PHENYLALANINE -
IT'S ROLE IN INFANT NUTRITION AND DISEASE

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Chloramphenicol, a known inhibitor of protein synthesis in the bacterial cell, is investigated for its action on amino acid metabolism and bone marrow morphology of infants and children. The interaction of phenylalanine and chloramphenicol is discussed in this section.

Many of the abnormal findings in animals deprived of a single amino acid, are analogous to the abnormalities found in children with the syndrome kwashiorkor. This analogy is pursued in the discussion, using both the relevant literature available and the findings described in Part II of the thesis. One important question raised is whether the ratio of essential amino acids contained in the diets at present force fed to the premature infant might be less than optimal for normal growth and brain development.
PREFACE

The object of this thesis is to examine in detail some aspects of phenylalanine metabolism in the infant and child. Specifically, the effects of a reduced dietary intake of phenylalanine are examined in the normal infant and the infant with phenylketonuria.

Chloramphenicol, a known inhibitor of protein synthesis in the bacterial cell, is investigated for its action on amino acid metabolism and bone marrow morphology of infants and children. The interaction of phenylalanine and chloramphenicol is discussed in relation to protein synthesis and the antibacterial properties of the drug.

Many of the abnormal findings in animals deprived of a single essential amino acid, such as phenylalanine, or given large doses of chloramphenicol, are analogous to the abnormalities found in children with the syndrome kwashiorkor. This analogy is pursued in the discussion, using both the relevant literature available and the findings described in Part II of the thesis. One important question raised is whether the ratio of essential amino acids contained in the diets at present force fed to the premature infant might be less than optimal for normal growth and brain development.
The young infant with phenylketonuria can be successfully treated by restricting his dietary intake of phenylalanine. Dietary management of two phenylketonuric infants diagnosed early is described, and the importance of a relative deficiency of phenylalanine as a critical limiting factor in protein synthesis is discussed. One possible method for controlling blood phenylalanine concentrations in the young phenylketonuric infant with a less critical dietary regimen is suggested.
HISTORICAL INTRODUCTION

The identification of carbon-dioxide or "fixed air" by Joseph Black (1728-1799), Professor of Chemistry and Physic at Edinburgh was perhaps the first step towards a scientific evaluation of nutrition (Kent, 1950). Daniel Rutherford (1749-1819), a pupil of Black, obtained his doctorate in Medicine from the University of Edinburgh for his "Dissertatio inauguralis de Aere Fixo Dicto, aut Mephitico" (Rutherford, 1772). In his thesis, he described the presence of another gas in the air which would not support life or fire and named it "aer malignus" or noxious air. There is argument as to whether Rutherford or Priestley (1772) was the first to describe this gas we now call nitrogen but McKie gives Rutherford the credit. The name nitrogen was given to the gas by Chaptel (1790) because it formed the radical of nitric acid.

Antoine Lavoisier continued work based on Black's original findings and describes experiments on human subjects in which he showed that oxygen is consumed and carbon dioxide evolved during respiration. He further demonstrated that the quantity of oxygen used increases by some 50% above the basal level after a meal (the specific dynamic action of food) and that exercise also increases oxygen consumption.
It was Magendie (1783-1855) who first demonstrated that nitrogenous compounds were essential constituents of the diet (Magendie, 1816). This conclusion was reached from experiments in which dogs fed solely a carbohydrate (sugar) or fat (olive oil) diet survived only a few weeks. Vitamins were unsuspected at this time and it is probable that vitamin deficiency played a major part in the death of these dogs. The experiments were the first long-term feeding experiments and were the forerunner to those described later in this thesis. Magendie expanded his work on dogs and made the distinction between nitrogenous and non-nitrogenous foods in his "Elementary Compendium of Physiology for the Use of Students" (1829).

The importance of nitrogen containing foods for the formation of blood and tissue was first recognized by Justus von Liebig. I quote from the English edition of his book, "Animal Chemistry, or Organic Chemistry in its Applications to Physiology and Pathology," *1842) (p. 95):

"... the substances of which the food of man is composed may be divided into two classes; into nitrogenized and non-nitrogenized. The former are capable of conversion into blood; the latter incapable of this transformation.

