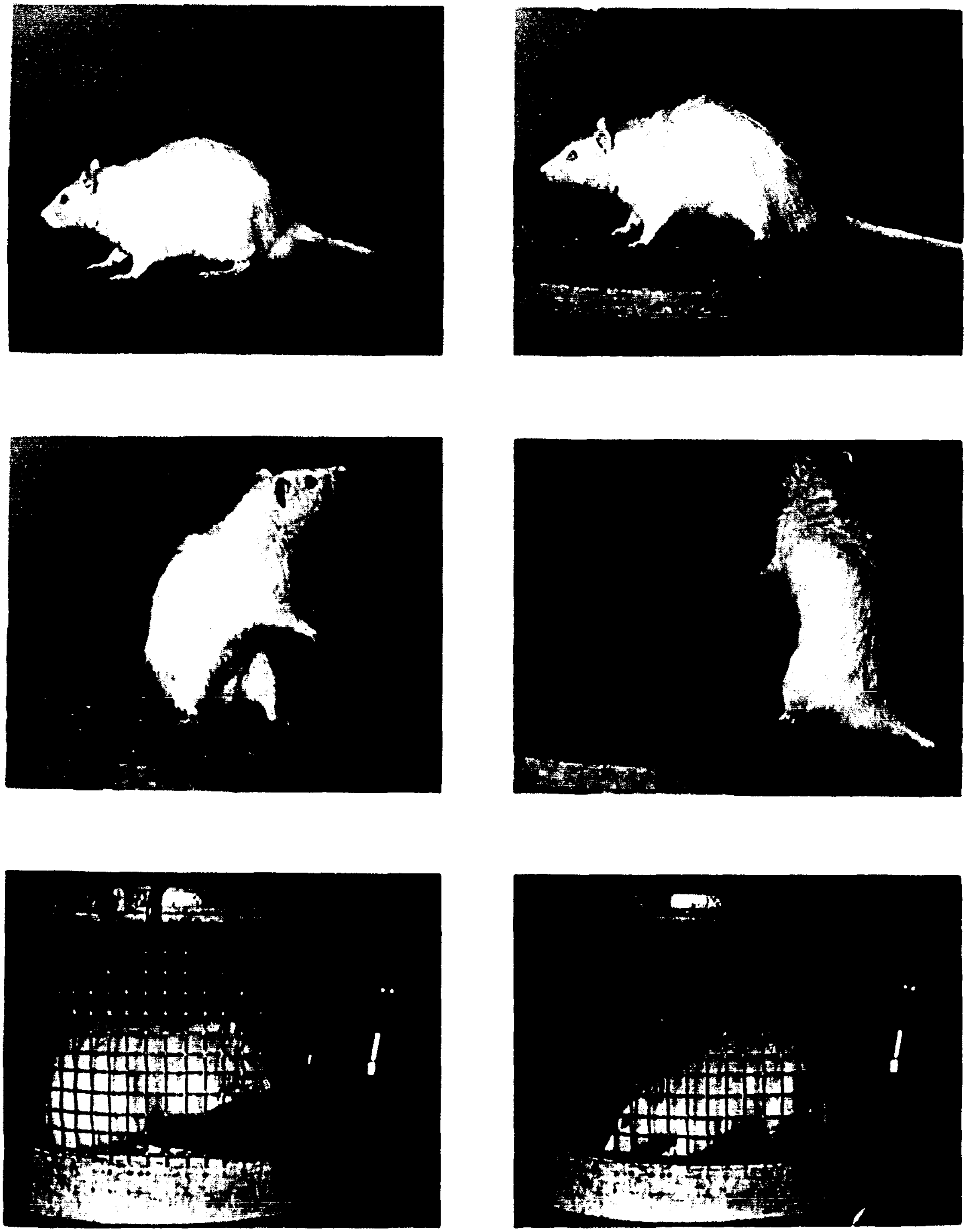

Plate 1. Rats fed a diet containing either 4.06 per cent of lactalbumin or the ten essential amino acids contained therein

apt to die if they did not recover within a few hours. On the day before the first balance test, the interval between feeding periods was reduced from 12 to eight hours in order to take care of the lag in the urinary excretion of nitrogen. The early administration of the second portion of food seemed to cause particular distress. Two animals succumbed after the second feeding, and others barely survived.

The rats consistently drank large amounts of water in addition to the quantity, 14 ml. per day, that was administered in the homogenized diet. The average total daily intake of four rats in Group A (Table 17) was 42 ml. in contrast to 28 ml. consumed by rats fed the same diet under different experimental conditions. The surviving rat in Group B drank 55 ml. or more in addition to the daily quota of water. Enuresis was observed in several animals.

Disturbances in water balance occurred in several cases. Between five and 15 ml. of thin serous fluid were found in the peritoneal cavity of three rats, one of which also had an excessive quantity of fluid in the pleural cavity. A light brown fluid began to flow from the mouth of another rat two hours after it was fed and continued until the animal died five hours after it was fed. At necropsy, 13 ml. of serous fluid were removed from the pleural cavity. The animals that survived the test did not have excessive quantities of fluid in the peritoneal or pleural cavities.

The livers of rats that died were pale and sometimes mottled. The livers of two rats were covered with a tenacious white film that could not be separated from the hepatic tissue. A peculiar "sweetish" odor was noted that was not present in other animals of this or later series. Also, large globules or even masses of material resembling butterfat were found on the diaphragm and/or mesentery of the two rats.

The adrenal glands of the rats that survived to the end of the experiment were conspicuously enlarged, 58 vs. 35 mg. (Table C, Appendix I). Those removed from rats that died were even larger. The kidneys were pale or hemorrhagic, seldom normal.

The cardiac portions of the stomachs of all animals in Series I were ulcerated, one-half or more of the entire area being covered with ulcers of moderate or severe degree. Even the rats that succumbed a few days after the initiation of the experiment were affected to some extent. This condition may have contributed to the resistance the animals exhibited to being fed.

Every rat in the series did not develop the syndrome to the same degree of intensity. All, however, displayed certain characteristics of the disorder at some stage of the experiment; none escaped. Some rats, for instance, only rested their heads on the fountain holders; others salivated excessively and had denuded chins; others had diarrhea but none of the other symptoms. In general, the rats that died showed all of the manifestations of the syndrome in its most severe form.

SERIES II

Evaluation of the Force-Feeding Technique

The Kuehl syndrome reproduced in Series I indeed represented a severe nutritional disorder, and it became important to determine whether or not its occurrence was related directly to the quality of the nitrogenous components of the ration. The possibility that the disorder was not specific was suggested by descriptions recently recorded by other investigators of symptoms that appeared when force-feeding was used in the administration of rations deficient in various nutrietns. For example, Spector and Adamstone (1950) ascribed characteristics that strikingly resembled the Kuehl syndrome to a deficiency of tryptophane induced by feeding an acid hydrolyzed casein diet. They wrote,

> . . . the tryptophane-deficient animals displayed the following peculiar behavior pattern after forced feeding: (1) pawing the face as though trying to withdraw the food; (2) crawling around the edge of the cage with the side of the face resting on the floor of the cage; (3) rearing up on the hind legs and pawing frantically with the front paws; (4) after 5 to 10 minutes they would go to sleep, with a tendency to sleep on the side. The latter may have been due to the fact that these rats were frequently bloated and the abdominal area was sensitive. . . . The stomach and intestines were found at autopsy to be greatly distended and filled with liquid and gas.
>
> The rats developed a nervous hyperexcitability which commonly manifested itself in convulsive fits accompanied by screeching, during which they bounced helplessly against all the sides of the cage. These fits frequently ended in death. (p. 216)

Photographs illustrating various positions assumed by Spector's animals are reproduced in Plate 2. They well might represent rats produced in the Iowa laboratory in the present study.

The chief difference in the behavior of the animals in the two laboratories was in respect to the convulsive fits. As noted earlier, none were observed by Kuehl or in Series I of this experiment. The fits attributed by Spector and Adamstone to a deficiency of tryptophane resembled those observed in pyridoxine-deficient animals (Lepkovsky and Krause, 1942). Pyridoxine was provided in adequate amounts, however, when the tryptophane-deficient diet was administered. (See Personal Communication from H. Spector, Appendix II.)

It should be noted that the tryptophane content of the diet fed in Series I of the present experiment was relatively high. It seemed rather that the syndrome was induced in the Iowa laboratory by the absence of the nonessential amino acids, since Kuehl had observed nitrogen equilibrium and normal behavior when the essential amino acids were supplemented with nonessential acids. She also called attention to the need of checking the adequacy of the mixture of essential amino acids in respect to valine, methionine, and phenylalanine before any conclusions were reached concerning the causal factor of the syndrome.

Morse also has described (1946) a disorder remarkably like the one induced by Kuehl which she believed was due to a deficiency of thiamine. She noted hyperexcitability, a disturbance of equilibrium, severe loss in weight, distension of the abdomen, and a negative nitrogen balance in the week preceding the death of the rats.

Since the symptoms produced in the three laboratories were ascribed to deprivation of different nutrients, it seemed likely to the author that the disorders were not related directly to the quality of the diet, but represented instead a response to the manner of its administration.

In this connection, certain questions may be pertinent. Was the total mass of food introduced into the stomach so large that it induced a severe physiological

Reproduced from plates prepared by H. Spector and F. Adamstone, J. Nutr. 40, 229 (1950)

3. Control rat fed 0.20% L-tryptophane

4-8. Rats fed a tryptophane-deficient diet containing acid hydrolyzed casein

7. Rat pawing the face while in a standing position

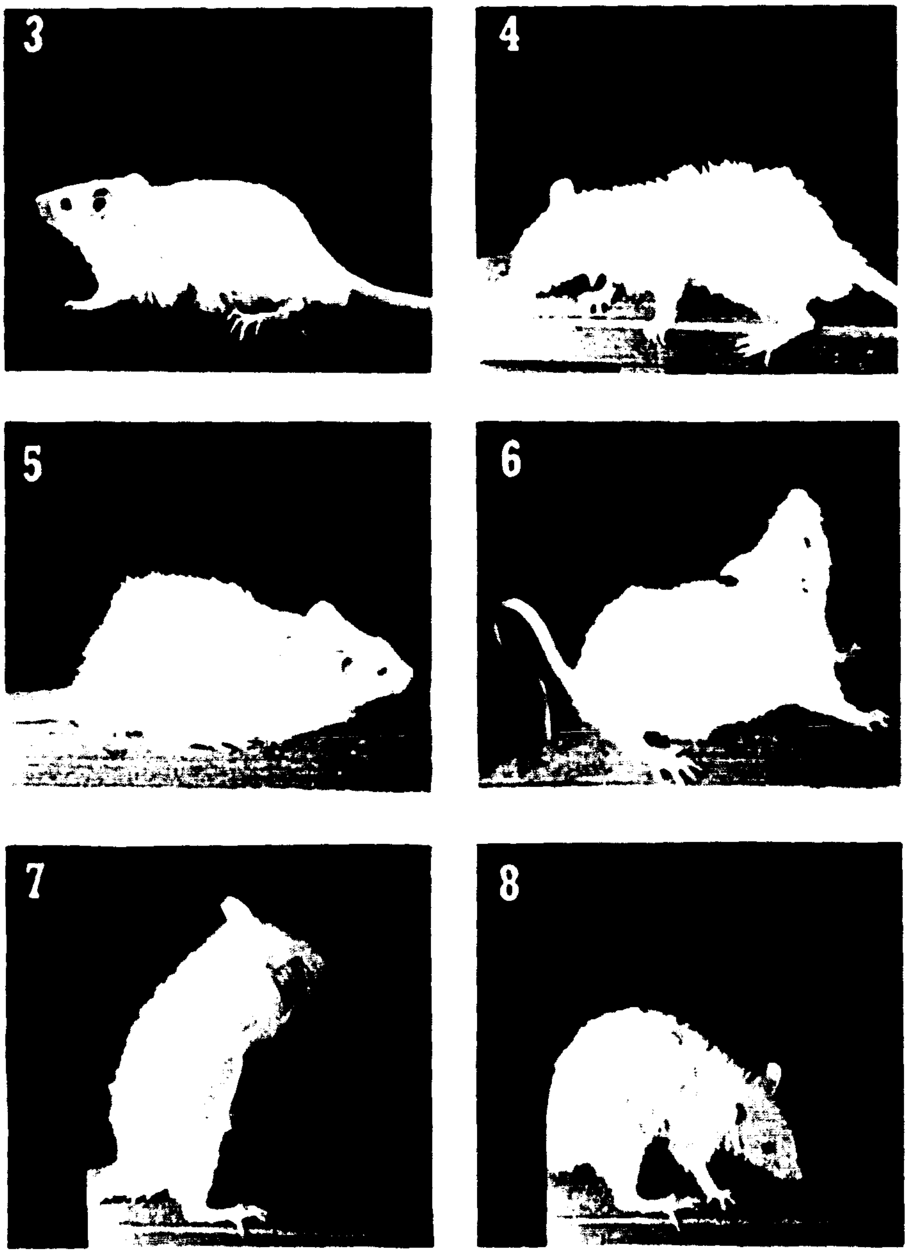

Plate 2. Tryptophane deficiency in the rat

or metabolic stress? Other workers have observed that bloating and diarrhea may accompany the feeding of excessive quantities of food unless the animals have been gradually accustomed to the treatment (Frazier et al., 1947; Denton et al., 1950). Was there an overabundance of calories? Was the system of the animals consequently overtaxed by an excess of metabolites arising from the utilization of carbohydrate and fat? With the influx of an unusually large quantity of food into the alimentary canal were renal thresholds for certain amino acids exceeded, following their absorption from the intestine, so that they were thrown away in the urine instead of being reabsorbed? Or, since the ration was dispersed in 14 ml. of water daily, was the syndrome a manifestation of water intoxication?

It should be noted, also, that initially Kuehl had offered the vitamin supplement apart from the basal ration, and had observed that the disorder was more severe in animals that failed to consume the supplement promptly. Such an observation suggested that the presence of the vitamins simultaneously with the test ration may be vital under conditions of force-feeding. The relative times of ingestion of the amino acids in respect to each other are important (Cannon, 1947; Geiger, 1947). A similar time relationship may exist also between amino acids and vitamins, particularly in view of the significance of certain vitamins in the intermediary metabolism of several amino acids (Umbreit *et al.*, 1948; Schaeffer *et al.*, 1950).

Furthermore, the administration of a definite volume of food in two portions in no way simulates the natural feeding habits of the rat. Entirely different results might be obtained if the same ration were offered *ad libitum*.

The experiments to be described in Series II, therefore, were planned to examine the influence that force-feeding *per se* may have on the outcome of the experiment--in this case specifically the appearance of the Kuehl syndrome--and to determine under what conditions it may be used effectively. The force-feeding technique has been studied from several angles, namely, the manner of supplying the vitamins, the number and size of daily feedings *ad libitum vs.* force-feeding, and the efficiency of delivery of the day's quota of the ration. The utilization of nitrogen and the well-being of the animals were evaluated.

It was necessary also in the development of the present research to determine the length of time that is required to establish relative constancy of nitrogen metabolism under a fixed experimental regime when force-feeding is employed.

Diet 2a, which was used in Series II, was identical with the ration used in Series I (See Table 7). Its sole source of nitrogen was a mixture of the ten essential amino acids simulating those present in a ration containing 4.06 per cent of lactalbumin.

The manner of feeding vitamins

In 1949, the vitamin supplements were offered apart from the basal ration in accordance with the procedure generally used in this laboratory in studies of protein utilization. Animals that are fed ad libitum almost invariably ingest the supplements promptly and completely. Kuehl observed that certain force-fed rats failed to consume the daily quota of vitamins regularly and concluded that such failure may have contributed to the development of the strange behavior pattern of the animals fed only the ten essential amino acids. For this reason, the vitamins were homogenized with the diet in the terminal phase of her experiment.

In the present series, the relationship between the intake of vitamins and the appearance of the Kuehl syndrome when the daily quota of food was administered in two portions was investigated. The vitamin mixture given to one group of rats (Group A, Table 17) was placed in the cage midway between the feeding intervals. An appropriate quantity of vitamins was homogenized with the diet for a second group (Group B, Table 17). Since the number of those animals was reduced by accidental and natural death, a third group was treated similarly (Group C, Table 17). The rats in this group were larger, however, than those in the first two groups, so the total quantity of nitrogen provided in the daily quota of the ration was increased in proportion to the average body weight of the animals (see amino acid mixtures for diets fed in Series I and II).

Data pertaining to changes in body weight and mortality of the animals are summarized in Table 17. The syndrome was marked. All animals at some time exhibited certain aspects of the disorder, although the intensity of the symptoms varied from rat to rat. Only eight animals from an original group of 16 survived.

One-half of the animals in Group A consistently refused to consume the vitamins within 24 hours, whereas the other half ate the supplement regularly and promptly. In view of the significance of several vitamins in amino acid metabolism, it seemed probable that their complete absence from the diet would vitiate the results of the experiment. For this reason, after the first week of the test the quantity of supplement that accumulated in the supplement cup of certain rats was administered by stomach tube every three days midway between feeding periods.

Although it was recognized that this method was not entirely satisfactory, it was considered preferable to complete failure on the part of certain rats to ingest the vitamins.

Refusal of the vitamin supplement was not always reflected in well-being. As shown in Table 16, two animals in Group A died, one of which (No. 49994) always consumed the supplement whereas the other (No. 50033) consistently refused it after the first four days.

No correlation was observed between the tendency to eat the vitamins and the absence of diarrhea. In general, the animals in all groups suffered severe losses in weight.

Sixty per cent of the rats in Groups B and C died. The data suggest that the manner of feeding the vitamins was not associated with the mortality rate. It was clear, however, that neither method of providing the vitamin supplement gave complete protection from the development of the Kuehl syndrome.

The nitrogen balances of the surviving animals in Groups A and C are shown in Table 16. The single value related to Group B was omitted because the animal was in a moribund condition at the termination of the test. The average nitrogen balance, in the interval between the twelfth and the eighteenth days of the experimental period, of the rats to which the vitamins were offered separately was -259 mg. per 300 gm. rat, the range in values being -171 to -326 mg. In contrast, the average value for the rats to which the vitamins were administered with the diet was -210 mg., the range in this case being -193 to -231 mg.

When the force-feeding technique was employed, therefore, retention of nitrogen seemed to be improved by administering the vitamins as part of the dietary mixture instead of offering them separately. Therefore, in all subsequent tests the vitamins were homogenized with the basal diet. This procedure also had an advantage because the animals varied in their willingness to ingest the supplement.

The number of daily feedings

Samuels (1946) has found evidence that hormonal adjustments are made in response to marked changes in the composition of the ration. That time for such adaptation was needed in the present experiment was indicated, since the rats were transferred directly from a stock diet containing a wide variety of natural foods to a synthetic ration consisting of simplified nutrients. Moreover, the volume of diet administered at one feeding was large, and digestive disturbances ensued. The capacity of the stomach may have been exceeded, especially during the first few days, so that possibly also a metabolic stress was imposed when the digested nutrients flooded the blood stream.

The influence upon the development of the Kuehl syndrome and upon nitrogen retention of altering the volume of food offered at a given time was therefore examined. The response of a group of rats given the daily ration in two portions of 12 ml. each was compared with that of another group of rats fed its daily quota of food in three portions of 8 ml. each. The first group was fed at 8 A.M. and 8 P.M.; the second at 8 A.M., 2 P.M., and 8 P.M.

The outcome of this simple alteration in experimental procedure was striking. None of the characteristic symptoms of the Kuehl disorder developed. All six of the rats fed thrice daily survived the experimental interval of 25 days, as shown in Table 18; one-half of those that received their food in two portions died. The animals that were fed three times a day lost only 5 gm. during the first week, and maintained their weight thereafter. In contrast, the surviving rats in the other group lost 20 gm. during the experiment; those that died suffered even greater losses.

All of the animals that were fed three times a day were free from diarrhea and distension even on the first day. After the first two days, in which they became accustomed to force-feeding, they were easily fed throughout the experiment. They rubbed their faces gently with their

Table 18. Body weights of rats in Series I and II at successive intervals of the experiment when a diet containing the ten essential amino acids was force-fed in three or two portions daily, the vitamins being homogenized with the diet

No. of feedings	Rat no.	Weight on different days				
		1st	7th	11th	14th	18th
		gm.	gm.	gm.	gm.	gm.
Three	49724	320	310	310	312	312
	49860	304	301	306	308	310
	49956	300	298	303	302	304
	49972	318	310	314	312	312
	50025	321	306	310	313	308
	50039	309	298	304	302	300
	Average	312	307	307	306	308
Two	50130	320	308	302	288*	---
	50185	325	320	316*	---	---
	50216	342	328	324	312*	----
	50177	366	360	352	346	343
	50201	338	336	327	320	320
	50209	350	340	330	330	330
	Average**	351	347	336	332	331

*Died

**of weights of surviving rats only

paws after they were fed, as though it were a normal reflex associated with swallowing. They also scratched their throats occasionally as if the introduction of the catheter caused slight discomfort. However, they did not exhibit any of the violent reactions so characteristic of the rats that were fed the same volume of diet in only two portions daily. They rested comfortably in normal positions after they were fed, and were alert and active between the feeding intervals.

The only occasion on which the rats of this group placed their heads or bodies on the holders of the fountain followed the administration of the third feeding of the day at 4 instead of 8 P.M., on the day prior to the first balance period. This observation, together with the behavior of certain animals in the group fed twice a day strengthened the idea that the Kuehl syndrome was related directly to the introduction of an excessive quantity of food into the stomach.

The nitrogen balance data in Table 19 are particularly informative in evaluating the respective responses of the rats fed the same quantity of food in either two or three portions. The balances of rats that were employed to test the influence of the manner of feeding the vitamin supplements and that were fed twice daily are also presented in this table. The average nitrogen loss of rats

Table 19. Nitrogen balances per 7 days of rats in Series II force-fed their daily quotas of Diet 2a containing only the ten essential amino acids in either two or three portions

No. of daily feedings	Rat no.	Body wt.	N in food	N in urine	N in feces	Total N excreted	N balance	N balance per 300 gm. rat
		gm.	mg.	mg.	mg.	mg.	mg.	mg.
Two*	49837	285	322	364	214	578	-256	-269
	49868	313	322	437	225	662	-340	-326
	49987	297	322	388	201	589	-267	-270
	50042	298	322	301	191	492	-170	-171
	Average	298	322	372	208	580	-258	-259
Two**	50041	274	322	441	310	751	-429	-470
Two**	50177	344	349	382	232	614	-265	-231
	50201	319	349	337	217	554	-205	-193
	50209	327	349	363	209	572	-223	-205
	Average	330	349	361	219	580	-231	-210
Three	49724	310	322	296	166	462	-140	-135
	49860	307	322	294	178	472	-150	-156
	49956	302	322	281	154	435	-115	-114
	49972	310	322	329	188	517	-195	-185
	50025	311	322	328	188	516	-194	-187
	50039	299	322	299	198	497	-175	-175
	Average	306	322	304	179	483	-161	-159

*Vitamins offered separately

**Vitamins homogenized with basal diet

fed thrice daily was 159 mg. between the twelfth and eighteenth days inclusive, the individual values varying from 114 to 187 mg. This datum stands in marked contrast to average negative values of 259 and 210 mg., respectively, that were characteristic of rats fed twice daily when the vitamins were provided separately and as a part of the homogenized diet. The average nitrogen balance for the seven rats fed twice daily was -238 mg. In other words, division of the daily quota of diet into three equal shares conserved 79 mg. of nitrogen per 7 days, a quantity representing 25 per cent of the total amount of nitrogen that was administered.

Further examination of the data reveals that the animals receiving 8-ml. portions absorbed nitrogen more efficiently than did those given 12 ml. per feeding. The animals that were fed twice daily excreted, on the average, 213 mg. of fecal nitrogen per 7 days, whereas those fed three times a day eliminated only 179 mg. by this route. The difference is highly significant. The group of rats that were fed the low-nitrogen ration in three equal portions also excreted, on the average, 179 mg. of nitrogen in a similar interval (Table 22). When the amino-acid-containing diet was fed, fecal nitrogen represented 37 per cent of the total quantity of nitrogen that was excreted, whether the ration was fed in two or three portions.

The idea that the introduction of a large volume of food represented an important factor in the development of the Kuehl syndrome was strengthened further by examining rats that were sacrificed as soon as possible after different amounts of slurry were administered. In the first test, the rats were killed after they were force-fed for the first time. When 12 ml. of food were given, the stomach was distended greatly, and undigested food had entered the small intestine to a distance of approximately two inches within a few minutes after the catheter was inserted. When 8 ml. of food were administered, however, no unaltered food was found in the intestinal tract.

If two 8-ml. portions were fed on the first day of force-feeding, some animals were apparently able to tolerate 12 ml. on the second day. Individual differences in the capacity of animals to adjust to force-feeding may explain partially certain variable results that were obtained when the daily allowance of food was divided into two portions. For example, in contrast to the majority of the animals, two rats tested in Series I and II never developed diarrhea, and two others lost only a few grams during the experiment. Probably certain rats, especially the larger ones, adapted quickly to the relatively large volume of food, whereas others immediately developed gastrointestinal disorders that were further aggravated by continued administration of the diet. A cumulative effect was suggested also by the fact that the animals generally died between the eleventh and fourteenth days of the test, rather than at the beginning of the experiment.

To observe the influence of administering extremely large quantities of food, 20 ml. of a low-nitrogen diet were given in a single dose to two rats that had never been force-fed. Two additional 10-ml. aliquots of the diet were given at two-hour intervals, the total quantity of diet being 40 ml. Diarrhea occurred in less than two hours after the first feeding, dizziness and instability in two hours, extreme lethargy in four hours, and death in seven hours. Apparently certain conditions that were associated with the Kuehl disorder could be precipitated by feeding very large volumes of a homogenized diet within a short interval of time. Nevertheless, the consequences of overfeeding comprised only part of the syndrome that developed when a diet containing only the ten essential amino acids was fed in two portions of 12 ml. each.

The tolerance to water of rats of approximately the same size as the test animals was investigated also, since the symptoms of water intoxication (Underhill and Salik, 1925) simulated those observed in the present test. However, when 60 ml. of water were administered within four hours by stomach tube to rats that had been fed the low-nitrogen ration for three days, diuresis was the only apparent effect. It seems unlikely, therefore, that the fluid intake of rats that were fed the homogenized diet supplying 14 ml. of water daily was an important factor in the production of the Kuehl syndrome.

The animals that were fed three times a day drank, on the average, 13 ml. of water in addition to the quantity that was administered by way of the diet. The total daily intake was distinctly below that of the animals fed twice daily, 27 *vs*. 42 ml., and was almost identical with that of rats that were fed the same diet *ad libitum*.

Evaluation of ad libitum vs. forced feeding

In experiments involving the utilization of proteins or amino acids, the quantity of basal diet that is ingested merits careful consideration because inadequate consumption may distort the outcome of the tests. Appetite is generally poor when the protein intake is inadequate; the resulting caloric restriction introduces a serious complication. Even the removal of a single amino acid from a mixture of sixteen amino acids will lead to a rapid decline in voluntary food intake (Frazier *et al*., 1947), which may be reversed readily by returning the missing amino acid. Wretlind (1949) also observed a close correlation between the concentration of certain amino acids in the diet and the appetite of growing animals.

The force-feeding technique offers a simple solution to the problem of ensuring a definite intake of specific nutrients, but it may not provide the most effective conditions for evaluating nitrogen utilization. It is possible that the rats may digest and absorb the various components of the diet differently when they consume the diet at will and when a definite quantity of the same diet is delivered at a specific time.

For this reason, Diet 2a was offered ad libitum to a group of rats in another experiment in Series II. The resulting data were compared with those secured from the group of rats to which 13 gm. of the same solid ration was administered in homogenized form in three daily portions of 8 ml. each. The quantity of food that was administered by stomach tube in Series I and II was based on data pertaining to the voluntary food intakes of rats fed natural proteins, as exemplified by data in Table 14.

The average daily food intakes in successive periods of the group that was fed ad libitum are reported in Table 20. During the first 7 days, the average food intake of six rats was 13.3 gm. Thereafter, it dropped rather sharply to little more than 10.0 gm., and remained practically constant for 18 days. The response of the

Table 20. Average daily food intakes in successive periods of rats in Series II when Diet 2a containing the ten essential amino acids as the only source of dietary nitrogen was fed *ad libitum*

Rat no.	Days				
	1-7	8-11	12-14	15-18	19-25
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
49809	12.8	9.8	9.9	10.3	10.6
49888	12.0	10.6	10.6	10.7	10.1
49899	13.3	9.9	10.4	10.9	11.3
49998	14.6	10.1	10.1	10.3	10.8
50026	13.5	10.9	11.1	9.9	11.1
50038	13.4	9.4	9.8	9.3	10.0
Average	13.3	10.1	10.3	10.2	10.6

animals to the diet containing amino acids was very similar to that of rats fed a lactalbumin-containing diet that just supported nitrogen equilibrium (compare data in Tables 14 and 20).

It is worth noting that 10 gm. of Diet 2a provided exactly the minimum caloric intake that was required by rats in Cannon's laboratory for the effective utilization of nitrogen by the hypoproteinemic rat, *i.e.*, 1240 K cal. per sq. m. (Benditt *et al.*, 1948). If the caloric requirement is the same for normal and depleted rats, the principal disadvantage of *ad libitum* feeding in the present test was in respect to a limitation of nitrogen rather than of calories.

The rats that were fed *ad libitum* lost one gram per day (Table 21) whereas the weight of the force-fed group remained virtually constant.

The only difference in the external appearance of the two groups of rats at the termination of the experiment was a tendency toward gauntness in the rats that were fed *ad libitum*. The muscle tone of both groups was good, but the hair was rougher and thinner than that of stock rats of a comparable age.

Unlike the animals that were force-fed twice daily, the animals to which both vitamins and diet were offered freely ingested the vitamins invariably within a few minutes after they were placed in the cage.

Table 21. Body weights of rats in Series II at successive intervals of the experiment when Diet 2a containing only the ten essential amino acids was either force-fed in three portions daily or fed *ad libitum*

Method of feeding	Rat no.	Pre-exp't'l weight	Weight on different days					
			1st	7th	11th	14th	18th	25th
		gm.	gm.	gm.	gm.	gm.	gm.	gm.
Force-fed in three portions	49724	324	320	310	310	312	312	313
	49860	318	304	301	306	308	310	308
	49956	312	300	298	303	302	304	306
	49972	324	318	310	314	312	312	312
	50025	321	321	306	310	313	308	312
	50039	317	309	298	304	302	300	298
	Average	319	312	307	307	306	308	308
Fed ad *libitum*	49809	324	318	314	308	306	303	296
	49888	318	303	305	296	294	288	284
	49899	315	308	303	304	296	296	290
	49988	328	324	320	314	310	303	294
	50026	320	313	308	310	309	303	296
	50038	318	312	306	308	305	304	288
	Average	320	313	309	307	303	299	291

Data pertaining to the nitrogen balances of the two groups, presented in Table 22, indicate that the group that was fed ad libitum ingested 7 mg. of nitrogen less per day during the metabolism period than the force-fed animals as a result of the voluntary limitation of food intake. The total quantity of nitrogen excreted by the two groups was almost identical in all periods. Consequently, the nitrogen balance clearly reflected the reduction in food intake, being -31, -29, and -25 mg. in successive periods for the ad libitum group and -24, -22, and -16 mg. for the force-fed group. In the interval between the twelfth and eighteenth days, inclusive, the average nitrogen balance of the group that was fed ad libitum, 210 mg., was almost identical with that of one group that was force-fed twice daily (Table 19).

The complete absorption of the nitrogen present in the amino acid-containing diets, whether fed freely or by stomach tube, is revealed in Table 22. The quantity of nitrogen, 179 mg., that was excreted in the feces by rats fed the basic low-nitrogen ration was almost identical with that excreted by rats receiving amino acids. No significant difference existed in respect to the absorption of nitrogen by rats fed ad libitum and those force-fed three times daily.

It seemed apparent, then, that the animals that were

Table 22. Nitrogen balances of individual rats in Series II from the twelfth to eighteenth days inclusive when Diet 2a containing only the ten essential amino acids was either offered *ad libitum* or force-fed in three portions daily

Source of N in diet	Method of feeding	Rat no.	Body wt.	N in food	N in urine	N in feces	Total N excreted	N balance	N balance per 300 gm. rat
			gm.	mg.	mg.	mg.	mg.	mg.	mg.
Ten essential amino acids only#	*Ad libitum*	49809	303	268	332	170	502	-234	-232
		49888	291	281	303	140	444	-163	-169
		49899	294	282	301	140	444	-163	-169
		49988	308	270	356	177	534	-263	-256
		50026	308	274	294	164	458	-185	-180
		50038	302	253	280	173	453	-199	-197
		Av.	301	271	311	169	480	-209	-208
Ten essential amino acids only#	Force-feeding	49724	310	322	296	166	462	-140	-135
		49860	307	322	294	178	472	-150	-156
		49956	302	322	281	154	435	-115	-114
		49972	310	322	329	188	517	-195	-185
		50025	311	322	328	188	516	-194	-187
		50039	299	322	299	198	497	-175	-175
		Av.	306	322	304	179	483	-161	-159
Low-nitrogen diet	Force-feeding	50589	321	53	272	161	433	-380	-355
		50594	347	53	276	190	465	-412	-356
		50605	311	53	264	178	442	-389	-375
		50705	349	53	287	196	482	-429	-369
		50712	329	53	258	170	429	-373	-340
		Av.	329	53	271	179	450	-397	-359

#In quantities furnished by a 4.06% lactalbumin ration force-fed three times a day utilized nitrogen as efficiently as the rats that were allowed to eat freely. Furthermore, rats fed ad libitum tended to decrease their food intake as the experiment progressed and to vary individually in respect to the quantity of nitrogen consumed. They also lost weight, whereas the force-fed rats maintained their original weight. Since force feeding therefore seemed to offer some advantages, it was used for the remaining phases of the present investigation. It is recognized that in the final analysis, however, the appetite of the test animal for a given diet may be indeed a function of its nutritive value. For this reason, pertinent data obtained by the force-feeding technique should be supported eventually by data obtained when the same diets are offered ad libitum.

An interesting observation was made in respect to the number of ulcers in the cardiac portion of the stomachs of rats tested in this phase of the study. All but one of the force-fed rats had from one to eight moderately severe ulcers; in contrast, only one of the rats that was fed ad libitum showed even slight evidence of such damage. The ulcers observed in the rats that were force-fed three times daily were, however, smaller and less severe than those observed in the rats that were given the same quantity of food in two portions.

Efficiency of delivery of the day's quota of diet by stomach tube

Experimental evidence is presented in Table 23 concerning the adequacy of delivery of the diet by stomach tube. Portions of the homogenized diet equivalent to those that were administered at each feeding were ejected at certain intervals from the stomach tube into flasks containing hydrochloric acid. A composite of either two or seven such aliquots was then analyzed for its content of nitrogen.

The first four values presented in Table 23 pertain to Diet 2a and represent samples containing two aliquots of 12 ml. each, all of which were taken from the same lot of homogenized diet. The homogenate was prepared after the conclusion of Series II from a smaller portion of diet than was used in routine preparation of the ration, and does not necessarily represent the concentration of nitrogen supplied to any group of rats. The data illustrate, however, that uniform deliveries were made by means of the technique employed in the present investigation.

Since a sufficient quantity of diet was homogenized at one time to feed each group of animals for three days, it seemed possible that the quantity of nitrogen actually administered to the rats might fluctuate from day to day.

Table 23. Quantities of nitrogen present in the daily quota (24 ml.) of various homogenized diets*

Diet no.	Mg. N in daily quota of homogenized diet, as analyzed	Mg. N calculated to be present on basis of solid diet and vitamins as analyzed	Per cent of N theoretically present on basis of solid diet and vitamins, as analyzed	Mg. N calculated to be present on basis of amino acid mixture as formulated, plus basal diets** and vitamins, as analyzed
2a	50.6	58.3	104.8	49.0
2a	50.4	48.3	104.3	49.0
2a	50.2	48.3	103.9	49.0
2a	50.4	48.3	104.3	49.0
Av.	50.4	48.3	104.3	49.0
1	7.9	7.8	101.3	7.8
8	103.7	102.0	101.7	101.3
9	111.2	111.5	100.2	110.3
10	90.5	91.0	99.4	91.3
11	107.5	101.1	106.3	94.8
12	90.6	88.6	102.3	91.3
13	90.7	90.6	100.1	91.3
14	92.5	91.1	101.6	91.3
20	77.7	79.6	97.6	81.3
21	90.5	90.7	99.8	91.3
Av.	86.3	85.4	101.0	85.2

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*Including 2.7 mg. of nitrogen from vitamin supplements

**The daily quota of basal portion of the low-nitrogen diet contained 5.1 mg. of nitrogen

If this were true, a single determination would be of little value as an index of nitrogen intake. The data reported in the second part of Table 23 were secured by analyzing a composite that contained one 8-ml. aliquot taken on each of the seven days of the balance period.

The analytical values presented in the first column were compared with the quantities of nitrogen shown to be present by analysis of the respective dry diets and the vitamin supplement. Data in the third column indicate that the average quantity of nitrogen delivered by stomach tube was 101 per cent of the theoretical value. The analytical results were compared also with the sum of the quantities of nitrogen supplied by the daily quota of the basic portion of the low-nitrogen diet, the vitamins, and the amino acids in the particular mixtures being tested. With the single exception of Diet 11, satisfactory agreement was found between the quantities of nitrogen theoretically present and those that were delivered by stomach tube in the various phases of the experiment.

Selection of the experimental interval

In applying the nitrogen balance technique to the evaluation of protein utilization, investigators have long recognized the necessity of allowing a period of time for adjustment to the test ration. The length of the preliminary period, however, varies from laboratory to laboratory. The nutritional history of the animals and the nature of the experimental diet constitute important factors in the capacity of the rats to adjust to a new regime. The introduction of the force-feeding procedure requires additional adaptation in terms of stomach capacity and of secretion of enzymes and hormones necessary for the digestion and mobilization of a large mass of food administered at a given time in contrast to the natural feeding habits of the species. It seemed advisable, therefore, to follow the excretion of nitrogen in successive periods so that the most suitable interval would be ascertained for the investigation of protein metabolism when the force-feeding technique was employed.

It is evident from data in Table 24 that the daily nitrogen balances of rats to which Diet 2a was force-fed in three daily portions between the twelfth and fourteenth days were almost identical with those observed between the fifteenth and eighteenth days. They were slightly higher than in the period between the nineteenth and twenty-fifth days. A similar trend was observed (Table 25) when animals were offered Diet 2a *ad libitum*.

When animals ingested a diet containing approximately 4 per cent of lactalbumin*, equilibrium was attained in

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*From Nutritional Biochemicals, Ltd.

Table 24. Daily nitrogen balances of rats in Series II force-fed Diet 2a containing only the ten essential amino acids in three daily portions during successive intervals of the experiment

Experimental period	Rat no.	Body wt.	N in food	N in urine	N in feces	Total N excreted	N balance
day		gm.	mg.	mg.	mg.	mg.	mg.
12th-14th inclusive	49724	309	46	43	23	66	-20
	49860	305	46	40	26	66	-20
	49956	299	46	43	22	65	-19
	49972	310	46	52	28	80	-34
	50025	312	46	46	29	75	-29
	50039	299	46	40	29	69	-24
	Average	306	46	44	26	70	-24
15th-18th inclusive	49724	312	46	42	24	66	-20
	49860	309	46	43	25	68	-22
	49956	304	46	38	22	60	-14
	49972	311	46	43	26	69	-23
	50025	310	46	47	25	72	-26
	50039	300	46	45	27	72	-26
	Average	308	46	43	25	68	-22
19th-25th inclusive	49724	313	46	40	24	64	-18
	49860	309	46	38	21	59	-13
	49956	304	46	40	23	63	-17
	49972	311	46	40	24	64	-18
	50025	310	46	38	23	61	-15
	50039	299	46	39	25	64	-18
	Average	308	46	39	23	62	-16

Table 25. Average daily nitrogen balances in successive intervals of rats in Series II when Diet 2a containing only the ten essential amino acids was either force-fed in three portions daily or offered *ad libitum*

Method of feeding	No. of rats	Interval	Body wt.	N in food	N excreted	N balance
		da.	gm.	mg.	mg.	mg.
Force-fed	6	12th to 14th	306	46	70	-24
Ad libitum	6	12th to 14th	302	39	70	-31
Force-fed	6	15th to 18th	308	46	68	-22
Ad libitum	6	15th to 18th	299	39	68	-29
Force-fed	6	19th to 25th	308	46	62	-16
Ad libitum	6	19th to 25th	294	40	65	-25

Table 26. Average daily nitrogen balances in successive intervals of rats in Series II fed *ad libitum* a diet containing approximately 4 per cent of lactalbumin*

Experimental period	Rats	Body wt.	N in food	N in urine	N in feces	Total N excreted	N balance
da.	no.	gm.	mg.	mg.	mg.	mg.	mg.
1-3	6	354	75	86	31	117	-42
4-7	6	347	79	60	35	95	-16
8-11	6	350	70	49	33	82	-12
12-18	5	348	72	42	30	72	0
19-25	5	347	67	43	30	73	-6

*From Nutritional Biochemicals, Ltd.

the interval between the twelfth and eighteenth days (Table 26). As a result of voluntary restriction of food intake, the nitrogen balance became slightly negative in the succeeding period.

On the basis of data herein presented and in view of the cost of feeding mixtures of amino acids to adult rats, the interval between the twelfth and eighteenth days inclusive, was selected as the balance period for all remaining phases of the experiment.

The Weights and Concentration of Nitrogen in Livers of Rats in Series II

Certain interesting observations were made in respect to changes in hepatic tissue extirpated from rats in the various groups of Series II (Table 27). For example, the average weight of both fresh and dry livers of rats that were force-fed twice daily and received the vitamins apart from the basal ration were markedly higher than those of any other groups, when expressed in terms of rats weighing 300 gm. Differences among the remaining groups were probably not significant. The weights of livers from individual rats that were fed three times daily were remarkably uniform in contrast to the group that was fed ad libitum (Table 28).

Table 27. Average weights and quantities of nitrogen in livers, and nitrogen balances of rats* in Series II fed Diet 2a containing only the ten essential amino acids equivalent to those present in a 4.06 per cent lactalbumin control ration

Group	Method of feeding	No. of rats	Body wt.	Wt. of fresh liver	Wt. of dry liver	Total N in liver	N balance/ 7 days
			gm.	gm.	gm.	mg.	mg.
A	Force-fed in two portions**	4	296	9.296	3.233	233	-259
B	Force-fed in two portions***	1	252	9.138	2.582	207	-470
C	Force-fed in two portions***	3	330	7.864	2.388	230	-210
D	Force-fed in three portions***	5	308	7.416	2.309	188	-161
E	*Ad libitum*	6	291	7.124	2.344	203	-209

*Per 300 gm. rat

**Vitamins offered separately

***Vitamins homogenized with basal diet

The total quantity of nitrogen in the livers of rats in Group A was also slightly higher than that of the other groups, suggesting that the nitrogen was stored instead of being utilized to meet immediate needs since the nitrogen balance was severely negative, -259 mg. per 300 gm. rat per 7 days. Although the average nitrogen balance of the rats that were fed three times daily indicated a more efficient utilization of nitrogen than was characteristic of other groups, the average hepatic stores were relatively low. The average value of 188 mg. includes two extremely low values of 148 and 168 mg. of nitrogen (Table 28). For this reason, it seems probable that the use of larger numbers of animals would be necessary before final conclusions could be drawn in any attempt to correlate the hepatic stores of nitrogen with the average nitrogen balances. The total quantity of nitrogen present in the livers of the rats that were fed *ad libitum* was not lower than that of the rats that were fed three times daily, although the nitrogen balance was less favorable.

In general, data obtained from examination of hepatic stores of nitrogen confirmed results derived by means of the nitrogen balance technique.