Out of those substances which are adapted to the formation of blood are formed all the organized tissues."
Liebig also drew attention to urea as the nitrogenous end-product of protein metabolism excreted in the urine. The name protein, derived from the Greek work "proteios" meaning "primary" or "first," was first used by the Dutch chemist Gerrit Jan Mulder (1802-1880) to describe nitrogenous compounds linked to sulphur and phosphorus (Mulder, 1839). Nitrogen balance studies were developed by Carl Voit (1831-1908) who had been a pupil of Liebig in Munich. He found in his long-term study of healthy mature animals that their nitrogen intake and output was equal, giving a state of equilibrium. He went on to demonstrate the protein-sparing action of carbohydrate and fat (Voit, 1869).

It was from this time on that the intensive study of nutrition and protein metabolism were to develop in many countries throughout the world. There is yet no final answer to what constitutes ideal nutrition but a great deal is now known of the quantitative and qualitative aspects of the problem.

Throughout infancy and childhood, while growth is taking place, there is retention of nitrogen in the body. If Voit had studied immature animals, his observations on nitrogen equilibrium would not have held true. The newborn and suckling periods of life are characterized by an extreme anabolic tendency with nitrogen retention.
Another Professor of Chemistry at Edinburgh, Playfair, first reported (1853 and 1865) the dietary requirements for protein in different groups of the population. He found that the normal labourer required 184 G of protein per day while the sedentary worker required only 57 G per day. The diet he recommended for the average healthy adult was one containing 119 G protein, 51 G fat, and 530 G carbohydrate. Results published by Voit (1881) and Atwater (1894) supported Playfair's findings although subsequent workers Swen (1901), Chittenden (1905), and Hindhede (1913) claimed that intake of from 30-55 G of protein per day were quite adequate. Cathcart (1921) and others noted, however, that there was a lowered resistance to disease in people maintained on low-protein diets. This observation is pertinent to this thesis and to present day observations on the syndromes of Kwashiorkor and marasmus which are the result of protein deficient diets. Watkin et al. (1963) recommend 1 G protein/Kg/day for the average healthy young adult.

Throughout infancy and childhood, while growth is taking place, there is retention of nitrogen in the body. If Voit had studied immature animals, his observations on nitrogen equilibrium would not have held true. The newborn and suckling periods of life are characterized by an extreme anabolic tendency with nitrogen retention.
This can be demonstrated even when there is not enough food available to allow growth to take place. The starving newborn infant derives only 4-5% of his total caloric requirement from protein breakdown, whereas the healthy adult male utilizes protein for about 20% of his metabolic needs (McCance and Strangeways, 1954). Normally, however, when food is provided, growth dominates and creates the great demand for protein and other nutrients which characterize the newborn period.

Bunge (1898) related the growth rate of the young of different animal species to the percentage protein of the mother’s milk. The higher the protein content found, the faster was the rate of growth for the species. A human baby retains 50% of the nitrogen from breast milk at 6-8 days of age and an average of 22% over the first 6 months (Slater, 1961). Babies on cow’s milk formula retain more nitrogen from the higher protein intake afforded. They do not, however, gain weight any faster than babies fed human milk. Although Bunge’s results hold true for each species, one cannot increase the growth rate in man by feeding the higher protein milk of another mammal. There is as yet no complete answer to the comparatively simple problem of comparing the relative merits for human infants of breast milk and substitutes based on cow’s milk (Platt et al, 1961).

Protein requirements are generally thought of in terms of so many grams per kilogram of body weight per day. On this basis, the human infant requires 2 G/kg/day while the young rat will require as
much as 20 G/kg/day. This intake of protein will maintain life and encourage growth but is not necessarily the optimum quantity. The intake of protein, which produces maximal growth rate, may not be optimal for health. Experiments performed by McCay et al (1935, 1939) showed that in animals severe restriction of food sufficient to interfere with growth allowed the animal to live longer. Ross (1959, 1961) confirmed these findings and suggested that protein restriction may contribute to a longer life in rats. The problem as to what constitutes the optimum protein intake for the maintenance of health remains as yet unanswered.

The concept of dietary amino-acid balance was first introduced by Osborne and Mendel (1919). A number of different amino-acids led them to conclude that the nutritive value of a protein was dependent upon the presence or absence of a particular amino-acid. However, it was not until the early 20th century that the relationship between the nutritional value of a protein and its amino-acid composition was further elucidated. Ross (1926; Wills, 1930; Wills and Wills, 1932) and others (Ratliff et al., 1936; Wills and Wills, 1938) demonstrated that the nutritive value of a protein was dependent upon the presence of a particular amino-acid. This concept was further developed by Block and Mitchell (1946-47) who developed the concept of Chemical Score to relate the nutritional value of a protein to its amino-acid composition. This score provided a means of predicting the nutritive quality of a protein. In essence, it compared
QUALITATIVE ASPECTS OF PROTEIN METABOLISM

Willcock and Hopkins (1906) reported that complement of Tryptophan would prolong the lives of mice fed a tryptophan free diet. This was the first demonstration that a dietary deficiency of an individual amino acid could seriously interfere with nutrition and health.

The concept of dietary amino-acid balance was first introduced by Osborne and Mendel (1914). Their observations on the body requirements for a number of different amino-acids led them to conclude that the nutritive value of a protein was dependent upon the proportions of the individual amino-acids it contained (Osborne and Mendel, 1915). During the years that followed, new amino-acids were discovered and the group of eight which are essential or indispensable dietary constituents for man were described and evaluated, (Rose, 1936; Whipple, 1940; Cannon, 1947; Rose et al, 1954 a-b; 1955 a-e; Rose and Wixom, 1955 a-b).

At the same time, Block and Mitchell (1946-47) developed the concept of Chemical Score to relate the nutritional value of a protein to its amino-acid composition. This score provided a means of predicting the nutritive quality of a protein. In essence, it compared
the proportions of essential amino-acids in a protein with those found in whole egg protein.

Concepts of relative nutritive values of proteins were further developed when it was realized how important was the ratio of essential to non-essential amino-acids (Harper and Kumta, 1959). If the ratio of amino-acids in a protein is such that a small amount of protein will provide each amino acid in the exactly required amounts, it is very efficient and will have a high Protein Efficiency Ratio or Chemical Score. On the other hand, if the ratio of amino-acids in a protein is such that a surplus of several amino-acids must be ingested to ensure an adequate intake of one or more of the others, then the protein will have a lower Protein Efficiency Ratio or Chemical Score.

Holt and co-workers at New York University have studied the amino-acid requirements of the normal and premature infants (Pratt et al, 1955; Snyderman et al, 1955, 1959 a-d; Holt, 1960). A synthetic diet in which nitrogen is supplied as a mixture of 18 L-amino acids in proportions present in breast milk was fed to infants. The quantity of the specific amino acid under consideration was reduced and replaced with an isonitrogenous quantity of glycine until a point was reached at which nitrogen retention and gain in weight suffered. Table I is summary of the findings of Holt et al and Albanese (1959).
### Table 1

**Minimum requirements of infants of essential amino acids (mg/kg/day)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Depletion Technique</th>
<th>Intake on Milk Minimum (1.65 gm protein/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>Histidine</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>(90) *</td>
<td>75</td>
</tr>
<tr>
<td>Leucine</td>
<td>150-229**</td>
<td>135</td>
</tr>
<tr>
<td>Lysine</td>
<td>105</td>
<td>83</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>90</td>
<td><strong>61</strong></td>
</tr>
<tr>
<td>Methionine</td>
<td>(65) *</td>
<td><strong>32</strong></td>
</tr>
<tr>
<td>Threonine</td>
<td>60</td>
<td><strong>51</strong></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>16-22***</td>
<td><strong>16</strong></td>
</tr>
<tr>
<td>Valine</td>
<td>105</td>
<td><strong>90</strong></td>
</tr>
</tbody>
</table>

*Data of Albanese et al.* (N furnished as degraded protein or protein hydrolysate).

**The usual leucine requirement did not exceed 150 mg/kg. One subject however required 229 mg/kg.

***The usual requirement did not exceed 16 mg/kg. One subject required 22 mg/kg.

The values for the essential amino acid requirements would seem applicable to the experimental conditions under which they were determined, but they may not be applicable to other situations. Furthermore, amino acid requirements are effected by the "balance" or pattern of the available amino acids of the diet. An unbalanced pattern may increase the requirement for one or another essential amino acid. Factors that spare essential amino acids will also affect the requirement; the intake of other calorigenic foodstuffs and of "unessential" nitrogen are of particular importance in this regard.