Table 28. Weights and quantitites of nitrogen in livers of rats in Series II fed Diet 2a

Group	Method of feeding	Rat no.	Wt. of fresh liver	Dry wt.	Wt.of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
A	Force-fed in two portions*	49837	8.128	31.92	2.594	2.63	214	8.619	2.751	227
		49868	9.043	34.52	3.122	2.49	225	8.721	3.020	218
		49987	10.165	36.75	3.735	2.42	246	10.511	3.811	253
		50042	9.242	36.47	3.371	2.50	231	9.335	3.361	233
		Average	9.144	34.91	3.205	2.51	229	9.296	3.233	233
B	Force-fed** in two portions	50041	7.676	28.24	2.169	2.27	174	9.138	2.582	207
C	Force-fed** in two portions	50177	8.378	31.08	2.604	2.90	243	7.330	2.278	212
		50201	8.877	30.02	2.665	2.81	250	8.320	2.498	234
		50209	8.737	30.06	2.626	3.06	267	7.942	2.387	243
		Average	8.664	30.39	2.632	2.90	253	7.864	2.388	230

(Continued on next page)

*Vitamins offered separately

**Vitamins homogenized with basal diet

Table 28 (Cont'd)

Group	Method of feeding	Rat no.	Wt. of fresh liver	Dry wt.	Wt.of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
D	Force-fed in three portions**	49724	7.621	31.14	2.373	2.02	154	7.307	2.275	148
		49860	7.651	31.52	2.383	2.82	216	7.450	2.320	210
		49956	7.618	31.40	2.392	2.79	212	7.469	2.345	208
		49972	7.507	31.42	2.359	2.86	215	7.218	2.268	207
		50039	7.583	31.44	2.321	2.56	167	7.636	2.337	168
		Average	7.538	31.38	2.366	2.55	193	7.416	2.309	188
E	Ad libitum	49809	7.543	31.35	2.365	2.36	178	7.643	2.396	180
		49888	7.024	35.48	2.492	278	195	7.417	2.631	206
		49899	6.374	30.84	1.965	2.97	189	6.590	2.032	195
		49988	7.306	30.39	2.220	2.92	213	7.455	2.265	217
		50026	6.367	31.33	1.995	3.24	206	6.451	2.021	209
		50038	6.901	37.81	2.609	2.99	206	7.188	2.718	214
		Average	6.917	32.87	2.274	2.88	196	7.124	2.344	203

**Vitamins homogenized with basal diet

Discussion

It has been demonstrated clearly in Series I and II that the external symptoms of the Kuehl syndrome are not only reproducible but also preventable. The disorder developed when a diet that contained a mixture of the ten essential amino acids in quantities equivalent to those in a diet containing 4 per cent of lactalbumin was force-fed in two daily portions of 12 ml. each; it did not appear when three portions of 8-ml. each were administered.

No attempt was made in the present investigation to delineate all of the pathological changes arising from the intake of an excessive quantity of food or to pursue the search for the fundamental cause or causes of the syndrome. It was deemed more pertinent at this time to attempt to formulate a mixture of amino acids that would support nitrogen equilibrium in the adult well-nourished rat. Such a composite represented a basic necessity for the elucidation of requirements for individual amino acids, of interrelationships between them, and of the etiology of the Kuehl syndrome.

Nevertheless, the observation by Kuehl (1949) that the disorder did not develop when the same mixture of amino acids was supplemented with nonessential acids or when the ration contained 4.06 per cent of lactalbumin indicated that more than the reaction to force-feeding was involved. The phenomenon merits careful investigation. That the addition of only 23 mg. of nitrogen in the form of nonessential acids to a mixture of essential amino acids that contributed 41 mg. of nitrogen daily should protect the animals completely and invariably from such a profound physiological and metabolic disorder is indeed remarkable. The nutritional implications of the observation should not be overlooked.

The fact that the rats that were fed twice daily lost 76 mg. of nitrogen per week more than did the rats fed thrice daily may be particularly significant. Such a quantity of nitrogen would reveal the excretion of 600 to 750 mg. of amino acids weekly, depending upon the particular assortment of amino acids that was present in the urine. The nitrogen eliminated in the urine might represent amino acids that were wasted because of the inability of the organism to absorb or metabolize the total quantity that was administered in each large doses; in addition, it might comprise amino acids discarded in the urine when certain tissues were broken down to release amino acids that were urgently required for vital processes. It would, therefore, be extremely interesting to compare the pattern of excretion of individual amino acids eliminated by rats in the groups fed in two and

and three portions. It is possible that reabsorptive mechanisms favored the retention of certain amino acids at the expense of others, thus permitting the loss of relatively large quantities of certain essential acids. It might be postulated that under conditions which did not favor nitrogen retention, such as the administration of a large mass of diet, the loss of certain acids might be such as to induce a state of deficiency in respect to certain acids, especially those which are irreversibly deaminated. A quantity of a given acid that would meet minimum requirements when provided in relatively small doses at frequent intervals might prove inadequate when administered in only two large feedings because of the inability of the animal to metabolize the acid efficiently.

Moreover, the nonessential amino acids play specific and vital roles, both in the synthesis of tissues and in the regulation of metabolic processes. Whether the basal diet was inadequate in respect to methionine and/or phenylalanine, for example, but was rendered effective by the presence in the mixture of cystine and/or tyrosine in the mixture of nonessential acids could be tested by providing those nonessential acids in graded quantities or by increasing the amount of either or both of the essential amino acids involved in the relationship. Both glutamic and aspartic acids facilitate the reamination of ketoacids arising from the deamination of certain amino acids. Whether their presence is particularly significant could be examined by supplying either or both of these acids in addition to the essential amino acids alone. Perhaps detoxification mechanisms failed because the quantities of cystine, glutamic acid, or glycine were inadequate for linkage with certain products or for the synthesis of glutathione. Furthermore, alamine, glycine, proline, and serine may perform additional functions over and beyond those that have been recognized to date. When the dietary supply of nitrogen is low, as in the ration employed in Series I and II, the organism may be incapable of synthesizing the nonessential acids at a sufficiently rapid rate to support good protein nutrition.

In attempting to further characterize the Kuehl syndrome, it would seem worthwhile to secure data concerning urinary excretion of individual amino acids and the partition of nitrogen in the blood and urine. Abnormal metabolites might be found that would suggest specific derangements on the degradation or utilization of certain amino acids or of fat or carbohydrate. For example, the concentration of pyruvic acid in the blood and the glucose tolerance of rats that were force-fed twice or thrice daily would reflect the state of carbohydrate metabolism. The concentration of nitrogen in the serum, coupled with values pertaining to the volume of blood, would be informative. In addition, such biochemical tests might be supplemented with histological examination of liver, kidneys, and adrenal glands.

It is suggested also that the findings of Spector and Adamstone (1950) be reexamined in the light of information gained in the present investigation. The quantities of food administered by them were relatively large in relation to the size of the test animals. Moreover, it seems unlikely that the symptoms which they described can be attributed specifically to a deficit of tryptophane, even though the addition of tryptophane to their basal diet prevented the appearance of the syndrome. The test protein, lactalbumin, which was selected as the basis for formulating the amino acid mixture used in this laboratory, contains almost twice as much tryptophane per gram as either casein or beef muscle. The total quantity consumed per day, however, was approximately half of the quantity supplied in the control diet fed by Spector and Adamstone.

The striking similarity of conditions developed in the laboratories of Spector, of Morse, and of Swanson suggest that several closely related factors are involved. It is clear that when the mechanisms involved in the production of the metabolic disorder described in the present investigation are elucidated, much information of a fundamental nature will be gained concerning specific roles of individual amino acids and their relationships to each other and to other nutrients.

SERIES III

Utilization of Nitrogen when a Mixture of the Ten Essential Amino Acids was Supplemented with Graded Quantities of Nonessential Amino Acids

The numerous experiments cited in the Review of Literature reveal the complex nature of the protein requirement. They illustrate clearly the many and varied factors influencing the utilization of nitrogen by the animal organism, and the paucity of information concerning even minimal needs for nitrogen.

Moreover, data obtained in Series II of the present investigation confirmed the earlier report from this laboratory (Kuehl, 1949) that a mixture of ten essential amino acids equivalent to those in a control ration containing 4.06 per cent of lactalbumin do not support nitrogen equilibrium in the normal adult rat. Kuehl has recorded, in addition, that the mixture of essential amino acids sustained nitrogen balance and prevented the appearance of the curious nervous symptoms when it was supplemented with the quantity of each of the nonessential amino acids that is present in a control ration like the one used herein. Only 23 mg. of nonessential nitrogen were added to the basal mixture of essential amino acids which contributed

41 mg. of nitrogen daily.

In Kuehl's experiment, the mixtures of amino acids were formulated on the basis of published values (Block and Bolling, 1945; Schmidt, 1944) of the composition of lactalbumin. Also, her experiment provided no information as to whether the striking differences in the nutritive properties of the mixtures containing ten or 13 amino acids was quantitative or qualitative.

The statement made by Rose in 1949 with reference to the human subject is applicable also to the adult rat, *i.e.*,

> No attempt has yet been made to ascertain the minimal total nitrogen content of a diet, carrying the recommended intakes of the ... essentials, which will sustain nitrogen equilibrium. (p. 551)

Such knowledge is necessary for a full understanding of the amino acid requirements of any species. In fact, it is basic, for optimum nutriture can be studied effectively only after minimal needs have been ascertained. Estimation of the *minimum* daily intake of total nitrogen that would sustain nitrogen equilibrium when a mixture of amino acids similar to that tested in Series I and II was provided, became one of the first objectives of the present series of experiments.

In these studies, however, the basal essential amino acid diet, which will be known as Diet 2, differed in two respects from the rations fed previously. Its composition was based (1) on the nitrogen content of a freshly prepared control and reference ration containing 4.27 per cent of the same lactalbumin that was used by Kuehl, and (2) on a microbiological assay of the amino acid content of the particular sample of lactalbumin that was incorporated in the control ration. The author is indebted to Dr. M. S. Dunn of the University of California at Los Angeles for these analyses, and wishes to record here her sincere appreciation of his generous assistance. Lactalbumin was chosen again as the reference protein because it seemed reasonable to assume that the proportions of amino acids in a natural protein of high biological efficiency are well balanced.

Furthermore, the role of the nonessential amino acids in the maintenance of nitrogen equilibrium in the adult rat has not been explored, particularly in respect to their influence upon the retention of nitrogen when the essential amino acids are present in low concentrations. Moreover, the capacity of cystine and tyrosine to replace part of the requirement of the growing rat for methionine and phenylalanine, respectively, is well known. Little attention has been given, however, to the significance of these relationships in estimating the amino acid needs of the adult rat. The only complete set of values recorded in the literature concerning the minimal requirements of the normal adult rat for the essential amino acids (Benditt, Woolridge, Steffee, and Frazier, 1950) was estimated when the rats consumed between 203 and 223 mg. of nitrogen daily, of which more than 80 per cent was provided by nonessential amino acids. Moreover, the total intake of nitrogen by these rats more than doubled the quantity that workers in the Iowa laboratory had shown would permit nitrogen equilibrium in normal adult male rats when nitrogen from several sources were fed, _i.e._, egg proteins (Mukhopadhay, 1949), lactalbumin (Swanson, 1948; Kuehl, 1949), and a mixture of amino acids simulating lactalbumin (Kuehl, 1949).

It seems almost certain that the uptake and utilization of the essential amino acids proceed differently when the nonessential amino acids are present in abundance, available only in low concentrations, or entirely lacking. Miller, Bly, Watson, and Bale (1950) found, for example, that the synthesis of plasma proteins in surviving rat liver was stimulated tenfold by the addition of the essential amino acids, and still further when the nonessential amino acids were provided simultaneously. The observation of Sprinson and Rittenberg (1949 a) that nitrogen from ammonia was utilized more efficiently by rats that were fed a diet low in protein than by those that received adequate quantities of protein likewise may have important implications in the establishment of minimal needs for nitrogen. Even the capacity of the organism to utilize the unnatural isomer of an amino acid may be altered by the concentration of nitrogen in the diet (Anderson and Nasset, 1950).

The second objective of the experiments recorded in Series III, therefore, became a study of the influence on nitrogen retention of supplementing the basal mixture of essential amino acids with graded quantities of a mixture of the nonessential amino acids found in lactalbumin. All of these amino acids, except serine and hydroxyglutamic acid, were available for the formulation of the mixtures. Again, the proportion of each amino acid was based on the microbiological analysis of the protein as provided by Dr. Dunn.

In determining the range in the quantities of supplementary nitrogen to be added to the basal essential amino acid diet, data pertaining to the minimal nitrogenous needs of the Wistar stock of rats used in the Nutrition Laboratory of the Iowa State College were considered. Recently, Rutman, Dempster, and Tarver (1949) demonstrated that hepatic tissue taken from offspring of rats from the stock colony belonging to the Nutrition Laboratory of the Iowa State College showed a consistently higher uptake of nitrogen than did that of another strain. Since animals of

Wistar stock were used in the present experiment, the observation of Rutman and associates suggested that data obtained in our laboratory pertaining to the nitrogenous requirements of Wistar rats would be more applicable to this study than would values taken from the literature.

It has, for example, been shown that a slightly positive balance results when normal adult male rats consume approximately 75 mg. of nitrogen daily from lactalbumin (Swanson, 1948; Kuehl, 1949), or 82 mg. from dehydrated defatted egg powder (Mukhopadhay, 1949). It seemed reasonable to expect, therefore, that an amino acid diet carrying approximately 75 mg. of nitrogen daily would be adequate. Possibly, under certain conditions, a smaller quantity would meet minimum requirements, since nitrogen balance was only slightly negative when a dietary mixture of eighteen amino acids simulating lactalbumin contributed 64 mg. of nitrogen daily (Kuehl, 1949).

Therefore, in Diets 4 to 9, the basal essential amino acid diet described in Table 8 was supplemented with 10, 20, 30, 40, 50, and 60 mg. of nitrogen per day from the nonessential amino acids (Table 10).

The test ration was force-fed in three portions of 8 ml. each, at 8 A.M., 2 P.M., and 9 P.M., the daily allowance of dry diet being 13 gm. as in Series II. On the day preceding the initiation or conclusion of a balance test or preceding the removal of tissues, the total intake of food was reduced to 20 ml. by restricting the second feeding to 4 ml. This feeding was given at noon instead of 2:00 P.M., and the third feeding, of 8 ml., was administered at 4:00 P.M. on that day. The metabolic materials were collected from the twelfth to the eighteenth days inclusive because data obtained in Series II indicated that the rats attained a relatively constant plane of nitrogen metabolism in 11 days.

Changes in body weight

The average weights of the animals in Series III at various intervals of the experiment are indicated in Table 29. In general, changes in weight were not extreme. Animals that were force-fed the low-nitrogen ration gradually declined in weight throughout the experiment, the average daily loss being 1.5 gm. In earlier experiments, in which the same diet was offered *ad libitum*, the rats lost approximately 2.5 gm. daily in a similar interval (Mukhopadhay, 1949; Clark, 1945). Apparently the provision of adequate calories partially protects the organism when the diet is almost devoid of nitrogen.

The rats that were fed the lactalbumin control ration maintained their body weight throughout the course of the experiment. On the other hand, animals receiving 43 mg. of

Table 29. Average body weights at certain intervals of rats in Series III fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration or equivalent and supplemented with graded quantities of nitrogen from nonessential acids (NEAA)

Diet no.	Description of diet	Pre-exp't'l weight	Initial wt.	Wt. on 11th day	Wt. in balance period	Change in balance period
		gm.	gm.	gm.	gm.	gm.
1	Low-nitrogen	366	353	338	331	-11
3	4.27% lactalbumin	373	354	358	357	+1
2	Ten EAA as present in Diet 3	364	356	347	344	-5
4	Diet 2 plus 10 mg. N from NEAA	363	355	350	344	-8
5	Diet 2 plus 20 mg. N from NEAA	362	353	352	350	0
6	Diet 2 plus 30 mg. N from NEAA	361	355	354	353	0
7	Diet 2 plus 40 mg. N from NEAA	366	356	359	357	0
8	Diet 2 plus 50 mg. N from NEAA	386	379	373	375	+5
9	Diet 2 plus 60 mg. N from NEAA	386	382	376	377	+5
10	Diet 7 with L-alanine, L-aspartic acid, and L-serine	377	365	361	358	0

Table 30. Body weights at certain intervals of individual rats in Series III fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration supplemented with graded quantities of nitrogen from the nonessential acids (NEAA)

Diet no.	Diet	Rat no.	Pre-exp't'l weight	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	gm.
1	Low-nitrogen	50589	353	340	326	316	-10
		50594	382	366	354	343	-11
		50605	342	330	315	305	-10
		50705	386	376	358	343	-15
		50712	366	352	334	327	-11
		Average	366	353	338	327	-11
3	4.27% lactalbumin (Harris)	50130	372	350	355	358	+1
		50139	370	358	362	362	0
		50146	378	350	354	351	-3
		50158	386	370	378	380	+2
		50184	374	356	356	360	+4
		50199	357	340	346	348	+2
		Average	373	354	358	359	+1
2	Ten EAA as present in Diet 3	50147	374	354	346	342	-4
		50161	384	376	370	361	-9
		50324	370	360	350	344	-6
		50330	358	355	344	348	+4
		50343	350	344	334	327	-7
		50359	350	345	336	329	-7
		Average	364	356	347	342	-5
4	Diet 2 plus 10 mg. N from NEAA	50162	377	366	360	354	-6
		50191	390	380	376	374	-2
		50265	346	340	343	336	-7
		50325	356	344	342	330	-12
		50342	360	354	342	332	-10
		50358	350	344	338	329	-9
		Average	363	355	350	342	-8

(Continued on next page)

Table 30 (Cont'd)

Diet no.	Diet	Rat no.	Pre-exp't'l weight	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	gm.
5	Diet 2 plus 20 mg. N from NEAA	50190	361	358	358	362	+4
		50263	346	334	343	344	+1
		50289	333	322	325	324	-1
		50329	376	366	363	358	-5
		50344	372	362	355	354	-1
		50366	384	376	370	370	0
		Average	362	353	352	352	0
6	Diet 2 plus 30 mg. N from NEAA	50292	370	363	368	370	+2
		50327	334	330	326	328	+2
		50333	373	366	358	352	-6
		50341	368	355	356	356	0
		50377	344	340	344	347	+3
		50392	378	374	374	376	+2
		Average	361	355	354	355	0
7	Diet 2 plus 40 mg. N from NEAA	50176	374	360	364	366	+2
		50295	343	336	343	344	+1
		50318	373	358	352	354	+2
		50334	356	346	355	356	+1
		50357	362	346	350	350	0
		50374	344	334	344	340	-4
		50422	377	366	360	362	+2
		50436	352	340	350	354	+4
		50444	399	390	390	386	-4
		50463	392	381	380	378	-2
		50464	370	357	358	356	-2
		Average	366	356	359	359	0

(Continued on next page)

Table 30 (Cont'd)

Diet no.	Diet	Rat no.	Pre-exp't'l weight	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	gm.
8	Diet 2 plus 50 mg. N from NEAA	50462	418	410	394	396	0
		50485	364	358	352	358	+6
		50537	426	416	406	412	+6
		50609	374	368	366	371	+5
		50620	348	343	347	357	+10
		Average	386	379	373	379	+5
9	Diet 2 plus 60 mg. N from NEAA	50536	364	357	355	362	+7
		50549	386	381	378	380	+2
		50553	420	416	408	412	+4
		50606	354	350	350	358	+8
		50621	406	404	388	394	+6
		Average	386	382	378	381	+5
10	Diet 7 with L-alanine, L-aspartic acid, and L-serine	50532	355	345	347	345	-2
		50548	390	380	374	370	-4
		50593	376	364	359	360	+1
		50601	395	376	374	375	+1
		50611	368	358	351	355	+4
		Average	377	365	361	361	0

nitrogen from the mixture of essential amino acids alone or the same mixture supplemented with 10 mg. of nitrogen from the nonessential acids lost 1.0 gm. of weight daily during the metabolism period. Rats maintained their initial weight when fed diets in which the nonessential nitrogen was increased to 20, 30, or 40 mg. of nitrogen per day. When the nonessential nitrogen was increased to 50 or 60 mg. daily, body weights increased slightly in the balance period.

Although none of the changes in weight were striking, they nevertheless indicated trends that were reflected clearly in the well-being of the animals and in the quantities of fat present in the various depots at necropsy. Thus, if they lost weight, the animals looked gaunt, had poor muscle tone, and possessed small reserves of fat. The rats that maintained their initial weight had good muscle tone and moderate depots of fat; and those that received 50 or 60 mg. of nitrogen were very plump and had large masses of fat in all the depots that were examined routinely. No marked differences in respect to muscle tone were noted between the rats that received the lactalbumin diet or essential amino acids supplemented with 40, 50, or 60 mg. of nitrogen from nonessential acids.

The data in Table 30 indicate that individual animals in the various groups responded uniformly to the test rations in respect to body weight.

Nitrogen balance

At the present time, agreement does not exist concerning the most suitable basis for the expression of data pertaining to nitrogen metabolism. It has been related to both body weight and surface area. The development of several different formulae for the calculation of surface area unfortunately has introduced considerable confusion. On the basis of data obtained from a wide variety of animals, Kleiber (1947) proposed that nitrogen metabolism is more closely correlated with metabolic body size, *i.e.*, the 3/4 power of body weight, than with either body weight or surface area. In the present discussion, values are presented in terms of 300 gm. body weight and also 100 sq. cm. of surface area.