The effects of omitting one essential amino-acid from the diet for only a few hours can be detected (Elman, 1939; Geiger, 1947-48; Cannon et al, 1947). Within one day (in young growing animals), this results in loss of appetite and growth failure, and, in mature animals, in a severe negative nitrogen balance.(Rose, 1938; Frazier et al, 1947; Kepkowsky, 1948). The animal is immediately unable to utilize the other dietary amino-acids except as a source of energy. These findings stem from the fact that there is essentially no storage of amino-acids in the body. It is this inability of the body to compensate, even for a short time for a deficiency of a single amino acid that makes amino-acid balance so critical and makes the growing infant so sensitive to alterations in the amino-acid pattern of his diet. Omission of most other essential nutrients, including vitamins and minerals for much longer periods of time, has little immediate effect on growth and food intake and may have none at all until body reserves are depleted.

Sidransky and co-workers in a series of studies (Sidransky, 1958 a-b, 1960, 1961, 1962, 1964 a-b, and Lyman and Wilcox, 1963) have studied in detail the effect of individual amino-acid deficiencies on the laboratory animal. Table II summarises the chemical and morphologic changes induced in young rats by force feeding purified diets devoid of single essential amino acids for three to eight days (Sidransky & Verney, 1964). The animals fed a diet deficient in phenylalanine
developed a periportal fatty liver, excess hepatic glycogen and atrophy of the pancreas, submaxillary gland, gastric mucosa, thymus and spleen within eight days of commencing the diet. Sidransky concluded that single deficiencies of all essential amino acids except arginine and leucine can produce most of the features of the kwashiorker-like experimental model. The results of experiments in which animals were force-fed diets containing plant proteins of poor quality and quantity (Deo and Ramalingaswami, 1960; Sidransky, 1960) were almost identical with those found with single essential amino acid deficiencies. No mention of bone marrow or peripheral blood changes resulting from isolated amino acid deficiencies is made in any of the animal studies quoted or in Holts' study of infants.

One purpose of this thesis is to explore further the concept of amino acid imbalance in the young infant with particular emphasis on the effect of a dietary deficiency of phenylalanine on amino metabolism and bone marrow morphology.
<table>
<thead>
<tr>
<th>Changes in body weight</th>
<th>Histidine, Isoleucine, Phenylalanine, Threonine, or Valine</th>
<th>Complete Diet Devoid of</th>
<th>Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Decrease</td>
<td>Leucine</td>
<td>Decrease</td>
</tr>
<tr>
<td>Weight</td>
<td>Increase</td>
<td>Lysine</td>
<td>Decrease</td>
</tr>
<tr>
<td>Protein</td>
<td>Decrease</td>
<td>Methionine</td>
<td>Decrease</td>
</tr>
<tr>
<td>Lipid</td>
<td>Increase</td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Glycogen</td>
<td>Increase</td>
<td></td>
<td>Increase</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Atrophy</td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Decrease</td>
<td></td>
<td>Increase</td>
</tr>
<tr>
<td>Weight</td>
<td>Decrease</td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Protein</td>
<td>Decrease</td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>Atrophy</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Stomach</td>
<td>Atrophy</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Spleen</td>
<td>Atrophy</td>
<td></td>
<td>Normal</td>
</tr>
</tbody>
</table>

ANAEMIA AND PROTEIN MALNUTRITION

In the tropics, cases of anaemia refractory to treatment with iron, vitamin B\textsubscript{12}, or folic acid are numerous. They are especially common during pregnancy, infancy and childhood (Woodruff, 1960). Hansen (1956) showed that the chief limiting nutrients in low protein diets that produce kwashiorkor are the amino acids. A study by Brock and Autret (1952) of protein malnutrition in Africa showed that the syndrome of kwashiorkor which is a result of protein malnutrition was associated with a mild normocytic or macrocytic anaemia. When parasitic infections were present, the anaemia could be severe. Woodruff (1951 and 1955) has also described such anaemia in association with protein malnutrition.

Some understanding of the mechanisms whereby protein malnutrition may cause anaemia was obtained from the experimental evidence provided by Whipple and Madden (1949) and Yule et al (1951). They demonstrated that haemoglobin may be synthesized from plasma proteins. In dogs, they showed that plasma could supply proteins from which the depleted animal could manufacture new haemoglobin and that 100 G casein digest enabled 25-40 G of new haemoglobin and plasma protein to be produced. This study was really a sophisticated demonstration of Liebig's original observation. The work of Wizet and Robscheit-Robbins (1950)
indicated (at the reticulocyte stage) the red cell is able to incorporate plasma proteins into itself or synthesize plasma proteins from amino acids. Furthermore, a deficiency or abnormality of plasma protein, delayed the maturation of red precursors.