The regression of nitrogen balance on nitrogen intake, in mg. per 100 sq. cm. of body surface per 7 days, is shown graphically in Figure 1, the regression equation being $Y = 0.8304 \; X - 118.7$. The regression was calculated on the basis of values up to and including 159 mg. of nitrogen consumed per 100 sq. cm. of body surface. The value pertaining to the last point in the figure, *i.e.*,

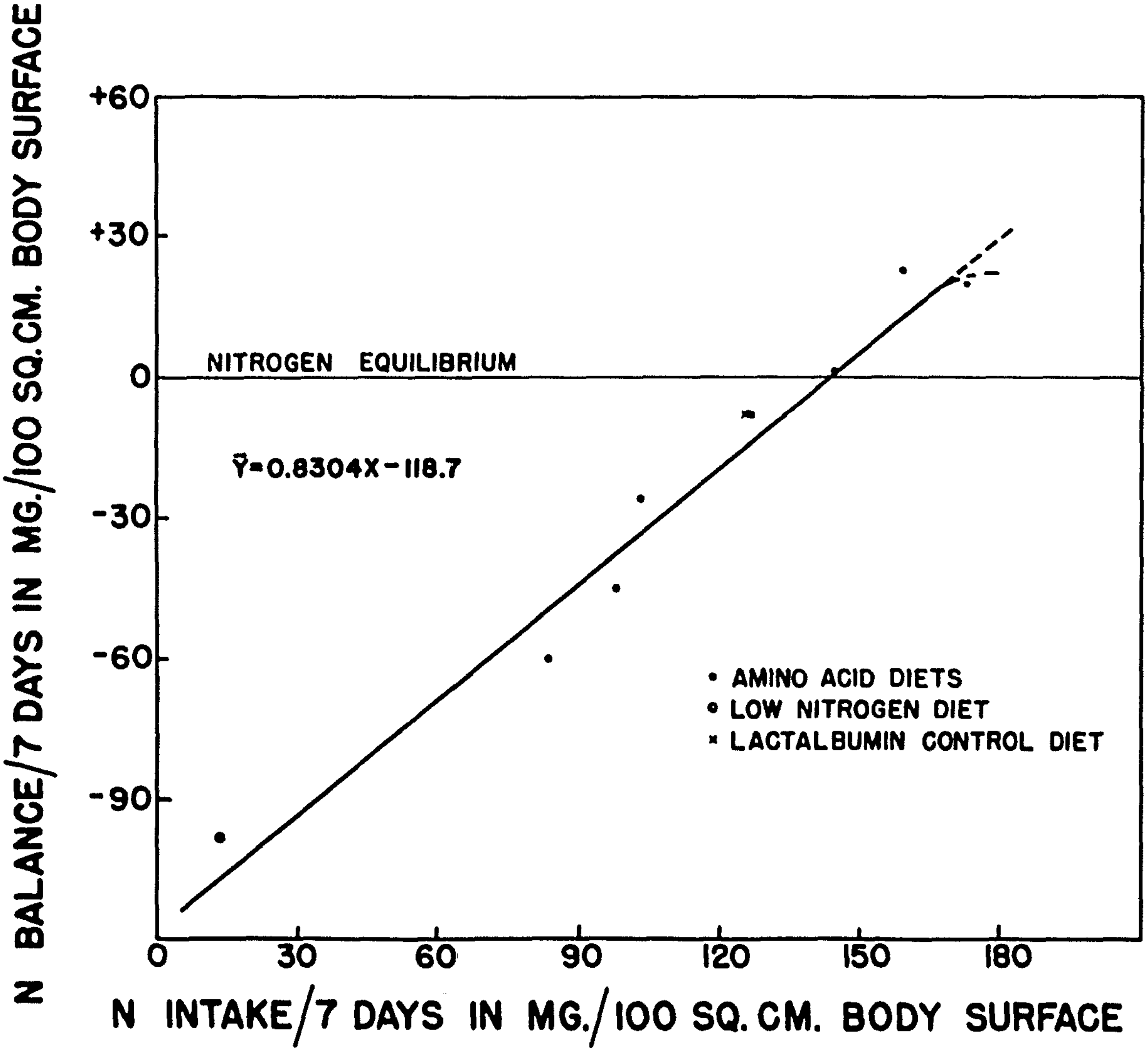

Figure 1. Regression of nitrogen balance on nitrogen intake in Series III when diets containing essential amino acids equivalent to those in a 4.37 per cent lactalbumin control ration were supplemented with graded quantities of nitrogen from nonessential acids

a nitrogen intake of 173 mg. per 100 sq. cm. which was provided by the diet that contributed 60 mg. of nitrogen from the nonessential acids, was omitted in calculating the regression equation because it appeared to be due to experimental variation. The data on which this figure is based are presented in Tables 31 and 32.

It is apparent from Figure 1 that the relationship between nitrogen intake and retention was linear until a daily intake of 159 mg. per 100 sq. cm., or 80 mg. per 300 gm. rat was attained. Whether the slope of the line would remain constant as the quantity of dietary nitrogen increased or whether the position of the last point indicated a real break in the linear relationship must await further experimentation. It would be interesting, indeed, to determine whether the retention of nitrogen could be enhanced still further by supplementing the basal essential amino acid diet with 70 or 80 mg. of nitrogen, or more, or whether the point of maximum efficiency for the particular assortment of essential amino acids that is present in lactalbumin was reached by providing 50 mg. of nitrogen per rat per day from nonessential sources.

The minimum requirement for nitrogen equilibrium, as revealed in Figure 1 and Table 31, was 145 mg. of nitrogen per 100 sq. cm. of body surface per 7 days. Such a quantity is equivalent to 520 mg. per 300 gm. rat per 7 days, or 74

Table 31. Average nitrogen balances per 7 days of rats in Series III fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration and supplemented with graded quantities of nitrogen from nonessential amino acids (NEAA)

No.	Diet	No. of rats	Body wt.	Body surface	N in food	N in food/ 100 sq.cm.	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	Low-nitrogen	5	331	408	53	13	271	179	450	-397	-359	-98
3	4.27% lactalbumin	6	357	427	536	126	337	232	569	- 33	- 28	- 9
2	10 EAA as present in Diet 3	6	344	417	349	84	400	201	601	-252	-220	-60
4	Diet 2 plus 10 mg. N from NEAA	6	344	417	408	98	382	213	595	-187	-163	-45
5	Diet 2 plus 20 mg. N from NEAA	6	350	421	476	113	372	212	584	-108	- 93	-26
6	Diet 2 plus 30 mg. N from NEAA	6	353	423	541	127	371	204	575	- 34	- 28	- 8
7	Diet 2 plus 40 mg. N from NEAA	11	357	427	618	145	404	206	610	+ 8	+ 7	+ 1
8	Diet 2 plus 50 mg. N from NEAA	5	375	439	697	159	402	194	597	+100	+ 82	+23
9	Diet 2 plus 60 mg. N from NEAA	5	377	441	762	173	466	207	673	+ 89	+ 72	+20
10	Diet 7 with L-alanine L-aspartic acid and L-serine	5	358	427	621	145	496	211	708	- 87	- 72	-20

Table 32. Nitrogen balances per 7 days of individual rats in Series III fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration and supplemented with graded quantities of nitrogen from nonessential amino acids (NEAA)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	Low-nitrogen	50589	321	400	53	272	161	433	-380	-355	95
		50594	347	418	53	276	190	466	-413	-356	99
		50605	311	393	53	264	178	442	-389	-375	99
		50705	349	419	53	287	196	483	-430	-369	103
		50712	329	406	53	258	170	428	-375	-340	92
		Average	331	407	53	271	179	450	-397	-359	98
3	4.27 per cent lactalbumin (Harris)	50131	353	424	536	353	200	553	- 17	- 14	- 4
		50139	361	429	536	331	223	554	- 18	- 15	- 4
		50146	350	420	536	343	233	576	- 40	- 34	-10
		50158	379	442	536	300	236	536	0	0	0
		50184	356	426	536	358	242	600	- 64	- 54	-15
		50199	345	417	536	335	259	594	- 58	- 50	-14
		Average	357	426	536	337	232	569	- 33	- 28	- 8

(Continued on next page)

Table 32 (Cont'd)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
2	Ten EAA as present in Diet 3	50147	344	417	349	410	206	616	-267	-233	-64
		50161	365	432	349	411	196	607	-260	-212	-60
		50324	347	418	349	375	214	589	-240	-207	-57
		50330	346	418	349	446	198	644	-295	-257	-70
		50343	329	406	349	373	193	566	-217	-198	-53
		50359	332	408	349	385	199	584	-235	-212	-58
		Average	344	417	349	400	201	601	-252	-220	-60
4	Diet 2 plus 10 mg. N from NEAA	50162	355	425	408	421	194	615	-207	-175	-49
		50191	373	438	408	402	214	616	-208	-167	-47
		50265	338	413	408	332	218	550	-142	-126	-34
		50325	333	409	408	408	211	619	-211	-190	-52
		50342	335	410	408	348	206	554	-146	-131	-36
		50358	331	408	408	382	233	615	-207	-188	-51
		Average	344	417	408	382	233	695	-187	-163	-45

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Table 32 (Cont'd)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
5	Diet 2 plus 20 mg. N from NEAA	50190	358	427	476	361	202	563	- 87	- 73	-20
		50263	341	414	476	369	218	587	-111	- 98	-27
		50289	323	402	476	417	207	624	-148	-137	-37
		50329	359	428	476	347	207	554	- 78	- 65	-18
		50344	351	422	476	358	210	568	- 92	- 79	-22
		50366	366	434	476	381	226	607	-131	-107	-30
		Average	350	421	476	372	212	584	-108	- 93	-26
6	Diet 3 plus 30 mg. N from NEAA	50292	368	434	541	364	207	571	- 30	- 24	- 7
		50327	327	404	541	360	192	552	- 11	- 9	- 3
		50333	353	424	541	389	205	594	- 53	- 45	-12
		50341	352	422	541	366	205	571	- 30	- 25	- 7
		50377	343	414	541	369	195	564	- 23	- 20	- 6
		50392	374	439	541	381	219	600	- 59	- 47	-13
		Average	353	423	541	371	204	575	- 34	- 28	- 8

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Table 32 (Cont'd)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
7	Diet 2 plus 40 mg. N from NEAA	50176	363	431	615	369	204	573	+42	+35	+10
		50295	343	414	615	383	191	574	+41	+36	+10
		50318	352	412	615	407	212	619	- 4	- 3	- 1
		50334	354	425	615	393	201	594	+21	+18	+ 5
		50357	348	419	615	392	211	603	+12	+10	+ 3
		50374	340	414	615	365	195	560	+55	+49	+13
		50422	358	427	621	435	206	641	-20	-17	-15
		50436	352	425	621	387	199	579	+42	+37	+ 9
		50444	385	446	621	427	216	643	-22	-17	- 5
		50463	376	440	621	482	223	705	-84	-67	-19
		50564	356	426	621	410	211	621	0	0	0
		Average	357	426	618	404	206	610	+ 8	+ 7	+ 1

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Table 32 (Cont'd)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
8	Diet 2 plus 50 mg. N from NEAA	50462	394	453	697	410	211	621	+76	+59	+17
		50485	355	425	697	373	168	541	+156	+132	+37
		50537	410	463	697	454	198	652	+45	+33	+10
		50609	366	434	697	392	215	607	+90	+73	+21
		50620	352	422	697	382	180	562	+135	+115	+32
		Average	375	439	697	402	194	597	+100	+82	+23
9	Diet 2 plus 60 mg. N from NEAA	50536	357	427	762	396	194	590	+172	+144	+40
		50549	379	442	762	480	197	677	+85	+66	+19
		50553	408	462	762	521	224	745	+17	+12	+4
		50606	353	424	762	437	200	637	+125	+107	+30
		50621	389	449	762	501	222	723	+39	+30	+9
		Average	377	441	762	467	207	674	+88	+72	+20
10	Diet 7 with L-alanine, L-aspartic acid and L-serine	50532	342	415	621	481	195	676	-55	-48	-13
		50548	367	433	621	519	205	724	-103	-83	-24
		50593	357	427	621	470	201	671	-50	-42	-12
		50601	372	437	621	510	228	738	-117	-94	-27
		50611	350	425	621	500	229	729	-108	-92	-25
		Average	358	427	621	496	212	708	-87	-72	-20

mg. per day, an amount almost identical with that observed in previous experiments (Swanson, 1948; Kuehl, 1949; Mukhopadhay, 1949).

The loss of nitrogen incurred by rats to which the low-nitrogen diet was force-fed differed little from that associated with ad libitum feeding in earlier studies. Thus, the negative nitrogen balance was 98 mg. per 100 sq. cm. per week (Table 31), whereas Brush (1946, p. 70) and Mukhopadhay (1949) reported losses of 96 and 110 mg. per 100 sq. cm. when the low-nitrogen diet was offered freely.

The lactalbumin control diet that was administered in this series was not entirely adequate for the support of nitrogen equilibrium, the average balance of the rats being -28 mg. per 300 gm. rat per 7 days. Kuehl (1949) reported a positive balance of 42 mg. per 300 gm. rat per 7 days. Although the animals tested in the present study were slightly larger than those used by Kuehl (344 vs. 306 gm.) the total intake of nitrogen per 300 gm. rat in the balance period differed little.

The ineffectiveness of the mixture that contained only the ten essential amino acids was demonstrated clearly by the negative nitrogen balance of 220 mg. per 300 gm. rat per 7 days, the range in values being 198 to 233 mg.. It is apparent from data obtained in this series that a mixture of amino acids which supplies only 34 mg. of nitrogen from the utilizable forms and 9 mg. of nitrogen from the unnatural isomers of the essential amino acids alone does not offset the daily loss of nitrogen by these rats. It is noteworthy that the total quantity of utilizable nitrogen from the essential amino acids in this diet was only one and one-half times the minimal quantity that Benditt and his associates (1950) found necessary to support nitrogen equilibrium when the supply of nonessential nitrogen was ample (_i.e._, at least eight times that furnished by the essential amino acids).

In view of the current interest in the influence upon nitrogen retention of an imbalance of amino acids, it is interesting that the mixture of 10 essential amino acids fed in the present series (III) was less effective in the maintenance of nitrogen equilibrium than the mixture used in Series II. In the first instance (Series III), the composite was formulated on the basis of a microbiological analysis of the lactalbumin used as the reference protein; in the second (Series II), according to the formula prepared by Kuehl from the amino acid composition of lactalbumin as reported in the literature.

Over a seven-day period, average nitrogen balances of the rats in Series II and III were - 159 and - 220 mg. per 300 gm. rat, respectively (Tables 22 and 32). The rats in

Series III also excreted 20 mg. more nitrogen in the feces than did the rats in Series II.

In an attempt to evaluate the difference in the response of the two groups, the amino acid contents of the two mixtures have been compared. It appears that the mixture (Diet 2) fed in Series III contained 20 and 25 per cent more leucine and valine, respectively, than did Diet 2a fed in Series II. On the other hand, the quantities of methionine and phenylalanine in Diet 2 were, respectively, 20 and 30 per cent lower than those in Diet 2a. The increases in the dietary intakes of leucine and valine, therefore, did not seem to enhance the utilization of the nitrogen of the total mixture in Diet 2 unless such effects were masked by the concomitant reductions in the amounts of methionine and phenylalanine present. Two explanations occur to the author: Either methionine and phenylalanine exert a specific beneficial effect on the overall utilization of nitrogen; or the quantities of leucine and valine present in Diet 2a, fed in Series II, represent maximal values and consequently no further benefit accrues when they are increased in the diet.

In light of other data reported in Table 32, it would be interesting to ascertain, in subsequent experiments, whether the concentrations of individual essential amino acids found in lactalbumin approach the minimal or maximal values for the retention of nitrogen when an additional source of nonspecific nitrogen is available in abundance.

Supplementation of the basal essential amino acid diet with the nonessential acids definitely enhanced nitrogen retention, negative balances dropping from 220 mg. per 300 gm. rat per 7 days to 163 and 93 mg., respectively, when the diets provided 10 and 20 mg. of nonessential nitrogen daily. The addition of 30 mg. of nonessential nitrogen per day almost permitted equilibrium. In fact, the nitrogen balance of -28 mg. per 300 gm. rat per 7 days was identical with that obtained when the lactalbumin control diet was given. Further supplementation of the essential amino acids in Diet 2 with 40 mg. of nitrogen from nonessential amino acids definitely improved nitrogen retention, the average nitrogen balance being + 7 mg. per 300 gm. rat per 7 days. To achieve this performance, the total amount of nitrogen needed for equilibrium was approximately twice the quantity that proved inadequate when Diet 2 containing only the essential amino acids was fed.

Further increases in the quantities of nonessential nitrogen to 50 and 60 mg. resulted in positive balances. The data indicate that maximum retention is attained in this range of feeding.

The experiments described above indicate clearly that the basal mixture became effective when supplemented appropriately with a sufficient quantity of nonessential amino acids. The results of the present test, however, did not agree with those reported by Frost (1949), who concluded that depleted rats could utilize effectively a mixture of amino acids in which not more than 20 to 25 per cent were provided from nonessential sources. The discrepancy in results may be due to differences in the composition of the basal mixture of essential amino acids, to the kinds of compounds chosen to supply the nonessential nitrogen, or to varied requirements of the normal and depleted organism.

In the experiments just described, DL-alanine and DL-aspartic acid were incorporated in the same proportion as their natural forms exist in lactalbumin; glycine was substituted isonitrogenously for serine. During the course of the experiment, it was possible to secure preparations of the L-forms of alanine, aspartic acid, and serine. Diet 10 differed from Diet 7 only because the L-forms of these three amino acids were substituted for DL-alanine, DL-aspartic acid, and glycine. Both diets contributed 40 mg. of nitrogen from the nonessential amino acids. It was thought that the animals might react favorably to their use in the ration. However, the response of the rats fed Diet 10, in which the L-forms of these three amino acids were incorporated, was surprising. Whereas Diet 7 supported equilibrium, the rats that were given Diet 10 lost 72 mg. of nitrogen per 300 gm. rat in 7 days. The retention of nitrogen, therefore, approximated that of rats receiving only 20 mg. of supplementary nitrogen daily from the mixture of nonessential acids. It should be noted that every precaution was taken to replicate experimental conditions in both tests. Body weights of the test rats were approximately the same, as were the sources of individual acids used in the formulation of the basal essential acid mixture and the composite of nonessential acids.

The only explanation that can be advanced at this time is the presence in the sample of serine supplied by Bios Laboratories of a contaminating substance that the animals could not tolerate. The particular lot of L-serine that was received in 1950 was yellowish-brown and slightly damp on arrival, and possessed an unpleasant acrid odor. It was very different in appearance from the crystalline sample of the L-preparation obtained from the same company for Kuehl's experiments in 1949.

Weights of livers and adrenal glands

The weights of the livers and of the adrenal glands were determined in the animals making up the respective groups of rats (Table 32) studied in Series III. Variations in the data obtained from these groups were not as conspicuous as might have been expected. On the fresh and dry bases, the average weights of livers representing various groups were not significantly different and apparently bore no relation to the range of dietary nitrogen imposed in the present experiment. It is particularly interesting that no marked decrement in the weight of the organ was associated with force-feeding of the low-nitrogen ration, a finding that stands in distinct contrast to observations made when the same diet was fed ad libitum for a similar interval in other experiments (Mukhopadhay, 1949; Clark, 1945).

It may be of interest at this point to call the reader's attention to data recorded in Table D of Appendix I relating to the size of the adrenal glands of the animals fed the various diets of this series. The average weights of the organs of the different groups were very similar, the values approximating 35.5 mg. per 300 gm. The glands removed from the rats fed Diet 6, containing 30 mg. of nitrogen from nonessential acids, were smaller than the average, 32.9 mg., whereas those fed the diet supplying the L-forms of alanine, aspartic acid, and serine were slightly larger. Kuehl (1949) has reported higher values than those presented

Table 33. Average weights and quantities of nitrogen in livers, and nitrogen balances of rats* in Series III fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration supplemented with graded quantities of nitrogen from the nonessential amino acies (NEAA)

Diet no.	Diet	Body wt.	Wt. of fresh liver	Wt. of dry liver	N in fresh liver	Total N	N balance/ 7 days
		gm.	gm.	gm.	%	mg.	mg.
1	Low-nitrogen	331	7.576	2.368	2.56	193	-359
3	4.27% lactalbumin	357	7.422	2.360	3.02	225	-28
2	EAA as present in Diet 3	344	7.231	2.270	2.96	214	-220
4	Diet 2 plus 10 mg. N from NEAA	344	7.268	2.250	2.96	215	-163
5	Diet 2 plus 20 mg. N from NEAA	350	7.309	2.301	3.09	226	-93
6	Diet 2 plus 30 mg. N from NEAA	353	7.369	2.384	3.02	222	-28
7	Diet 2 plus 40 mg. N from NEAA	357	7.712	2.487	2.98	231	+6
8	Diet 2 plus 50 mg. N from NEAA	375	7.142	2.357	2.83	209	+82
9	Diet 2 plus 60 mg. N from NEAA	377	7.196	2.456	2.88	207	+72
10	Diet 7 with L-alananine, L-aspartic acid, and L-serine	358	7.682	2.363	3.13	240	-72

*Per 300 gm. rat

herein for rats fed a 4 per cent lactalbumin ration and one containing eighteen amino acids, *i.e.*, 42.6 and 44.7 mg. respectively.

Concentration of nitrogen in livers

Data pertaining to the quantity of nitrogen present in the livers of the individual rats in each group of the series are shown in Table 34. They are summarized in Table 33. Information concerning the composition of the livers will be discussed in terms of total nitrogen only, since no data are available at this time in respect to the concentration of fat in the organs of the various groups. In general, however, relative values correspond with absolute values.

The difference between the total quantity of nitrogen in livers of rats deprived of nitrogen for 18 days and those fed Diet 2, the basal essential amino acid diet, were highly significant. The respective values were 193 and 214 mg. A further significant increment occurred when the basal essential amino acid diet was supplemented with 20 mg. of nitrogen. No further changes in hepatic nitrogen were observed as the animals passed into nitrogen equilibrium following successive additions of the nonessential amino acids to the ration, *i.e.*, 30 and 40 mg. daily. When the 30 mg. dose was provided, the concentration of nitrogen in

Table 34. Weights and quantities of nitrogen in livers of rats in Series III fed diets containing the ten essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration supplemented with graded quantities of nitrogen from the nonessential acids (NEAA)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
1	Low-nitrogen	50589	8.534	31.01	2.646	2.50	214	7.975	2.472	200
		50594	8.953	32.35	2.896	2.46	220	7.738	2.503	190
		50605	7.855	30.34	2.383	2.62	206	7.575	2.298	199
		50705	8.002	30.82	2.466	2.72	218	6.880	2.120	187
		50712	8.462	31.71	2.683	2.48	210	7.713	2.446	191
		Average	8.361	31.25	2.615	2.56	214	7.576	2.368	193
3	4.27 per cent lactalbumin	50131	8.853	31.77	2.813	2.97	263	7.522	2.390	223
		50139	8.834	32.60	2.880	2.98	263	7.343	2.394	219
		50146	8.694	31.40	2.730	3.13	272	7.450	2.339	234
		50158	8.946	32.68	2.924	2.94	263	7.084	2.315	208
		50184	9.222	29.66	2.735	2.98	275	7.769	2.304	232
		50199	8.467	32.88	2.784	3.10	262	7.363	2.421	228
		Average	8.836	31.83	2.811	3.02	266	7.422	2.360	225

(Continued on next page)

Table 34 (Cont'd)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
2	100 EAA as present in Diet 3	50147	8.276	31.00	2.566	2.98	247	7.216	2.237	214
		50161	9.078	31.56	2.865	2.99	271	7.460	2.354	223
		50324	8.226	31.91	2.625	2.92	240	7.110	2.269	208
		50330	8.139	32.16	2.617	3.05	248	7.059	2.270	216
		50343	7.640	31.31	2.392	3.07	234	6.965	2.180	213
		50359	8.387	30.65	2.571	2.75	231	7.577	2.322	209
		Average	8.291	31.43	3.606	2.96	245	7.231	2.270	214
4	Diet 2 plus 10 mg. N from NEAA	50162	8.628	30.29	2.614	2.83	244	7.294	2.209	206
		50191	9.175	30.34	2.784	2.95	270	7.381	2.239	217
		50265	8.424	31.05	2.616	2.92	246	7.475	2.321	218
		50325	8.576	31.09	2.666	2.73	235	7.726	2.402	212
		50342	7.354	31.54	2.320	3.19	235	6.584	2.076	210
		50358	7.885	31.50	2.484	3.15	248	7.149	2.252	225
		Average	8.340	30.97	2.581	2.96	246	7.268	2.250	215

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Table 34 (Cont'd)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
5	Diet 2 plus 20 mg. N from NEAA	50190	8.850	31.25	2.766	3.14	278	7.418	2.318	233
		50263	8.272	30.36	2.511	3.05	252	7.275	2.209	222
		50289	8.063	31.85	2.568	3.09	249	7.486	2.383	231
		50329	8.534	31.94	2.726	2.89	247	7.129	2.277	206
		50344	8.184	32.35	2.647	3.35	274	6.994	2.262	234
		50366	9.214	31.21	2.876	3.03	279	7.552	2.357	229
		Average	8.519	31.49	2.682	3.09	263	7.309	2.301	226
6	Diet 2 plus 30 mg. N from NEAA	50292	9.242	32.63	3.016	3.07	284	7.532	2.458	231
		50327	8.365	33.31	2.786	2.75	230	7.674	2.556	211
		50333	8.283	31.88	2.641	3.10	257	7.037	2.244	218
		50341	8.543	32.43	2.770	3.08	263	7.282	2.362	224
		50377	8.240	32.02	2.638	3.01	248	7.209	2.308	217
		50392	9.331	31.78	2.965	3.08	287	7.482	2.378	230
		Average	8.667	32.34	2.803	3.02	261	7.369	2.384	222

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Table 34 (Cont'd)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
7	Diet 2 plus 40 mg. N from NEAA	50176	9.289	32.37	3.007	2.83	263	7.677	2.485	217
		50295	8.916	34.00	3.031	2.88	257	7.805	2.652	225
		50334	9.347	32.26	3.015	2.95	276	7.921	2.555	234
		50357	9.448	32.16	3.039	3.02	285	8.145	2.620	255
		50374	8.834	33.38	2.949	2.98	263	7.797	2.603	232
		50422	8.957	32.50	2.911	3.14	282	7.509	2.440	236
		50436	8.924	32.08	2.863	2.98	266	7.608	2.441	227
		50444	10.189	29.00	2.955	2.83	288	7.941	2.303	224
		50463	9.249	32.45	3.001	3.04	281	7.382	2.395	224
		50564	8.706	32.46	2.826	3.16	275	7.334	2.381	232
		Average	9.186	32.27	2.960	2.98	274	7.712	2.487	231

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Table 34 (Cont'd)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
8	Diet 2 plus	50462	9.356	32.11	3.004	2.96	277	7.126	2.288	211
	50 mg. N	50485	8.553	35.61	3.046	2.82	241	7.230	2.575	204
	from NEAA	50537	9.629	33.22	3.199	3.04	293	7.044	2.340	214
		50609	8.504	32.80	2.789	3.02	257	6.971	2.286	211
		50620	8.610	31.25	2.691	2.80	241	7.340	2.294	205
		Average	8.931	32.50	2.946	2.93	262	7.142	2.357	209
9	Diet 2 plus	50536	8.985	34.66	3.114	2.76	248	7.550	2.615	208
	60 mg. N	50549	9.287	34.70	3.222	2.79	259	7.353	2.549	206
	from NEAA	50553	9.718	34.00	3.304	2.85	277	7.146	2.429	204
		50606	8.138	33.84	2.754	3.03	246	6.914	2.340	209
		50621	9.104	33.40	3.041	2.98	271	8.019	2.345	209
		Average	9.046	34.12	3.087	2.88	260	7.196	2.456	207
10	Diet 7 with	50532	8.963	31.28	2.804	3.03	272	7.863	2.460	239
	L-alanine,	50548	9.468	30.92	2.927	3.10	294	7.742	2.393	240
	L-aspartic	50593	8.984	31.58	2.837	3.07	276	7.549	2.384	232
	acid, and	50601	9.505	31.40	2.984	3.19	303	7.665	2.406	244
	L-serine	50611	8.858	28.61	2.534	3.24	287	7.590	2.171	246
		Average	9.156	30.76	2.817	3.13	286	7.682	2.363	240

the liver and the nitrogen balance were identical to those characteristic of the rats fed the reference diet containing 4.27 per cent of lactalbumin. The addition, however, of 50 mg. of supplementary nonessential nitrogen to the ration was reflected in a reduction in hepatic nitrogen from 220 mg. in livers of control rats to 209 mg., despite the attainment of a positive nitrogen balance of 32 mg.

This last observation is of particular interest. May it not signify that nitrogen deposited in the liver, perhaps temporarily, may be utilized when the supply of nonessential acids becomes ample for the synthesis of body proteins and metabolites and that thereby positive balance becomes possible? Some support for this hypothesis may be gained from the data describing the hepatic nitrogen of the group of rats fed the L-forms of alanine, aspartic acid, and serine (Diet 10, Table 33). These rats while failing to attain nitrogen balance with a nitrogen intake equivalent to that of the animals fed Diet 7, nevertheless, had livers that contained approximately the same amount of nitrogen.

Preliminary examination of data in Table 31 revealed differences in respect to the absorption of dietary nitrogen from various diets. For this reason, data relative to excretion of fecal nitrogen were summarized in Table 35.

Table 35. The distribution between urine and feces of nitrogen excreted by rats* in Series III fed the essential amino acids supplemented with graded quantities of nonessential acids

Diet no.	Diet	Total N excreted	N in feces	% of total N in feces
		mg.	mg.	mg.
1	Low-nitrogen	408	162	39.7
3	Lactalbumin (4.27%)	478	195	40.8
2	10 EAA as present in Diet 3	524	175	33.4
4	Diet 2 plus 10 mg. N from NEAA	519	186	35.8
5	Diet 2 plus 20 mg. N from NEAA	500	182	36.4
6	Diet 2 plus 30 mg. N from NEAA	488	173	35.4
7	Diet 2 plus 40 mg. N from NEAA	513	173	33.7
8	Diet 2 plus 50 mg. N from NEAA	478	159	33.3
9	Diet 2 plus 60 mg. N from NEAA	535	163	30.8
10	Diet 7 with L-alanine, L-aspartic acid, and L-serine	593	177	30.0

*Per 300 gm. rat

They are expressed on the basis of a uniform body weight of 300 gm. Rats that were fed the lactalbumin-containing diet excreted on the average 195 mg. of nitrogen in the feces per 7 days, in contrast to 162 mg. by those fed the low-nitrogen ration. The quantities of nitrogen lost in the feces by rats fed the amino acid-containing diets varied from 159 to 186 mg. per 300 gm. rat. The animals that were fed diets containing 50 or 60 mg. of nitrogen from the amino acids absorbed nitrogen completely, the respective amounts of nitrogen in the feces of such rats being 159 and 163 mg.

It seems reasonable to believe, therefore, that nitrogen derived from the amino acids was absorbed quantitatively, or nearly so, when the animals were in positive nitrogen balance or in equilibrium. The low coefficient of digestibility for lactalbumin may account for the slightly negative balance characterisitc of rats fed the 4.27 per cent lactalbumin diet.

In summary, it may be recorded that data secured in Series III have revealed (1) that nitrogen equilibrium in well-nourished adult albino rats could be achieved by supplementing a basal mixture of essential amino acids, which simulated lactalbumin and contributed 43.5 mg. of nitrogen daily, with a particular composite of nonessential

SERIES IV

Utilization of Nitrogen from a Mixture Containing Essential Amino Acids Only

The experiments described in Series III showed that a diet whose nitrogen was derived only from the essential amino acids induced a severe negative nitrogen balance when fed to well-nourished adult rats. In this instance, the ration contained the amino acids in the proportions in which they occur in lactalbumin and in the quantities that would be supplied by a diet containing 4.37 per cent of the protein.

The observation led to interesting speculation because other workers had demonstrated that the essential amino acids were capable of fulfilling the nitrogenous requirements of the adult repleting rat as measured by nitrogen balance (Brush, Willman, and Swanson, 1947), by weight recovery (Frazier et al., 1947), and by regeneration of serum proteins (Benditt et al., 1947). Later (1948) Wissler and associates fed (to normal adult rats) the same mixture containing nine of the ten essential amino acids, excluding arginine, as was used by Frazier and Benditt in Cannon's laboratory. They concluded that such a ration in the normal rat also was capable of maintaining appetite, weight, and nitrogen balance. The composition of the ration is presented in Table 1. It is apparent that the total quantities of each of the acids in this mixture greatly exceeded those supplied by Kuehl in 1949 or by the present investigator.

On the other hand, literature showing that nonessential nitrogen exerts growth-stimulatory properties in several species when added to a diet containing only the essential amino acids has been reviewed by Swanson and Clark (1950).

It seemed worthwhile to determine whether or not the results obtained in the present experiment could be ascribed to the relatively small amount of essential nitrogen provided by Diet 3. Perhaps, in light of the limited supply, too great a burden was imposed upon the chemical resources of the cells in the concurrent synthesis of the nonessential amino acids and the maintenance of metabolic processes to permit the establishment of equilibrium. It was decided, therefore, to study the effect of feeding a ration in which the quantity of the amino acid mixture containing only the essential amino acids was doubled (Diet 11, Table 11).

Several relationships exist between essential and nonessential amino acids which suggest that nitrogen equilibrium might well occur in animals fed a diet supplying the essential amino acids only. In the growing rat, methionine and phenylalanine, for example, can perform the roles played respectively by dietary cystine (Womack, Kemmerer, and Rose, 1937) and tyrosine (Womack and Rose, 1934). There is no reason to believe that this relationship is altered in the mature organism. Rose and Womack (1947) also suggest on the basis of feeding experiments that arginine, proline, and glutamic acid are mutually convertible although at different rates. Furthermore, ornithine exists in equilibrium with proline, which in turn is readily converted to glutamic acid (Stetten and Schoenheimer, 1944). It seemed, therefore, that potential sources of at least four nonessential amino acids would be provided if the quantities of arginine, methionine, and phenylalanine present in the basal essential amino acid diet (Diet 2) were increased.

That the D-forms of certain amino acids can be utilized for the synthesis of nonessential acids but not for fabrication of tissues has been assumed by many investigators including Frazier and others (1947), Anderson and Nasset (1948), and Ramasarma, Henderson, and Elvehjem (1949). Very recently Lardy and Feldott (1950) reported that growing rats fed diets containing either the natural forms of the essential amino acids or the racemic mixtures thereof retained the same percentage of ingested nitrogen. If normal adult rats also utilize the D-isomers effectively, the presence of additional quantities of D-isoleucine, D-threonine, and D-valine introduced through the incorporation of DL-forms in the proposed mixture should facilitate the synthesis of nonessential amino acids.

The diet containing twice the quantities of the essential amino acids present in the basal synthetic ration no. 2 provided 87.0 mg. of nitrogen per day. In total nitrogen content, it compared favorably to Diet 7., i.e., 87.0 mg. vs. 83.5 mg. Approximately one-half (40 mg.) of the nitrogen of this latter diet, it may be recalled, had its origin in a supply of nonessential nitrogen.

The average nitrogen balances per 7-day experimental period of the rats fed these three diets appear in Table 36. The data show that doubling the quantity of the essential amino acid mixture decreased body catabolism. The negative balance shifted from -220 mg. per 300 gm. rat per 7 days to -70 mg. However, the animals did not utilize their dietary supply of nitrogen nearly as effectively as they did when the ration contained both dispensable and indispensable acids, as shown by the respective balances of the two groups, i.e., -70 and + 7 mg., respectively. It is rather interesting that the extent of utilization was roughly equivalent to that occurring when the essential acids in Diet 2 were supplemented with only 20 mg. of nitrogen from nonessential acids (Diet 5, Table 31). In other words, 43.5 mg. of essential nitrogen and 20 mg. of nonessential nitrogen improved

Table 36. Average nitrogen balances per 7 days of rats in Series IV

Diet no.	Diet	Body wt.	N in food	Total N excreted	N balance/ 300 gm. rat	N balance/ 100 sq. cm.
		gm.	mg.	mg.	mg.	mg.
2	Basal essential amino acid diet	344	349	601	-220	-60
11	Twice the quantity of essential amino acids in Diet 2	369	691	778	- 70	-16
7	Diet 2 plus 40 mg. N from NEAA	357	618	610	+ 7	+ 1
8	Diet 2 plus 50 mg. N from NEAA	375	697	597	+ 82	+23

Table 37. Nitrogen balances per 7 days of individual rats in Series IV fed Diet 11 containing twice the quantity of the ten essential amino acids provided by the basal essential amino acid diet

Diet no.	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
		gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
11	50429	351	422	691	549	201	750	- 59	- 50	-12
	50435	371	436	691	611	216	827	-136	-110	-25
	50443	384	446	691	523	220	743	- 52	- 40	- 9
	50477	382	444	691	606	241	847	-156	-123	-28
	50483	359	428	691	528	196	724	- 33	- 27	- 6
	Average	369	435	691	564	215	778	- 87	- 70	-16

the value of the indispensable acids in the basic mixture to about the same extent. The addition of 50 mg. of nonessential acids to Diet 2 (Diet 8, Table 36) threw the animals into decided positive balance, although the total quantity of dietary nitrogen was only 6 mg. higher per day than in experimental Diet 11.

During the period of observation, the experimental rats fed Diet 11 did not show any consistent or marked changes in body weight (Table 38). The nitrogen balances of the individual rats, however, were more variable (Table 37) than those recorded in Series III. The extent of the negativity of the balances corresponded to changes in body weight.

The elevated excretion of nitrogen by rats fed Diet 11 was accounted for almost entirely by an accentuated loss of nitrogen in the urine. Thus, the rats that received Diet 11 excreted 564 mg. of urinary nitrogen per 7 days, whereas those that were fed Diets 6 and 7, containing, 20 and 40 mg. of nonessential nitrogen, eliminated 372 and 404 mg., respectively. The quantity of fecal nitrogen characteristic of rats fed Diet 11 was almost identical with that of rats fed the low-nitrogen diet or the amino-acid containing diets fed in series III. This fact indicated satisfactory absorption supplied as essential amino acids only.

Table 38. Body weights at certain intervals of rats in Series IV fed Diet 11 containing twice the quantity of the ten essential amino acids provided by the basal essential amino acid diet

Diet no.	Rat no.	Pre-exp't'l wt.	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
		gm.	gm.	gm.	gm.	gm.
11	50429	357	350	354	350	-4
	50435	401	394	374	375	+1
	50443	392	382	386	386	0
	50477	390	386	382	380	-2
	50483	372	358	360	362	+2
	Average	382	374	371	370	0

A metabolic disturbance associated with the administration of only the essential amino acids was indicated on several occasions by a reddish genital exudate that was not observed in other groups. Whether it resulted from irritation of the alimentary or genito-urinary tracts or whether it represented an abnormal metabolite was not ascertained. Rat 50435 which had a severely negative balance showed the exudate more frequently than did other members of the group.

No alterations in the weights of the adrenal glands followed the feeding of the essential amino acids alone, the average weight being 35.5 mg. (Table E, Appendix I).

The external appearance of the animals was less satisfactory than that of rats in the control group that were fed 40 mg. of additional nitrogen from the nonessential acids. Their muscle tone was poorer and bald patches appeared on the backs of certain animals. Necropsy showed that the fat depots were filled more completely, however, than were those of rats fed Diet 7, and resembled those of rats fed 50 mg. of nitrogen from nonessential acids. Data that will be available at a later date pertaining to the concentrations of fat in hepatic tissue of rats in the various groups will contribute to an understanding of the mobilization of fat when the essential amino acids represented the sole source of nitrogen. It seems possible that the supply of certain ketogenic amino acids such as isoleucine, leucine, and phenylalanine exceeded the requirement for the synthesis of tissues and was diverted, therefore, to pathways leading to the deposition of fat.

Only slight differences were observed in the weights of livers of rats fed the essential amino acids only and the control diet, on either the fresh or dry basis (See Table 39). Also, the average total quantities of nitrogen found in the livers of rats from the two groups were almost identical when expressed in terms of rats weighing 300 gm. Again, as in Series III, the quantity of the element in the organ was greater than that in the tissue of animals in which positive nitrogen balance was successfully established (Diet 3, Table 39). The fact that Rat 50435, for example, which had a severe negative balance and also lost weight (Tables 37 and 38) possessed larger reserves of nitrogen than did any other animal in the group is further indication of inefficient utilization of dietary nitrogen derived from the essential amino acids. The question may be raised again concerning the significance of hepatic nitrogen under the specific experimental conditions imposed in the present experiment.

In summary, the data suggest definitely that the adult rat in a good state of nutriture cannot synthesize the non-

Table 39. Average weights and quantities of nitrogen in liver, and nitrogen balances of rats* in Series IV

Diet no.	Diet	Body wt.	Wt. of fresh liver	Wt. of dry liver	Total N	N balance/ 7 days
		gm.	gm.	gm.	mg.	mg.
2	Basal essential amino acid diet	344	7.231	2.270	214	-220
11	Twice the quantity of essential amino acids present in Diet 2	369	7.152	2.218	228	- 70
7	Diet 2 plus 40 mg. N from NEAA	357	7.712	2.487	231	+ 7
8	Diet 2 plus 50 mg. N from NEAA	375	7.142	2.357	209	+ 82

*Per 300 gm. rat

Table 40. Weights and quantities of nitrogen in livers of rats in Series IV fed Diet 11 which contained twice the quantity of the ten essential amino acids provided by the basal essential amino acid diet

Diet no.	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm.		
							Fresh liver	Dry liver	Total N
		gm.	%	gm.	%	mg.	gm.	gm.	mg.
11	50429	8.588	31.24	2.683	3.01	258	7.340	2.293	220
	50435	9.919	30.69	3.044	3.19	316	8.018	2.460	255
	50443	8.439	30.92	2.615	3.15	266	6.608	2.043	208
	50477	8.443	30.36	2.563	3.34	282	6.632	2.031	221
	50483	8.571	31.85	2.730	3.30	282	7.160	2.282	237
	Average	8.796	31.01	2.727	3.20	281	7.152	2.218	228

essential acids rapidly enough to support normal nitrogen metabolism from a supply of essential amino acids approximately equivalent to those in an 8 per cent lactalbumin diet. The author had rather expected that this would occur. Data presented in this series suggest that normal adult rats need both the essential and nonessential acids in their diet at a level of total nitrogen intake that will just support nitrogen equilibrium. Apparently under these conditions, the essential acids cannot substitute for the nonessential. The normal adult animal apparently responds like the repleting animal in this respect. Frost in 1949 found, for example, that such a rat could not utilize fully the nitrogen supplied by twice the minimal quantities of essential amino acids recommended by Cannon and associates, whereas supplementation of the original mixture with the nonessential amino acids or some inorganic source of nitrogen was beneficial. He concluded that the ability of the organism to convert essential to nonessential acids was limited. Similarly, Rose, Oesterling, and Womack (1948) observed that growing rats fed ten essential amino acids gained only 70 to 75 per cent as much weight as did their littermates which consumed an equivalent amount of nitrogen from nineteen amino acids.

The possibility remains that an imbalance of amino acids existed in the mixture tested in Series IV. Van Pilsum and Berg (1950), for example, have demonstrated that in the growing rat excessive quantities of the natural isomer of certain amino acids, especially methionine, may be even more deleterious than are the same amounts of the D-configuration of the same amino acid.

Evidence from the laboratories of Wretlind (1949) and of Berg (Van Pilsum and Berg, 1950) also suggest that investigators have tended to ascribe to the presence of unnatural isomers certain physiological responses that are associated more specifically with the nature of the acid than with its configuration. Or perhaps, in the present investigation, The capacity of the organism to deaminate the D-amino acids provided in the diet was exceeded. Only when the L-forms of all of the essential amino acids are obtainable will it become possible to evaluate conclusively the response of the adult rat to a mixture containing different proportions of the essential amino acids alone.

Further information could be gained by adding individual nonessential amino acids or inorganic nitrogen in varying concentrations to graded quantities of essential amino acids tested in Diet 11, or by modifying the proportions of the individual essential amino acids represented therein.

SERIES V

Utilization of Nitrogen when Methionine and Phenylalanine Were Increased to Replace Cystine and Tyrosine Isonitrogenously in a Mixture of Amino Acids that Supported Nitrogen Equilibrium

That a ration containing an assortment of amino acids equivalent in quality and quantity to those present in a diet containing 4.27 per cent of an analyzed preparation of lactalbumin is adequate for the maintenance of nitrogen equilibrium in the adult well-nourished rat has been demonstrated. Whether or not the assortment represented the most efficient mixture for this purpose was not known, however. Since such description is the ultimate objective of one of the research programs of the department with which the author has been associated, it seemed important that the potential nutritive value of the mixture be examined critically at this point. Its composition in respect to the constituent essential amino acids has been re-evaluated, therefore.

This phase of the study was approached first by comparing the amino acid content of Diet 7 with that of other diets described in the literature which, according to the authors, were just capable of supporting equilibrium in the adult *well-nourished rat*. Data were available in respect to two such rations.

As stated earlier, Diet 7 which was used in the present study simulated a reference diet containing 4.37 per cent of a lactalbumin preparation in regard to the kind and quantity of amino acids herein. All of the amino acids occurring in lactalbumin except serine and hydroxyglutamic acid could be secured for incorporation in the mixture.

The first diet against which comparisons could be made was tested by Mukhopadhay in 1949 and contained 32 mg. of nitrogen that was derived solely from dehydrated defatted egg proteins. She showed that healthy adult rats remained in nitrogen equilibrium when fed this ration. The particular sample of egg powder that was incorporated in her ration was distributed as a reference protein in a collaborative assay sponsored by the Bureau of Biological Research of Rutgers University. Data relative to its amino acid composition as indicated by microbiological assay were made available to the investigators who cooperated in the project.*
From this information, the concentrations of the individual amino acids in the egg-containing diet were estimated.

The second diet to be examined was formulated in Cannon's laboratory in 1950 by Benditt and his coworkers

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*On file in the Nutrition Laboratory of the Iowa State College who used a mixture simulating casein as a starting point (See Table 1, Wissler). By feeding graded quantities of each of the essential amino acids in the presence of ample quantities of the other essential and nonessential acids, these investigators developed a composite of essential amino acids which, in their opinion, represented the minimal quantities of each of the essential amino acids that would meet the nitrogenous needs of the mature well-nourished rat. The composition of the mixture is described in Table 2.