The anaemia, as stated, may be normocytic or slightly macrocytic. Mean cell volumes (MCV) are commonly at the upper limit of normal and the mean corpuscular diameter (MCD) is often increased above the normal range which results in a corresponding reduction in the mean corpuscular average thickness (MCAT) (Woodruff, 1955). The broadening and thinning of erythrocytes, described by Trowell (1949) as dimorphic anaemia might well be due to this type of cell where the thinness gives the appearance of hypochromia while broadening makes them appear macrocytic (Woodruff, 1961).

Pigs fed protein deficient diets show a reduced haemoglobin and red cell count accompanied by a fall in unsaturated iron-binding capacity and a rise in serum iron (Platt et al, 1964). The limiting factor for haemoglobin synthesis in these animals was shown clearly to be protein.

One type of anaemia which is probably directly related to protein deficiency has been observed in children with severe kwashiorkor (Viteri et al, 1964). These children come from the highland areas of Guatemala where there is no hookworm and where vitamin B₁₂ and folic acid deficiencies are uncommon. The anaemia is normocytic or slightly
macrocytic and not usually severe although the bone marrow is severely hypoplastic, especially for the erythroid series. When the children are treated with milk alone, the bone marrow rapidly becomes hyperplastic and a good reticulocyte response is observed within a week. This type of anaemia appears to be due to an arrest in erythrocyte formation at a very early stage in haematopoiesis because of the severe deficiency of protein.

Vacuolizations of early erythroblasts has been reported in African children during the early stages of marasmus and kwashiorkor, (Kondi and Foy, 1964). This change in marrow morphology disappears with increased protein intake. Sometimes the vacuoles develop later in the illness and are associated with intercurrent infection; vacuolization is then accompanied by hypoplasia of the erythroid series. Kondi and Foy comment that "In marasmus and kwashiorkor, vacuolization may be associated with a dietary deficiency of phenylalanine or other amino-acids for low protein intake is usually regarded as an aetiological factor in these conditions." They demonstrated similar morphologic changes in the erythroblasts of baboons made deficient in riboflavine. The riboflavine deficiency might well have acted through an interference with amino acid metabolism because the animals had an abnormal amino aciduria. Reference to a possible deficiency of phenylalanine is based on the findings of Sherman et al (1964) who reported anaemia and red cell precursor maturation arrest with vacuolization in an infant with phenylketonuria on a low phenylalanine diet.
Tryptophan deficiency in animals will produce anaemia (Albanese et al, 1953). Supplements of tryptophan will prevent this anaemia even when the diet of the animals is poor.

There is then good evidence linking protein deficiency states to a normocytic or macrocytic anaemia. The bone marrow is hypoplastic and the few red cell precursors present may show cytoplasmic vacuoles. This anaemia in children can be reversed by protein feeding.

Host reaction to an infectious agent involves the study of a great many factors. Genetic variations in host resistance, the virulence of the infectious agent, host age, sex and coexistent disease render the interpretation of the effect of protein deficiency very difficult in man. Moreover, the host may have previously developed sufficient immunity from contact with the infecting agent so that he is no longer susceptible.

Effect on Antibody Response: Madden and Whipple (1940) found that dogs fed low protein diets had poor antibody responses to a variety of antigens. Miller et al (1940) demonstrated that the same dogs
INFECTION AND PROTEIN MALNUTRITION

Severe protein deficiency interferes with the resistance of the body to infection (Scrimshaw et al, 1939). Mild protein deficiency has little or no effect on the adult animals' resistance to infection (Metcoff et al, 1948-49). A degree of protein deprivation which will not affect the adult response to infection might, however, seriously interfere with the response in the growing infant. The high mortality which is seen in young children in technically underdeveloped countries is due directly to the synergism of malnutrition and infective disease (Scrimshaw, 1961).

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Effect on Antibody Response: Madden and Whipple (1940) found that dogs fed low protein diets had poor antibody responses to a variety of antigens. Miller et al (1940) demonstrated that the same dogs
were much more susceptible to infections. When the dogs were fed a good protein diet, their antibody responses and their response to infection returned to normal. Protein deficiency in the rabbit reduces its capacity to produce agglutinins to Salmonella (Cannon et al, 1943) and to the Pneumococcus (Wissler, 1947 a). A group of rats normally resistant to a particular strain of pneumococcus lost this resistance when fed a protein deficient diet (Wissler, 1947 b). The antibody response to the infection was very poor but it improved markedly when a mixture of amino acids was fed. Loss of antibody response in rats to injected sheep red blood cells can be directly correlated to the duration of protein deprivation (Benditt et al, 1949).