The quantities of individual essential amino acids in these three diets, expressed as the number of milligrams provided daily per 100 sq. cm. of body surface, are depicted graphically in Figure 2.

It is evident that the concentrations of histidine, threonine, and valine in the three rations are very similar. The diet that was fed in the present investigation contained approximately 25 per cent more tryptophane than did the other two diets since the proportion of tryptophane in lactalbumin is higher than in either egg proteins or casein. The quantities of isoleucine and leucine in Diet 7 resembled those in the diet used by Mukhopadhay but differed strikingly from Cannon's mixture in respect to the relative amounts of each amino acid. The total amount of nitrogen provided

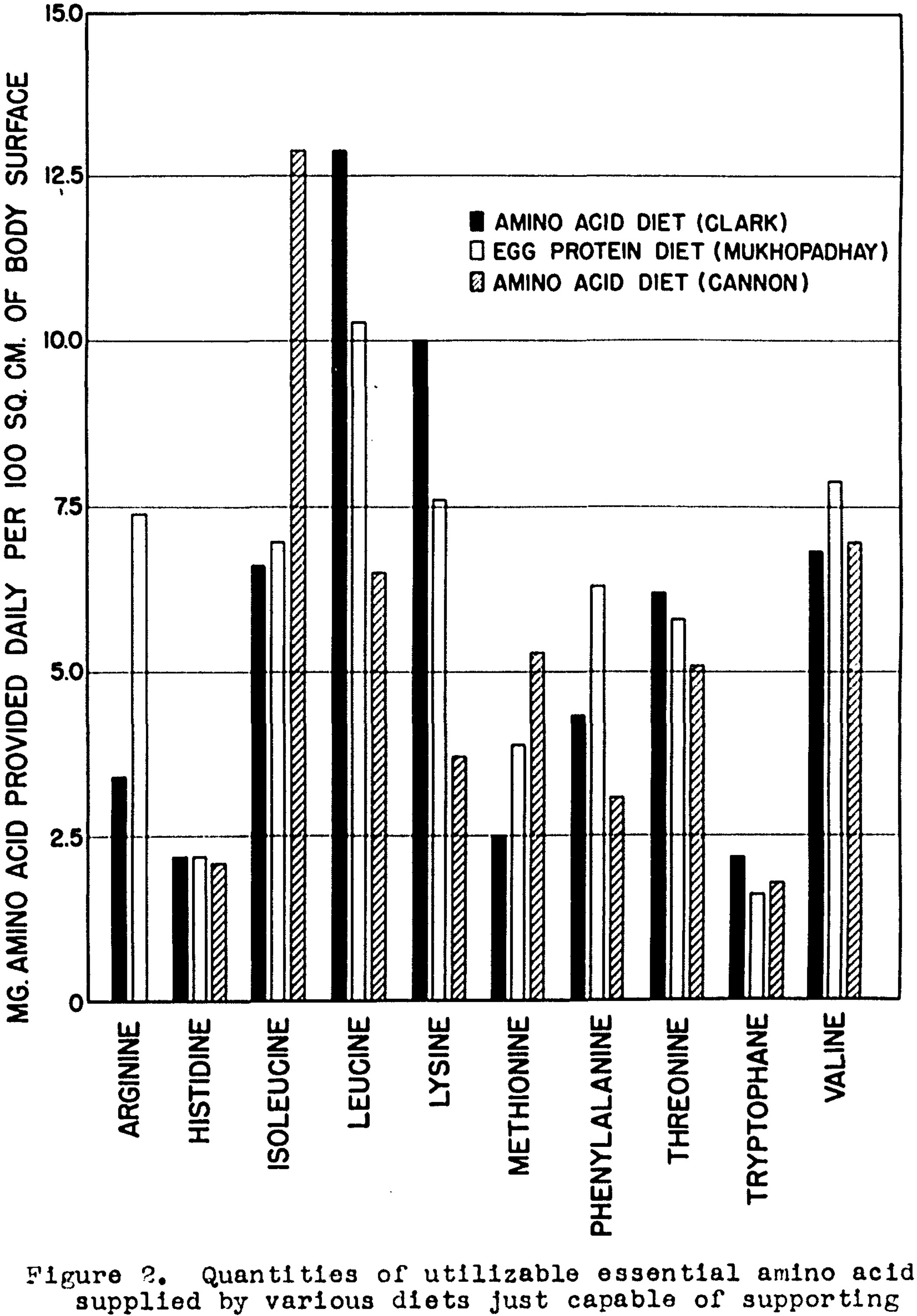

Figure 2. Quantities of utilizable essential amino acids supplied by various diets just capable of supporting nitrogen equilibrium in the normal adult male rat

by the two amino acids was approximately the same, however. The amount of leucine in Diet 7 was twice as high as in Cannon's diet. Diet 7, on the other hand, provided isoleucine in only one-half the amount which Cannon's group considered necessary. Recent information concerning interaction between leucine and isoleucine (Shemin, 1950) and transamination reactions involving ketoglutaric acid and leucine (Tanenbaum and Shemin, 1950) suggest, however, that the rat may possess the capacity to adjust to moderate variations in the proportions of these two amino acids in the diet, although both are truly essential. Probably, therefore, the three rations may be considered similar in respect to these six-carbon amino acids.

Variations in the concentration of methionine in the three rations were marked. The quantity that was provided in the daily quota of diet fed in the present investigation was only a little more than one-half the amount present in egg proteins and less than one-half of the minimal quantity recommended by Cannon. This relationship is apparent from data presented in Table 41 and in Table I of Appendix I, as well as from Figure 2. The key position of the sulfur-containing amino acid in the utilization of nitrogen and fat has been revealed in many laboratories. However, its functions have not been fully elucidated. It seemed probable,

Table 41. Quantities of utilizable essential amino acids supplied daily by various diets just capable of supporting nitrogen equilibrium in the adult well-nourished rat (mg. amino acid/100 sq. cm./day)

Amino acid	Investigator		
	Clark	Mukhopadhay	Cannon*
Arginine	3.4	7.4	---
Histidine	2.2	2.2	2.1
Isoleucine	6.6	7.0	12.9
Leucine	12.9	10.3	6.5
Lysine	10.0	7.6	3.7
Methionine	2.5	3.9	5.3
Phenylalanine	4.4	6.3	3.1
Threonine	6.2	5.8	5.1
Tryptophane	2.2	1.6	1.8
Valine	6.8	7.9	7.0
Total	57.2	60.0	47.5

*Benditt _et al_., J. Nutr. _40_, 341 (1950) therefore, that the diet simulating lactalbumin was low in this vital compound; whether or not it was critically so could only be determined by experimentation. However, the total quantity of sulfur-containing amino acids in Diet 7 very closely approximated that in the other two diets (Table 42). It is also evident from the data in this table that the nonessential acid, cystine, contributed two-thirds of the total amount of nitrogen that was available from the sulfur-containing acids incorporated in Diet 7. The omission of the nonessential amino acids from the dietary mixture used in Diet 2 thus drastically reduced the total supply of nitrogen from the sulfur-containing amino acids. The ineffectiveness of the essential amino acids alone in sustaining nitrogen balance might be due to such a

Table 42. Quantities of nitrogen supplied daily by methionine, cystine, phenylalanine, and tyrosine in diets that supported nitrogen equilibrium (in mg./300 gm. rat/da.)

	Investigator		
Amino acid	Clark	Mukhopadhay	Cannon*
Methionine	1.0	1.4	1.9
Cystine	1.9	1.5	.7
Phenylalanine	1.5	2.1	1.0
Tyrosine	2.1	1.3	7.6

*Calculated from data presented in Tables 1 and 2.

deficit, the amount of methionine being too small to meet the body's demands for both cystine and methionine.

In the diet containing egg proteins, cystine contributed one-half of the total amount of nitrogen coming from the sulfur acids. In Dr. Cannon's mixture, however, the quantity of methionine was considerably higher than in the other two diets.

Comparisons of the phenylalanine and tyrosine contents of the three diets are interesting also. The quantity of phenylalanine also was lower in Diet 7 than in the ration used by Mukhopadhay but it was not below Cannon's minimal value. From Table 42 it may be seen that tyrosine provided two-thirds of the nitrogen available from the two aromatic acids in Diet 7; two-fifths of that from these sources in Mukhopadhay's egg-containing ration and nearly nine-tenths of that in Cannon's diet. In fact, Benditt and his associates (1950) have written of this diet

> The diets in the present investigation probably contained sufficient tyrosine to reduce the phenylalanine requirement for synthesis of tyrosine to its minimum. The quantity of cystine, similarly, was probably sufficient to reduce the methionine requirement for conversion to this acid nearly to its minimum. (p. 345)

In summary, then, it would appear that if the amino acid mixture used in Diet 7 were deficient at all, such deficiencies probably lay in the respective quantities of methionine and phenylalanine in that portion of the mixture that provided the essential amino acids. However, it was possible that the introduction of nonessential tyrosine and cystine in Diet 7 relieved the need for the synthesis of these units, the mixture thereby becoming adequate as indicated in Series III. The foregoing analysis however certainly raises a question as to its optimal efficiency.

The experiments in Series V were planned, therefore, to determine whether the relatively low amounts of methionine, or phenylalanine, or both in Diet 7 influenced its nutritive value. The author was interested also in demonstrating whether or not the needs for maintenance could be met when methionine and phenylalanine were incorporated in the diet in the absence of cystine and tyrosine. Diet 7, which successfully fulfilled the nitrogenous requirements for maintenance in Series III, was selected as the control ration. It was modified so that it provided larger quantities (1) of methionine only, (2) of phenylalanine only, and (3) of both methionine and phenylalanine. Diet 12, for example, differed from the control ration only by virtue of the isonitrogenous replacement of cystine with methionine (Table 12). The total quantity of nitrogen contributed by both methionine and cystine in Diet 7 was present only as methionine in Diet 12. No other alteration was made in the

Table 43. Body weights at certain intervals of rats in Series V fed diets in which methionine and phenylalanine of Diet 7 were increased, cystine and tyrosine being replaced isonitrogenously (Diets 12 to 14)

Diet no.	Diet	Rat no.	Pre-exp't'l wt.	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	gm.
12	Methionine of Diet 7 increased, cystine replaced isonitrogenously	50414	369	366	372	370	-2
		50421	354	354	357	360	+3
		50433	396	388	385	383	-2
		50451	379	372	380	382	+2
		50464	385	383	381	382	+1
		50468	354	342	350	350	0
		Average	373	367	371	371	0
13	Phenylalanine of Diet 7 increased, tyrosine replaced isonitrogenously	50416	370	370	368	368	0
		50423	378	373	376	378	+2
		50434	370	364	367	366	-1
		50442	354	352	352	354	+2
		50450	396	386	386	384	-2
		50470	382	376	382	383	+1
		Average	375	370	370	372	0
14	Methionine and phenylalanine increased, cystine and tyrosine replaced	50448	431	426	414	414	0
		50476	370	369	370	374	+4
		50502	383	370	366	372	+6
		50535	408	389	386	391	+5
		50588	346	342	346	353	+7
		Average	388	379	376	381	+5

composition of the test mixture. Similarly, tyrosine was replaced isonitrogenously with phenylalanine in Diet 13. Finally, in Diet 14, both methionine and phenylalanine were increased simultaneously, replacing both cystine and tyrosine.

Body weight

Data presented in Table 43 indicate that the body weights of the rats fed the diets in which methionine or phenylalanine was increased remained constant. The same was true of the weights of animals fed Diet 7 (Table 29). The average increment of 5.0 gm. per rat during the balance period suggested, however, that replacement in Diet 14 of both cystine and tyrosine by the appropriate essential amino acids was beneficial.

Nitrogen balance

The average nitrogen balances of the rats, as shown in Tables 44, indicated that the respective replacements of either cystine or tyrosine individually by methionine and phenylalanine did not enhance the retention of nitrogen. The nitrogen balance in the 7-day period per 300 gm. rat was almost identical with that associated with the control ration when methionine was increased. It was slightly lower

Table 44. Average nitrogen balances per 7 days of rats in Series V

Diet no.	Diet	Body wt.	N in food	Total N excreted	N balance/ 300 gm. rat	N balance/ 100 sq. cm.
		gm.	mg.	mg.	mg.	mg.
7	The basal essential amino acid diet plus 40 mg. N from NEAA	357	618	610	+ 7	+ 1
12	Methionine of Diet 7 increased, cystine replaced isonitrogenously	369	605	599	+ 5	+ 1
13	Phenylalanine increased, tyrosine replaced isonitrogenously	371	619	638	-15	- 4
14	Methionine and phenylalanine increased, cystine and tyrosine replaced	379	622	552	+58	+16

Table 45. Nitrogen balances per 7 days of individual rats in Series V fed diets in which methionine and phenylalanine of the basal essential amino acid diet were increased, cystine and tyrosine being replaced isonitrogenously (Diets 12 to 14)

Diet no.	Diet	Rat no.	Body wt.	Body surface	N in food	N in urine	N in feces	Total N excreted	N balance	N balance/ 300 gm rat	N balance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
12	Methionine increased, cystine being replaced isonitrogenously	50414	368	434	605	377	206	583	+22	+18	+ 6
		59421	357	427	605	380	209	589	+16	+14	+ 4
		50433	380	443	605	439	210	649	-44	-34	-10
		50451	380	443	605	405	194	599	+ 6	+ 5	+ 1
		50464	380	443	605	402	201	603	+ 2	+ 2	0
		50468	349	419	605	362	216	578	+27	+23	+ 6
		Average	369	435	605	394	205	599	+ 5	+ 5	+ 1
13	Phenylalanine increased, tyrosine being replaced isonitrogenously	50416	367	433	619	429	216	645	-26	-21	- 6
		50423	375	439	619	429	219	648	-29	-22	- 6
		50734	365	432	619	404	202	606	+13	+11	+ 3
		50442	352	422	619	401	228	629	-10	- 9	- 2
		50450	384	446	619	443	205	648	-29	-23	- 6
		50470	381	443	619	425	227	652	-33	-26	- 7
		Average	371	436	619	422	216	638	-19	-15	- 4
14	Methionine and phenylalanine increased, cystine and tyrosine being replaced	50448	413	465	622	437	198	635	-13	-10	- 3
		50476	371	436	622	355	203	558	+64	+52	+15
		50502	371	436	622	319	166	485	+137	+111	+31
		50535	390	450	622	381	195	576	+46	+35	+10
		50588	349	419	622	328	175	505	+117	+101	+28
		Average	379	441	622	364	188	552	+70	+58	+16

when phenylalanine was increased at the expense of tyrosine, being -15 mg. in contrast to the positive balance of 7 mg. that was associated with ingestion of the control ration. It is impossible to determine whether this finding resulted from experimental variation or whether a relatively high proportion of phenylalanine in the presence of other amino acids may have stimulated the catabolism of tissues as it did in the absence of other amino acids (Brush, Willman, and Swanson, 1947). Nevertheless, when both methionine and phenylalanine replaced cystine and tyrosine, the average nitrogen retention was improved distinctly, to reach a positive balance of 53 mg. per 300 gm. rat per 7 days.

The observation that nitrogen balances were almost identical when the sulfur-containing amino acids were incorporated as methionine alone or as a mixture which supplied one-third of the total nitrogen in the form of methionine and two-thirds as cystine is in harmony with results obtained by Dr. H. B. Lewis* with growing rats. Both experiments have indicated that the provision of cystine as such does not ensure greater physiological economy provided the total supply of sulfur-containing moities is adequate. The

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*Personal communication from Dr. H. B. Lewis, Professor of Biological Chemistry, University of Michigan, Ann Arbor, May 1, 1950 to Helen E. Clark.

significance of the nonessential acid, serine, which provides the necessary carbon chain to accept the sulfur atom from methionine is emphasized by such a relationship.

No clear-cut explanation of the increased retention of nitrogen that was associated with the simultaneous replacement of cystine and tyrosine by isonitrogenous quantities of methionine and phenylalanine, respectively, can be given at the present time. The fact that nitrogen metabolism was altered when both essential amino acids were increased but not when either was elevated alone suggests the existence of an intricate relationship, possibly involving hormonal influences. In this connection, the recent statement by Keller, Boissonnas, and Du Vigneaud (1950) may be pertinent

> The present interest in the interrelationship between arterenol and epinephrine makes the involvement of transmethylation in the biosynthesis of epinephrine all the more intriguing. (p. 631)

Arterenol, which is believed to be an intermediate in the conversion of hydroxytyramine to adrenaline, is elaborated in the adrenal medulla and possesses many of the properties of epinephrine (Holz and Schumann, 1949).

Weights and concentrations of nitrogen in livers

Data pertaining to weights and hepatic stores of nitrogen generally confirmed the conclusions drawn from nitrogen balances. For example, the livers of rats receiving additional phenylalanine were slightly heavier on the fresh basis than were other livers in the series, although the difference was less marked when dry weights were considered (Tables 46 and 47).

The total quantity of nitrogen in the livers of rats fed both methionine and phenylalanine in the absence of cystine and tyrosine was significantly lower than that of rats fed diets in which either methionine or phenylalanine alone replaced cystine or tyrosine. The concentration of nitrogen in the livers of rats which received increased quantities of both essential amino acids at the expense of cystine and tyrosine corresponded to that in the livers of rats fed the control ration. Again, the low concentration of nitrogen in the livers of rats fed Diet 14 coupled with the distinctly positive nitrogen balance suggest an accelerated mobilization of nitrogen involving the withdrawal of nitrogen from the liver and its efficient utilization.

In pursuing the investigation of the roles of these two essential amino acids, further information might be gained by increasing the concentrations of both methionine and phenylalanine beyond the highest quantities incorporated in any of the diets so far tested here and elsewhere, and to do so in the absence of cystine and tyrosine. The incre-

Table 46. Average weights and quantities of nitrogen in livers, and nitrogen balances of rats* in Series V

Diet no.	Diet	Body wt.	Wt. of fresh liver	Wt. of dry liver	Total N	N balance/ 7 days
		gm.	gm.	gm.	mg.	mg.
7	Basal essential amino acid diet plus 40 mg. N from NEAA	357	7.712	2.487	231	+ 7
12	Methionine of Diet 7 increased, cystine being replaced isonitrogenously	369	7.782	2.450	246	+ 5
13	Phenylalanine of Diet 7 increased, tyrosine replaced isonitrogenously	371	8.155	2.627	246	-15
14	Methionine and phenylalanine of Diet 7 increased, cystine and tyrosine replaced	379	7.350	2.267	223	+58

*Per 300 gm. rat

Table 47. Weights and quantities of nitrogen in livers of rats in Series V fed diets in which methionine and phenylalanine were increased, cystine and tyrosine being replaced isonitrogenously

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
12	Methionine increased; cystine replaced isonitrogenously	50414	9.449	31.40	2.967	3.18	300	7.700	2.418	244
		50421	9.912	30.47	3.020	3.07	304	8.330	2.538	258
		50433	9.621	32.09	3.087	3.28	316	7.593	2.436	249
		50451	9.536	32.14	3.065	3.08	294	7.526	2.419	232
		50464	0.893	31.32	3.098	3.12	309	7.808	2.445	246
		50468	8.993	31.59	2.841	3.20	288	7.733	2.443	248
		Av.	9.567	31.50	3.013	3.16	302	7.782	2.450	246
13	Phenylalanine increased; tyrosine replaced isonitrogenously	50416	9.649	32.14	3.101	2.73	263	7.890	2.536	216
		50423	10.116	33.09	3.347	3.11	315	8.093	2.678	252
		50434	8.802	32.39	2.851	3.27	288	7.232	2.343	235
		50442	9.766	32.06	3.131	3.09	302	8.326	2.669	258
		50450	10.571	33.22	3.512	3.04	321	8.258	2.744	251
		50470	11.598	30.55	3.543	2.91	337	9.132	2.790	265
		Av.	10.084	32.24	3.247	3.03	304	8.155	2.627	246
14	Methionine and phenylalanine increased; cystine and tyrosine being replaced isonitrogenously	50448	9.891	31.29	3.095	3.24	320	7.183	2.247	232
		50476	9.351	31.20	2.918	2.91	272	7.559	2.359	220
		50502	9.203	31.40	2.890	3.05	281	7.440	2.336	227
		50535	9.935	29.14	2.895	2.84	282	7.642	2.227	217
		50588	8.056	31.27	2.519	3.19	257	6.927	2.166	221
		Av.	9.287	30.86	2.863	3.04	282	7.350	2.267	223

ments might also be made at the expense of nonessential amino acids other than cystine and tyrosine. It seems possible that the retention of dietary nitrogen might be enhanced still further until that point is reached when methionine becomes deleterious.

SERIES VI

Utilization of Nitrogen when Certain Nonessential Amino Acids Were Removed from a Ration that Supported Nitrogen Equilibrium

That the utilization of a certain mixture of essential amino acids patterned after the amino acid composition of lactalbumin was enhanced more efficiently by the addition of a composite of nonessential amino acids than by an equivalent amount of nitrogen supplied by the basal mixture of essential amino acids was revealed by tests conducted in Series III and IV. Data secured in Series V pointed to the efficacy of an increased intake of methionine as a substitute for cystine and of phenylalanine instead of tyrosine when these two nonessential amino acids were replaced individually. However, the retention of nitrogen was improved distinctly when methionine and phenylalanine were increased simultaneously to permit the isonitrogenous replacement of cystine and tyrosine, respectively. Either a metabolic relation governs the utilization of the two former acids or they are not present in sufficient quantities to permit adequate synthesis of the equally important cystine and tyrosine.

Thus, the quantities of particular nonessential amino acids may determine specifically the nutritional effectiveness of a given mixture of amino acids. It seemed important, therefore, to study the part played by members of the nonessential group of acids in the establishment of nitrogen equilibrium when synthetic amino acids were offered to well-nourished adult rats as the sole source of dietary nitrogen. The resources of the laboratory did not permit the testing of individual nonessential amino acids. Therefore, they were divided into groups according to the general classification of amino acids. The effect of the omission of the following acids, respectively, from Diet 7, the control ration, was studied: the monoaminomonocarboxylic acids, the dicarboxylic acids, cystine, proline, and tyrosine. Diet 7 was chosen as the control ration because experiments in Series III demonstrated that it met the minimal needs of the organism for nitrogen equilibrium. In the formulation of all test rations, Diets 15 to 19, the basal mixture containing only the essential amino acids was incorporated without alteration (Table 13). Also, the total quantity pf nitrogen in the daily quota was held constant when any nonessential moiety was omitted since the normal rat apparently required 40 mg. of nitrogen from nonessential amino acids in addition to 43.5 mg. of nitrogen provided by the essential amino acids. Accordingly, the concentrations of the remaining amino acids were elevated to compensate for the acid or acids that were removed.

When these diets were fed, certain striking responses were observed in respect to body weight. The rats that were deprived of the dicarboxylic acids or of proline gained approximately 10 gm. in the metabolism period (11th to 18th days), as shown in Table 48. These rats, therefore, gained more than did the animals fed 50 or 60 mg. of supplementary nitrogen in Series III (Table 29). Animals in other groups maintained their weight throughout the experiment. Certain individual variations in response may be observed in Table 49.

When the influence upon nitrogen balance of omitting the various nonessential amino acids was examined (Table 50), it became evident that only the feeding of the cystine-free diet produced a definitely deleterious effect. A marked destruction of tissues occurred as revealed by an average nitrogen balance of -156 mg. per 300 gm. rat in 7 days. Values ranged from -134 to -183 mg. (Table 51). The negative balances were not reflected by concurrent losses in weight, as might have been expected. The nitrogen from a mixture of amino acids that supplied 40 mg. of nitrogen in nonessential forms but was devoid of cystine was utilized with the same degree of efficiency as a diet containing only 10 mg. of nitrogen from the nonessential acids in addition

Table 48. Average body weights at certain intervals of rats in Series VI

Diet no.	Diet	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
		gm.	gm.	gm.	gm.
7	Basal essential amino acid diet plus 40 mg. N from NEAA	356	359	359	0
15	Monoaminomonocarboxylic nonessential acies of Diet 7 replaced	366	366	369	+3
16	Dicarboxylic acids of Diet 7 replaced	364	362	371	+9
17	Cystine of Diet 7 replaced	358	354	354	0
18	Proline of Diet 7 replaced	356	356	365	+9
19	Tyrosine of Diet 7 replaced	351	354	358	+4

Table 49. Body weights at certain intervals of rats in Series VI

Diet no.	Diet	Rat no.	Pre-exp't'l wt.	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	
15	Monoamino-monocarboxylic acids replaced	50475	413	394	385	388	+ 3
		50501	384	368	368	369	+ 1
		50565	355	350	350	354	+ 4
		50603	370	360	366	366	0
		50614	369	360	363	368	+ 5
		Av.	378	366	366	369	+ 3
16	Dicarboxylic acids replaced	50534	351	342	350	363	+13
		50604	423	412	396	400	+ 4
		50610	330	326	326	334	+ 3
		50619	387	374	372	380	+ 8
		50706	371	364	364	376	+12
		Av.	372	364	362	371	+ 9
17	Cystine replaced	50533	383	364	356	362	+ 6
		50554	379	374	356	356	0
		50613	354	346	345	345	0
		50653	386	376	378	374	- 4
		50675	340	332	334	330	- 4
		Av.	368	358	354	354	0
18	Proline replaced	50543	390	373	372	370	- 2
		50590	342	334	335	349	+14
		50595	368	348	347	355	+ 8
		50654	380	374	376	390	+14
		50710	359	350	348	362	+14
		Av.	368	356	356	365	+ 9
19	Tyrosine replaced	50544	396	384	381	386	+ 5
		50622	334	316	326	336	+10
		50674	359	352	358	360	+ 2
		50713	357	352	350	351	+ 1
		Av.	361	351	354	358	+ 4

Table 1, Wissler). These workers indicated that 23 mg. of phenylalanine were required for maintenance of weight on this basis.

In contrast to the omission of cystine or tyrosine, the removal of the monoamonomonocarboxylic acids did not affect the retention of nitrogen adversely. The average positive nitrogen balance was 18 mg. when glycine, alanine, and serine were omitted simultaneously (Table 50). If the single negative balance were omitted, the balance would become + 34 mg. (Table 51). No conspicuous changes occurred in body weight or in weight of the livers or in the concentration of nitrogen therein (Table 52). Apparently this group of acids is easily and efficiently synthesized.

The omission of the dicarboxylic acids or of proline resulted in a very definite conservation of nitrogen as indicated by positive balances of 52 and 53 mg., respectively. Greater individual variation was associated with the dietary absence of aspartic and glutamic acids than with the omission of proline, the values in the former case ranging from + 8 to + 110 mg. Both groups of animals gained approximately 10 gm. during the balance period. Weights of dry livers did not differ significantly from those removed from the control group, although the content of nitrogen in livers of rats deprived of proline was only 205 mg. in contrast to 231 mg.

Table 53. Weights and quantities of nitrogen in livers of rats in Series VI fed diets in which certain nonessential amino acids of Diet 7 were replaced isonitrogenously with other nonessential acids (Diets 15 to 19)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
15	Monoamino-monocarboxylic acids replaced	50475	9.854	30.08	2.763	3.03	299	7.698	2.158	234
		50501	8.854	31.13	2.756	2.99	265	7.275	2.265	218
		50565	7.979	31.60	2.521	2.84	227	6.820	2.154	194
		50603	9.776	30.35	2.866	2.79	273	8.079	2.368	226
		50614	8.663	32.31	2.799	2.93	254	7.160	2.313	210
		Average	9.025	31.09	2.741	2.92	264	7.406	2.251	216
16	Dicarboxylic acids replaced	50534	8.475	36.32	3.078	2.90	246	7.163	2.601	221
		50604	9.388	37.44	3.515	2.98	280	7.112	2.663	212
		50610	7.858	36.45	2.864	2.96	232	7.163	2.511	211
		50619	8.918	34.87	3.110	3.00	267	7.151	2.494	214
		50706	8.638	34.81	3.007	2.85	246	7.022	2.445	200
		Average	8.709	35.98	3.115	2.94	254	7.122	2.563	211
17	Cystine replaced	50533	7.952	30.75	2.445	2.57	204	6.665	2.049	171
		50554	8.261	31.20	2.577	2.89	239	6.959	2.171	201
		50613	8.062	29.92	2.412	3.02	243	7.029	2.103	212
		50653	7.814	30.72	2.400	3.04	238	6.266	1.925	191
		50675	6.963	30.27	2.108	3.10	216	6.330	1.916	196
		Average	7.810	30.57	2.388	2.92	228	6.650	2.033	194

(Continued on next page)

Table 53 (Cont'd)

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
18	Proline replaced	50543	8.578	32.25	2.766	2.81	241	6.918	2.231	194
		50590	8.205	32.72	2.685	2.93	240	7.153	2.340	210
		50595	7.951	41.81	3.324	2.95	235	6.778	2.834	200
		50654	8.653	33.24	2.876	2.99	259	6.760	2.247	202
		50710	8.701	32.30	2.810	2.97	258	7.336	2.367	218
		Average	8.418	34.46	2.892	2.93	247	6.989	2.404	205
19	Tyrosine replaced	50544	8.683	32.55	2.826	3.07	267	6.798	2.213	209
		50622	8.273	33.77	2.794	2.74	227	7.453	2.517	204
		50674	8.404	33.13	2.784	3.06	257	7.061	2.339	217
		50713	7.831	32.77	2.566	3.20	251	6.710	2.199	215
		Average	8.296	33.05	2.742	3.02	250	7.005	2.317	211

in the control group. Thus, in this Series diminishing hepatic nitrogen again is associated with improvements in the utilization of dietary nitrogen.

The phenomenon of the particularly favorable response that resulted from the omission of the dicarboxylic acids and of proline demands explanation. The nitrogen balances were almost identical with those obtained in Series V when both methionine and phenylalanine were increased in Diet 14 to replace both cystine and tyrosine. The question may be raised as to whether the enhancement of nitrogen retention could be attributed to the increased quantities of other nonessential acids that were incorporated in the diet to keep the total nitrogenous intake constant when aspartic and glutamic acids and proline were withdrawn. The composition of the resulting mixtures (Table 13) suggests that concomitant increases in the quantities of available cystine and tyrosine may result in a synergism similar to that which was manifested when both methionine and phenylalanine were increased in Diet 14 which was fed in Series V.

Pertinent information concerning specific effects of a single amino acid might be obtained by comparing the results obtained when the concentration of nitrogen was adjusted as in Series VI to compensate for the omission of a given acid with values obtained when the amino acid was replaced with nitrogen from inorganic sources without altering the concentrations of remaining acids. The test might be extended by increasing individual amino acids and including sufficient inorganic nitrogen to provide a total quantity of 40 mg. of nitrogen from nonessential sources. It would be interesting also to increase the concentration of methionine beyond the quantities that have been provided in the present tests, without altering the amounts of other amino acids. It is possible that the improved nitrogen balance ascribed to graded quantities of nonessential acids in Series III resulted more specifically from the presence of increasing concentrations of cystine and tyrosine than from the total quantity of nitrogen supplied in each case.

SERIES VII

Utilization of Nitrogen when a Single Nonessential Amino Acid Was Substituted for Other Nonessential Amino Acids

Ultimately the manifold roles of the individual nonessential amino acids in intermediary metabolism, their relationship to the essential amino acids, and their significance in the maintenance of a sound nutriture will be revealed by means of metabolic tests and isotopic studies. At present, however, available information concerning these points is limited and conflicting. Apparent discrepancies undoubtedly arise from differences in experimental procedures, particularly in respect to the composition of the basal mixture of essential amino acids. The experiments herein described emphasize the importance of the latter point.

The capacity of cystine and tyrosine to replace methionine and phenylalanine at least partially has been demonstrated, as well as the ability of the essential amino acids to substitute for cystine and tyrosine. The improvement, however, that resulted from the removal of the dicarboxylic acids from the mixture was surprising. In many experiments reported to date, glutamic acid has been shown to play a particularly significant role in metabolism.

It participates actively in transamination, and is closely related to both the ornithine and tricarboxylic acid cycles. Moreover, Frost (1949) concluded that it could replace all other nonessential acids as a source of nitrogen for protein-depleted rats. Rose, Oesterling, and Womack (1948) proposed that arginine, glutamic acid, and proline were mutually interconvertible. Frost also observed that glycine was an effective source of nitrogen for the supplementation of the essential amino acids (1949). It was, however, less satisfactory than glutamic acid (Frost, 1949). It has been recorded, too, that even urea and diammonium citrate can be utilized successfully by both growing rats (Lardy and Feldott, 1950; Rose, Smith, Womack, and Shane, 1949) and adult rats (Frost, 1949).

In attempting to devise a mixture of amino acids that would express the minimal requirements for the maintenance of nitrogen equilibrium, a reduction in the number of amino acids present in the ration would simplify the problem and hasten its ultimate solution. If a single nonessential amino acid could fulfill the functions of all the other nonessential acids, investigation of the roles of individual essential amino acids would be facilitated greatly. Of all the nonessential amino acids represented in the present mixture, glutamic acid was obviously the one that would be most likely to replace all other nonessential acids effectively.

Therefore, in the present series, VII, the basal essential amino acid diet no. 2 was supplemented with 30 mg. of nitrogen daily from glutamic acid to constitute Diet 20. The total intake of nitrogen equalled that furnished by the 4.27 per cent lactalbumin diet or by Diet 6.

The inability of glutamic acid to replace the other nonessential acids as a source of nitrogen in the supplementation of the essential amino acids simulating those present in lactalbumin was evident even in an early phase of the experiment. All animals in the group lost weight (Table 54), the average loss in the balance period being 9.0 gm. In this respect, the behavior of the animals resembled that of rats fed the ten essential amino acids either alone or supplemented with only 10 mg. of nitrogen from nonessential acids.

The nitrogen balance data were even more striking, as shown in Table 55. The average nitrogen balance was -194 mg. per 300 gm. rat per 7 days. In other words, the addition of 30 mg. of nitrogen from glutamic acid was almost without effect, since the balance differed little from that of rats fed only the essential amino acids. The results obtained when glutamic acid was added alone contrasted

Table 54. Body weights at certain intervals of rats in Series VII fed diets in which methionine, phenylalanine, and glutamic acid were increased, certain nonessential acids being replaced isonitrogenously (Diets 20 and 21)

Diet no.	Diet	Rat no.	Pre-exp't'l wt.	Initial wt.	Wt. on 11th day	Wt. on 18th day	Change in balance period
			gm.	gm.	gm.	gm.	
20	Basal essential amino acid diet plus 30 mg. N from glutamic acid	50286	350	346	350	343	- 9
		50302	344	328	334	328	- 6
		50328	385	362	360	351	-10
		50345	360	352	350	346	-10
		50373	378	372	373	365	- 9
		50405	365	353	354	347	- 8
		Average	364	352	353	346	- 9
21	Methionine, and phenylalanine of Diet 7 increased, glutamic acid replacing NEAA except cystine and tyrosine	50449	412	406	402	406	+ 4
		50503	379	354	352	358	+ 6
		50516	336	326	326	340	+14
		50538	402	394	386	381	- 5
		50550	370	348	350	354	+ 4
		Average	380	366	363	368	+ 5

Table 55. Average nitrogen balances per 7 days of rats in Series VII

Diet no.	Diet	Body wt.	N in food	Total N excreted	N balance/ 300 gm. rat	N balance/ 100 sq.cm.
		gm.	mg.	mg.	mg.	mg.
2	The basal essential amino acid diet	344	349	601	-220	-60
6	Diet 2 plus 30 mg. N from NEAA	353	541	575	- 28	- 8
20	Diet 2 plus 30 mg. N from glutamic acid	346	543	768	-194	-53
7	Diet 2 plus 40 mg. N from NEAA	357	618	610	+ 7	+ 1
14	Methionine and phenylalanine of Diet 7 increased, cystine and tyrosine being replaced isonitrogenously	379	622	552	+ 58	+16
21	Diet 14, with glutamic acid replacing all NEAA except cystine and tyrosine	365	620	564	+ 47	+13

strikingly with those associated with the feeding of 30 mg. of nitrogen from a mixture of nonessential acids, the value for the latter group being -28 mg.

The unsatisfactory results obtained when glutamic acid was incorporated in Diet 20 possibly could be attributed to the presence of a large proportion of the dicarboxylic acids in the ration. The fact that no attempt was made to neutralize the glutamic acid might also make it undesirable in a dietary mixture, although this potential problem was recognized before the diets were formulated. However, Ramasarma, Henderson, and Elvehjem (1949) had reported that the growing rat could utilize effectively a diet containing as much as 14 per cent of glutamic acid. Moreover, Maddy and Elvehjem (1949) did not observe any benefit from neutralization of diets containing aspartic and glutamic acids that were fed to mice.

Another experiment was planned, therefore, to determine whether or not the unfavorable outcome of replacing all of the dietary nonessential nitrogen with glutamic acid could be ascribed to the relatively low amounts of methionine, cystine, phenylalanine, and tyrosine in the test mixture. The study was approached by increasing and quantities of the two essential amino acids which serve as precursors of cystine and tyrosine. To do this, methionine and phenylalanine replaced the two nonessential acids isonitrogenously, as in

Table 56. Nitrogen balances per 7 days of individual rats in Series VII fed diets in which methionine, phenylalanine, and glutamic acid were increased, certain nonessential amino acids being replaced isonitrogenously (Diets 20 and 21)

Diet no.	Diet	Rat no.	Body wt.	Body sur-face	N in food	N in urine	N in feces	Total N ex-creted	N bal-ance	N bal-ance/ 300 gm. rat	N bal-ance/ 100 sq.cm.
			gm.	sq.cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
20	Basal essential amino acid diet plus 30 mg. N from glutamic acid	50286	343	416	543	537	218	755	-212	-185	-50
		50302	328	405	543	543	195	738	-194	-177	-48
		50328	351	422	543	569	210	779	-235	-201	-55
		50345	342	415	543	592	198	789	-246	-216	-59
		50373	365	432	543	578	194	772	-228	-187	-53
		50405	347	418	543	549	227	776	-232	-201	-55
		Average	346	418	543	561	207	768	-224	-194	-53
21	Methionine and phenylalanine and glutamic acid of Diet 7 increased	50449	403	459	620	375	210	585	+ 35	+ 26	+ 8
		50503	356	426	620	332	207	539	+ 81	+ 68	+19
		50516	334	410	620	331	167	498	+122	+110	+30
		50538	380	443	620	395	225	620	0	0	0
		50550	350	420	620	365	211	576	+ 44	+ 30	+10
		Average	365	432	620	360	204	564	+ 56	+ 47	+13

Diet 14. To this composite, 36 mg. of nitrogen from glutamic acid were added in Diet 21. This quantity was selected because cystine and tyrosine together contributed 4.0 mg. of nitrogen in Diet 7 which was selected as the control ration. Thus, in Diet 21 any deleterious effects that might arise from a large proportion of glutamic acid would be accentuated.

To the surprise of the author, instead of reacting unfavorably, the rats responded very well to the dietary modification (Diet 21, Table 55). The average nitrogen balance was + 47 mg., and the range from 0 to 122 mg. (Table 56). Evidently, glutamic acid could replace all other nonessential acids except tyrosine and cystine. The results also lend support to the idea that the original mixture of essential acids was inadequate in respect to methionine and phenylalanine. Time did not permit further investigation of the efficacy of glutamic acid as a source of nitrogen when the quantity of phenylalanine in the basal ration was held constant while methionine was increased to replace cystine. Such data would be informative, as would the effect of substituting diammonium citrate or some other source of inorganic nitrogen for the quantity of glutamic acid that was present in Diet 21.

No striking alterations occurred in the weights of the

Table 57. Average weights and quantities of nitrogen in livers, and nitrogen balances of rats* in Series VII

Diet no.	Diet	Body wt.	Wt. of fresh liver	Wt. of dry liver	Total N	N balance/ 7 days
		gm.	*gm.*	*gm.*	*mg.*	*mg.*
2	The basal essential amino acid diet	344	7.231	2.270	214	-220
6	Diet 2 plus 30 mg. N from NEAA	353	7.369	2.384	222	- 28
20	Diet 2 plus 30 mg. N from glutamic acid	346	7.383	2.313	225	-194
7	Diet 2 plus 40 mg. N from NEAA	357	7.712	2.487	231	+ 6
14	Diet 7, with methionine and phenylalanine increased to replace cystine and tyrosine isonitrogenously	379	7.350	2.267	223	+ 58
21	Diet 14, with glutamic acid replacing all NEAA except cystine and tyrosine	365	7.377	2.266	221	+ 47

*Per 300 gm. rat

Table 58. Weights and quantities of nitrogen in livers of rats in Series VII fed diets in which methionine, phenylalanine, and glutamic acid were increased, certain nonessential acids being replaced isonitrogenously

Diet no.	Diet	Rat no.	Wt. of fresh liver	Dry wt.	Wt. of dry liver	N in fresh liver	Total N	Per 300 gm. rat		
								Fresh liver	Dry liver	Total N
			gm.	%	gm.	%	mg.	gm.	gm.	mg.
20	The basal essential amino acid diet plus 30 mg. N from glutamic acid	50286	8.629	31.42	2.711	3.03	261	7.549	2.372	229
		50302	8.634	31.09	2.684	3.00	259	7.899	2.456	237
		50328	8.308	31.26	2.597	3.12	259	7.101	2.220	221
		50345	7.986	31.37	2.505	3.09	247	7.006	2.197	217
		50373	9.006	32.12	2.893	2.98	268	7.400	2.377	220
		50405	8.497	30.72	2.610	3.07	261	7.344	2.256	225
		Average	7.510	31.33	2.667	3.05	260	7.333	2.313	225
21	Methionine and phenylalanine of Diet 7 increased, glutamic acid replacing NEAA except cystine and tyrosine	50449	10.075	30.76	3.099	2.89	291	7.502	2.308	217
		50503	8.622	31.23	2.692	2.97	256	7.263	2.268	216
		50516	7.934	29.92	2.374	3.02	240	7.128	2.132	215
		50538	9.700	31.05	3.012	3.05	296	7.656	2.377	234
		50550	8.553	30.64	2.620	3.02	258	7.329	2.246	221
		Average	8.977	30.72	2.759	2.99	268	7.377	2.266	221

livers or in the concentrations of nitrogen therein (Tables 57 and 58), or in the weights of the adrenal glands (Table H, Appendix I), when the rats were fed either Diet 20 or 21.

SUMMARY

Eventually the protein needs of the adult animal organism will be expressed in terms of the dietary amino acids present in its normal food supply. Tentative values indicating the quantities of the ten essential amino acids that are required for the maintenance of body weight and for the establishment of nitrogen balance in two species, *i.e.*, the human being and the rat, have been reported in the literature. Whether or not the requirements have been expressed quantitatively is a question that may be debated, because the utilization of nitrogen from any dietary source is influenced by many varied and interlocking factors.

It has seemed to workers in the Home Economics Research Department at the Iowa State College that a sound approach to a study of the amino acid requirements of the well nourished albino rat could be made by observing the response of the animals to the incorporation into a balanced ration of a mixture simulating those in a "pure" protein whose biological efficiency was well established. Lactalbumin represented such a protein. Kuehl, in a preliminary experiment in 1948-49 was able with the 18 amino acids then available to formulate a diet that supplied approximately 75 per cent of the total nitrogen present in a 4 per cent lactalbumin ration. When this diet was force-fed to rats, nitrogen equilibrium was attained. Upon the withdrawal of the nonessential nitrogen, however, the force feeding of the ration not only resulted in a marked destruction of body tissue as shown by a highly negative nitrogen balance but in the development of a curious syndrome characterized by hyperactivity and hyperexcitability.

The present investigation represents an extension of this study, and is a critical evaluation of the nutritive efficiency of a mixture of amino acids alike in quality and quantity to those in a diet containing just enough lactalbumin to support nitrogen equilibrium in adult well nourished rats under conditions of rigidly controlled caloric intake (force feeding). To answer questions that seemed pertinent in this attempt to establish the nitrogenous requirements of the rat in terms of the amino acids, seven experiments were conducted. The problem underlying each series and the experimental results are summarized in the following paragraphs.

Series I and II

Is the syndrome described by Kuehl the direct outcome of a deficiency of specific amino acids or groups of amino acids?

The Kuehl syndrome was replicated successfully by force-feeding 13 gm. of a diet containing only the ten essential amino acids in two daily portions of 12 ml. each. The quantities of amino acids were those present in a diet that contained sufficient lactalbumin to permit only a slightly positive nitrogen balance. Results were the same when the vitamins were offered apart from the basal diet and when they were incorporated in the homogenized diet. The nervous disorder and digestive disturbances did not develop, however, when the same total quantity of the same basal diet was force-fed in three daily portions of 8 ml. each, or when it was offered *ad libitum*. Despite the absence of symptoms, the nitrogen balance remained strongly negative. Therefore, it seems that the method used in the administration of the test diet has a profound influence on the nature of the response elicited from the test animal, and that the syndrome described by Kuehl and by others cannot be referred to as amino acid deficiency disease.

Series III

If the nonessential acids are needed to prevent degradation of body tissue, what is the minimal quantity that will supplement successfully the essential acids present in a 4 per cent lactalbumin diet?

Since the nitrogenous mixtures used in the experiments just described were formulated in accordance with published values of the amino acid composition of lactalbumin, it seemed possible that they did not represent accurately the make-up of the particular preparation of lactalbumin used in the ration. In this series, therefore, two amino acid mixtures were formulated that were based on the microbiological assay of a preparation of lactalbumin. In the first, the essential acids provided by the control diet containing 4 per cent of purified lactalbumin were incorporated; in the second, the nonessential acids. All amino acids present in lactalbumin were available except serine. Additional glycine replaced it in the formula.

The mixture of essential acids thus prepared again failed to support nitrogen equilibrium, a negative balance of 220 mg. per 300 gm. rat per seven days resulting. When this mixture was supplemented with 30 mg. of nitrogen per rat per day from the composite of nonessential acids patterned after the 4 per cent lactalbumin diet, it nearly sustained nitrogen equilibrium (-28 mg.). In fact, the response to the feeding of the combined mixtures was identical to that induced by feeding the control lactalbumin diet.

Thus, it seems that the nonessential amino acids play an important part in establishing nitrogen equilibrium when minimal amounts of a natural protein are fed to the adult rat. To find the minimal dose of nonessential acids required for the maintenance of this balance, graded doses of the mixture of nonessential amino acids were next fed in conjunction with the essential amino acids, the qualitative characteristics of the nonessential nitrogen of the lactalbumin preparation being maintained. Six different diets were force-fed to rats; the daily doses of nonessential nitrogen provided by these diets were 10, 20, 30, 40, 50, and 60 mg. Nitrogen balance, weight of the liver, and the concentration of nitrogen in the liver were determined. The same studies were made with rats deprived of all sources of dietary nitrogen, and with rats fed the lactalbumin control diet.

The regression of nitrogen balance/7 days in mg./100 sq. cm. body surface on nitrogen intake/7 days in mg./100 sq. cm. of body surface was linear. Nitrogen equilibrium was attained when the diet provided approximately 150 mg.

of nitrogen per 7 days per 100 sq. cm. of body surface. This was equivalent to a total of 83 mg. of nitrogen per 300 gm. rat per day, 40 mg. of which represented nonessential nitrogen per day and 43 mg. essential nitrogen. The latter mixture contributed 34 and 9 mg. of nitrogen from the L- and D- forms, respectively, of the essential acids.

Positive balance was attained when the nonessential acids provided 50 and 60 mg. of nitrogen daily. The influence of larger proportions than those tested should be investigated because the curve of response did not demonstrate clearly whether or not maximum utilization had been obtained.

The weights of the dry livers were not influenced by any of the dietary regimes imposed. The livers of the rats fed the low nitrogen diet (negative control) contained less total nitrogen than did the livers of the lactalbumin-fed rats. It was very interesting, however, when graded doses of nonessential acids were fed that the total quantities of nitrogen in the livers were similar to those in the organ of the positive control rats until nitrogen equilibrium was reached. However, as soon as nitrogen balance became positive in response to the addition of nonessential nitrogen, the concentration of hepatic nitrogen declined.

Series IV

Is a mixture of essential amino acids as effective in maintaining nitrogen equilibrium as a mixture of essential and nonessential acids if the same total quantity of nitrogen is provided?

To study this problem, a diet was formulated which furnished twice the amount of each essential amino acid that was present in the unsupplemented mixture tested in Series III. The nitrogen balance of rats fed this ration was -70 mg. per 300 gm. rat per day in contrast to +7 mg. when the basal mixture of essential amino acids tested in Series III was supplemented with nonessential acids. Both diets yielded 83 mg. of nitrogen daily.

Series V

Does the mixture of essential amino acids formulated for the experiments described in Series III represent the most efficient combination of amino acids for the support of nitrogen equilibrium?

Feeding tests in the present series indicated that cystine and tyrosine in the nonessential amino acid mixture were augmenting the respective supplies of methionine and phenylalanine in the mixture of essential acids. They showed that the isonitrogenous replacement of methionine with cystine and of tyrosine with phenylalanine did not alter the nitrogen balance significantly. But very interestingly, the simultaneous substitution of the two nonessential acids with methionine and phenylalanine increased the nitrogen retention by 51 mg. per 300 gm. rat in the 7-day test period.

It would seem that the minimum requirement of the normal adult rat weighing 300 gm. for the sulfur-containing acids may be met by the daily inclusion in the diet of 26 mg. of methionine in the absence of cystine, or by 9 mg. of methionine and 14 mg. of cystine, provided that an adequate supply of the other nonessential acids is present. It should be emphasized that these values were obtained when the caloric value of the diets were constant and adequate. The data likewise indicate that the daily requirement of phenylalanine for nitrogen balance is approximately 16 mg. under similar conditions. The data also suggest the importance of testing the response of animals to the supplementation of this new mixture containing increased methionine and phenylalanine with graded doses of nonessential nitrogen. This work is needed to clarify the contribution of the nonessential amino acids in meeting the minimal requirement for dietary nitrogen.

Series VI

Are any of the nonessential amino acids in the mixture simulating those present in a 4 per cent lactalbumin diet of particular importance in the establishment of nitrogen balance?

This question became particularly interesting in light of the observation that the nutritive value of a mixture of essential amino acids only did not equal that of a mixture supplying the same quantity of nitrogen from both essential and nonessential sources. To study this problem, the different nonessential acids were divided into groups according to their chemical classification, i.e., the sulfur acid, cystine; the aromatic acid, tyrosine; the monoaminomonocarboxylic acids, glycine and alanine; the dicarboxylic acids; and the heterocyclic acid, proline. When any group was omitted from the dietary mixture, the total quantity of nitrogen was held constant by increasing the quantities of the other nonessential acids. These modified mixtures of nonessential acids were then fed as supplements to the mixture of essential acids simulating the composition of lactalbumin (Series III).

The omission of cystine resulted in marked nutritive failure as measured by nitrogen balance. In fact, the response of the rats was similar to that of rats receiving no supplementary nonessential nitrogen. Thus, while the methionine content of lactalbumin seems to be inadequate, it can be successfully supplemented by cystine in the maintenance of nitrogen equilibrium.

The omission of the monoaminomonocarboxylic acids or of the dicarboxylic acids or of proline did not affect the retention of nitrogen adversely. Indeed, some evidence was obtained of an improvement in the utilization of nitrogen when proline or aspartic and glutamic acids were absent. It is possible that the results may be attributable to increased quantities of cystine and/or tyrosine that were added to compensate for the omission of these three acids.

Series VII

Can one nonessential amino acid effectively supplement the mixture of essential amino acids patterned after lactalbumin in meeting the needs for body maintenance?

Glutamic acid was selected for study. When this acid was added alone to the mixture of essential amino acids formulated for use in Series III, the animals passed into acute negative balance. However, when it was used to supplement the second mixture tested in Series V in which methionine and phenylalanine were substituted isonitrogenously for cystine and tyrosine, respectively, very satisfactory balance ensued. It approximated that attained by rats fed the same mixture of essential acids fortified with a variety of nonessential acids providing the same total quantity of nitrogen. Thus, it seems that, in the first test of this series, a deficiency of cystine and tyrosine was being measured rather than a deleterious effect of a superabundance of one nonessential acid. Thus, evidence is obtained from another angle of the indispensbility of cystine and tyrosine unless the diet provides sufficient quantities of those essential amino acids which serve as their precursors.

CONCLUSIONS

1. The method of feeding of an experimental ration may alter profoundly the response of the organism to an experimental diet. If force-feeding is used, the food must be administered at several intervals in small doses. Otherwise symptoms may appear that may be ascribed erroneously to a nutritional deficiency.

2. Nitrogen equilibrium cannot be supported in the adult well-nourished male rat by a mixture of essential amino acids equivalent to those in that quantity of the intact protein, lactalbumin, which will sustain nitrogen balance. Its supplementation with nonessential amino acids is necessary. These nonessential acids cannot be replaced with an equivalent quantity of nitrogen originating from the essential acids alone. At this level of nitrogen intake, the rat evidently is unable to meet its requirements for the essential amino acids and to synthesize the nonessential amino acids.

3. In evaluating the nutritive efficiency of any protein or mixture of amino acids, the relative quantities of nonessential acids as well as essential acids must be considered.

4. When a fixed level of essential nitrogen is fed,

balance is related directly to the quantity of nonessential nitrogen ingested.

5. When an assortment of amino acids patterned after those in lactalbumin is fed, the minimum requirement of the adult well-nourished rat for nitrogen, as measured by balance tests, is 83 mg./300 gm. rat. Approximately one-half of this quantity may be provided by the essential and one-half by nonessential acids.

6. Lactalbumin does not contain the ideal assortment of essential amino acids for the attainment of nitrogen equilibrium in the adult rat. It appears to contain too little methionine and phenylalanine.

7. When minimal quantities of nitrogen are fed, hepatic nitrogen may reflect dietary nitrogen in either of two ways. A decrease in the total nitrogen content of the liver may indicate, on one hand, active mobilization of the element for metabolic processes and, on the other, a state of depletion of body stores of nitrogen.

8. The essentiality of cystine when mixtures of essential amino acids simulating lactalbumin are fed has been demonstrated. Indeed, the response to graded doses of nonessential acids may be attributable partially to increments in the supply of this particular acid.

9. Glycine and alanine can be replaced by other nonessential acids without an adverse effect on nitrogen

balance. Glutamic acid, on the other hand, seems important. Its ability to contribute toward nitrogen retention is related definitely to the composition of the mixture of essential amino acids provided in the diet.

10. Glutamic acid can effectively replace a complex mixture of nonessential acids, such as naturally occurs in common foods, only when methionine and phenylalanine are present in quantities adequate for nitrogen retention.

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APPENDIX I

Table A. Concentration of nitrogen in amino acids as fed in the experiment*

Amino acid		Nitrogen theoretically present	Nitrogen found in Lot 1	Nitrogen found in Lot 2
		%	%	%
Arginine.HCl		26.6	26.6	----
Histidine.HCl.H_2O		20.0	19.5	----
Isoleucine		10.7	10.4	10.2
Leucine		10.7	10.4	10.5
Lysine.HCl.H_2O		13.9	14.6	13.9
Methionine		9.4	9.2	----
Phenylalanine		8.5	8.3	----
Threonine		11.8	11.6	11.4
Tryptophane		13.7	13.3	----
Valine		11.9	11.7	11.7
Alanine	(DL)	15.7	15.4	----
Alanine	(L)	15.7	13.8	----
Aspartic acid	(DL)	10.5	10.2	10.3
Aspartic acid	(L)	10.5	10.3	----
Cystine		11.6	11.4	----
Glutamic acid		9.5	9.3	----
Glycine		18.6	18.2	----
Proline		12.2	11.8	----
Serine	(L)	13.3	10.8	----
Tyrosine		7.7	7.6	----

*All amino acids were obtained from Merck and Co., except tryptophane from General Biochemicals, and L-alanine, L-aspartic acid, and L-serine from Bios Laboratories

Table B. Recovery of nitrogen from a standard solution of ammonium sulfate distributed at intervals over metabolism cages

Cage no.	Quantity of nitrogen theoretically present	Quantity of nitrogen recovered	Recovery of nitrogen
	mg.	mg.	%
1	398.14	397.70	99.86
		397.70	99.86
		398.16	100.00
2	398.14	398.16	100.00
		397.04	99.72
		397.70	99.86
3	398.14	397.70	99.86
		397.70	99.86
		397.04	99.72
4	398.14	396.48	99.58
		397.04	99.72
		396.48	99.58

Table C. Weights of adrenal glands of rats in Series I and II, fed Diet 2a containing only the ten essential amino acids equivalent to those in a 4.06 per cent lactalbumin control ration

Method of feeding	Rat no.	Wt.	Wt./300 gm. rat
		mg.	mg.
Force-fed, in two daily portions*	49837	50.3	55.3
	49868	57.4	55.6
	49987	62.8	64.9
	50042	54.3	55.4
	Average	56.2	57.8
Force-fed, in two daily portions**	50041	67.4	80.9
Force-fed, in two daily portions	50177	46.9	41.5
	50201	48.8	46.3
	50209	55.4	50.7
	Average	50.4	46.2
Force-fed, in three daily portions	49724	37.6	35.9
	49860	36.2	34.6
	49956	36.0	35.0
	49972	36.7	35.3
	50025	36.0	34.8
	50039	37.4	37.4
	Average	36.7	35.5
Fed <u>ad libitum</u>	49809	31.4	32.4
	49888	32.1	34.4
	49899	29.0	30.8
	49988	31.7	32.3
	50026	36.1	40.7
	50038	34.9	37.4
	Average	32.5	34.7

*Vitamins separate from basal diet
**Vitamins homogenized with basal diet

Table D. Weights of adrenal glands of rats in Series III, fed diets containing essential amino acids (EAA) equivalent to those in a 4.27 per cent lactalbumin control ration supplemented with graded quantities of nitrogen from the non-essential amino acids (NEAA)

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
1	Low-nitrogen	50589	37.6	35.7
		50594	36.1	31.6
		50605	35.9	35.3
		50705	40.1	35.1
		50712	40.2	36.9
		Average	38.0	34.9
3	4.27% lactalbumin	50131	42.3	35.9
		50139	41.3	34.2
		50146	45.4	38.9
		50158	40.6	32.0
		50184	42.7	36.0
		50199	41.0	35.6
		Average	42.2	35.4
2	10 EAA as present in Diet 3	50147	38.9	33.9
		50161	41.7	34.3
		50324	39.1	33.8
		50330	43.3	37.6
		50343	40.2	36.6
		50539	41.0	37.0
		Average	40.7	35.5
4	Diet 2 plus 10 mg. N from NEAA	50162	43.8	37.0
		50191	37.1	29.7
		50265	36.7	32.6
		50325	42.3	38.1
		50342	35.4	31.8
		50358	41.5	37.6
		Average	39.5	34.5

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Table D (Cont'd)

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
5	Diet 2 plus 20 mg. N from NEAA	50190	36.7	30.7
		50263	39.1	34.7
		50289	40.8	37.9
		50329	36.0	38.4
		50344	39.4	33.9
		50366	42.7	35.2
		Average	40.8	35.1
6	Diet 2 plus 30 mg. N from NEAA	50292	39.4	32.1
		50327	41.0	37.7
		50333	38.1	32.4
		50341	37.5	32.0
		50377	38.5	33.8
		50392	36.5	29.3
		Average	38.5	32.9
7	Diet 2 plus 40 mg. N from NEAA	50176	40.3	33.2
		50295	38.7	33.9
		50318	45.6	38.5
		50334	37.6	31.9
		50357	40.9	35.2
		50374	43.1	38.4
		50422	41.2	34.5
		50436	40.6	34.6
		50444	37.0	28.8
		50463	45.9	36.6
		50564	49.2	41.4
		Average	41.8	35.2

(Continued on next page)

Table D (Cont'd)

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
8	Diet 2 plus 50 mg. N from NEAA	50462	43.8	33.3
		50485	43.5	36.8
		50537	46.7	34.2
		50609	42.5	34.8
		50620	42.7	36.4
		Average	43.8	34.8
9	Diet 2 plus 60 mg. N from NEAA	50536	46.9	38.9
		50549	44.2	35.0
		50553	46.1	33.6
		50606	41.2	34.5
		50621	47.7	36.3
		Average	45.2	35.7
10	Diet 7 with L-alanine, L-aspartic acid and L-serine replacing DL-forms	50532	40.7	35.7
		50548	41.4	33.9
		50593	42.2	35.5
		50601	49.4	39.8
		50611	45.0	38.6
		Average	43.7	36.7

Table E. Weights of adrenal glands of rats in Series IV fed Diet 11 which contained twice the quantity of the ten essential amino acids provided by the basal essential amino acid diet

Rat no.	Wt.	Wt./300 gm. rat
	mg.	mg.
50429	34.7	30.0
50435	46.5	37.6
50477	45.6	35.8
50483	36.1	38.5
Average	43.2	35.5

Table F. Weights of adrenal glands of rats in Series V in which methionine and phenylalanine of Diet 7 were increased, cystine and tyrosine being replaced isonitrogenously (Diets 12 to 14)

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
12	Methionine increased, cystine replaced isonitrogenously	50414	39.6	32.3
		50421	40.2	33.5
		50433	44.1	34.8
		50451	47.5	37.5
		50464	40.0	31.6
		50468	39.7	34.1
		Average	41.8	34.0
13	Phenylalanine increased, tyrosine replaced isonitrogenously	50423	42.8	34.2
		50434	41.0	33.7
		50442	41.4	35.3
		50450	43.5	34.0
		50470	38.8	30.5
		Average	41.5	33.5
14	Methionine and phenylalanine increased, cystine and tyrosine being replaced isonitrogenously	50448	47.6	34.5
		50476	46.8	37.5
		50502	42.9	34.6
		50535	42.6	32.7
		Average	45.0	34.8

Table G. Weights of adrenal glands of rats in Series VI fed diets in which certain nonessential amino acids of Diet 7 were replaced isonitrogenously with other nonessential acids

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
15	Monoamino-monocarboxylic acids omitted	50475	45.6	35.6
		50501	44.8	36.8
		50565	42.5	36.3
		50603	44.8	37.0
		50614	43.4	35.9
		Average	44.2	36.3
16	Dicarboxylic acids omitted	50534	35.5	39.3
		50604	39.1	29.3
		50610	39.6	35.6
		50619	44.6	35.2
		50706	39.9	31.8
		Average	39.7	34.2
17	Cystine omitted	50533	34.5	28.6
		50554	45.1	38.0
		50613	39.6	34.5
		50653	42.8	34.3
		50675	40.7	37.0
		Average	40.5	34.5

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Table G (Cont'd)

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
18	Proline omitted	50543	39.2	31.6
		50590	37.5	32.2
		50595	45.1	38.4
		50654	46.4	35.7
		50710	40.5	33.6
		Average	41.7	34.3
19	Tyrosine omitted	50544	40.9	31.8
		50602	45.0	37.1
		50622	42.3	38.1
		50674	44.2	37.1
		50713	42.3	36.2
		Average	43.0	36.1

Table H. Weights of adrenal glands of rats in Series VII fed diets in which methionine, phenylalanine, and glutamic acid were increased to replace certain nonessential acids

Diet no.	Diet	Rat no.	Wt.	Wt./300 gm. rat
			mg.	mg.
20	Basal essential amino acid diet plus 30 mg. N from glutamic acid	50286	43.2	38.6
		50302	42.4	39.0
		50328	39.1	34.1
		50345	40.3	36.2
		50373	37.8	31.6
		50405	42.7	37.4
		Average	40.9	36.1
21	Methionine, phenylalanine, and glutamic acid of Diet 7 increased	50449	46.2	34.1
		50503	43.8	36.7
		50516	37.2	32.8
		50538	42.6	33.5
		50550	39.2	33.2
		Average	41.8	34.0

Table I. Quantities of utilizable essential amino acids supplied daily by various diets just capable of supporting nitrogen equilibrium in the adult well-nourished rat (mg. amino acid/350 gm. rat/day)

	Investigator		
Amino acid	Clark	Mukhopadhay	Cannon
	mg.	mg.	mg.
Arginine	14.2	32.7	----
Histidine	9.1	9.9	8.8
Isoleucine	27.7	31.2	54.2
Leucine	53.8	45.6	27.3
Lysine	41.9	33.9	15.5
Methionine	10.3	17.2	22.3
Phenylalanine	18.2	27.8	13.0
Threonine	25.7	25.9	21.4
Tryptophane	9.2	7.2	7.6
Valine	28.4	35.1	29.4
Total	238.5	266.5	199.5

APPENDIX II

A. Steenbock XVII Diet

This diet was fed to all male rats prior to the initiation of the experiment. Its composition was:

Yellow cornmeal, finely ground	560 gm
Casein (technical)	50 gm
Linseed meal	160 gm
Alfalfa leaf meal	20 gm
Sodium chloride	5 gm
Calcium carbonate and trace elements	5 gm
Yeast, brewers' irradiated	5 gm
Yeast, brewers'	95 gm
Wheat germ	100 gm
Klim	160 gm

To prepare the mixture of calcium carbonate and trace elements, the following quantities were added per gm of calcium carbonate: 0.80 mg potassium iodide, 3.16 mg manganese sulfate, 0.98 mg potassium aluminum sulfate, and 4.07 mg copper sulfate.

B. Personal communication to Dr. Pearl Swanson from Dr. M. S. Dunn

June 24, 1949

. . . the amino acid values reported to you were those obtained on the samples exactly as received from you. In other words, they were calculated as the percentages of the listed amino acids on these samples without any corrections for ash or moisture. Of course, you could calculate them on an ash and moisture free basis, using the data which you enclosed in your letter . . . Or, you could calculate them to a sixteen per cent nitrogen basis, since the values reported to you were for your products containing, it appears from your table of data, around twelve or thirteen per cent nitrogen.

M. S. Dunn
Professor of Chemistry
University of California
at Los Angeles

C. Personal Communication from Dr. H. Spector to H. Clark

3 August 1950

Thank you for your letter of July 29th regarding our paper on tryptophan deficiency in the rat, which appeared in the Journal of Nutrition. I very much appreciate your calling to my attention the absence of pyridoxine from the list of vitamins in Footnote 5. In fact, I see that nicotinic acid also was not included in this list. However, the sentence preceding Footnote 5 states that "all vitamins were supplied in adequate amounts" and both pyridoxine (0.25 mg. per 100 grams) and nicotinic acid (1.0 mg. per 100 grams) were provided. The error of omission probably arose by copying the list of vitamins from my report in J.B.C. 173, 659 (1948). In the investigation of the metabolic interrelation between tryptophan, pyridoxine, and nicotinic acid, these three vitamins were omitted from the vitamin mixture and were provided, where indicated, as daily supplements.

Although the conversion of tryptophan to nicotinic acid is not dependent upon the presence in the diet of adequate amounts of pyridoxine, the need for this vitamin for the metabolism of tryptophan and its function as codecarboxylase in the enzymic decarboxylation of amino acids as well as the coenzyme of transaminases is well established.

Very truly yours,

HARRY SPECTOR
Chief, Nutrition Division
Food Laboratories
Quartermaster Food & Container
Institute for the Armed Forces