Evidence for a delayed or poor antibody response to a number of antigens in man in the presence of a poor dietary intake of protein has been reported. Wohl et al (1949) noted a markedly delayed antibody response to typhoid vaccine in 88 patients with low serum albumin levels. Cannon (1944) had reported the same findings in a group of 15 patients with low total serum proteins. A fifteen year old girl reported by Krebs (1946) showed no response to thyroid vaccine while she had a total protein of 3.1 G/100 ml. serum of which 2.0 G/ml was albumin.

More recently, Olarte et al (1956) reported a retarded response to diphtheria antitoxin in five two-year old Mexican children with severe malnutrition. Budiansky and Da Silva (1957) found that twelve
of fifteen children with kwashiorkor showed practically no antibody response to typhoid vaccine.

**PHENYLALANINE AND PROTEIN SYNTHESIS**

The antibody is an important body defence against the invading pathogen but it is only one of several defence mechanisms which lose their efficiency in the face of protein deficiency. It is a response, however, which is readily measured and thus lends itself to study. Phagocytic activity is equally important in body defence and the lack of leucocytic response to infection in children with kwashiorkor is probably a significant factor in their reduced resistance to infection (Trowell et al, 1954; Scrimshaw and Behar, 1961).

Erlemeyer and Lipp (1882) synthesised phenylalanine from phenylacetaldehyde and Schulze and Barbieri (1883) proved that their protein constituent was structurally identical with the synthetic compound.

![Phenylalanine](image)

**Fig. 1** Phenylalanine (α-amino β-phenylpropionic acid)

Phenylalanine, like all the other amino acids, is not stored in the body. Once it has been absorbed from the intestine into the portal
Phenylalanine is a nutritionally essential amino acid. Schulze and Barbieri (1879) first isolated phenylalanine from the sprouts of lupine. They later isolated it from acid and alkaline hydrolysates of squash seed proteins and showed it to be a constituent of protein (1883). The empirical formula was established by these same authors and they demonstrated that oxidation yielded benzoic acid and that dry distillation yielded what appeared to be phenylethylamine (1881).

Erlenmeyer and Lipp (1882) synthesized phenylalanine from phenylacetaldehyde and Schulze and Barbieri (1883) proved that their protein constituent was structurally identical with the synthetic compound.

CH -CH-COOH

\[ \text{NH}_2 \]

Fig. 1 Phenylalanine (L-amino \( \text{\beta} \)-phenylpropionic acid)

Phenylalanine, like all the other amino acids, is not stored in the body. Once it has been absorbed from the intestine into the portal circulation one of several pathways of metabolism is open to it. First, in the growing infant, it is essential for the formation of many types of new protein and is not available for utilization. Second, it is necessary for the production of melanin, the black pigment of the skin. In addition, it is a constituent of the melanin, the black pigment of the eyes. It is also found in the blood, as well as in the hair and nails. This amino acid appears to have a structural similarity with other amino acids that have been isolated from the body, such as those found in phenylketonuria.

The presence of an asymmetric carbon atom in phenylalanine, as in all other amino acids except glycine, allows the property of stereo-isomerism. D- and L- optically active forms of phenylalanine are present in dietary proteins but it is the L-form which is of major importance in protein metabolism.
circuit one of several pathways of metabolism is open to it. First, in the growing infant, it is essential for the formation of many types of new protein as well as the continuous renewal of the existing pool of body protein. The functions and types of protein required by the infant are many, e.g., haemoglobin, antibody, digestive enzymes, plasma proteins, hormones and body tissue protein.

A second pathway is the conversion of phenylalanine to tyrosine which in turn is necessary for the production of melanin, the pigment which affects skin, hair and eye colour, noradrenaline and adrenaline as well as the thyroid hormones (iodotyrosines). It is a block to this metabolic pathway which results in the clinical syndrome of Phenylketonuria and will be discussed in detail later.

A third possible route is the conversion of phenylalanine into a source of energy by its breakdown and entry into the glycogenic and ketogenic pathways of the liver. Small quantities of phenylalanine appear normally in the urine. This loss is insignificant in amount unless there is renal overflow from high blood levels of phenylalanine such as that found with phenylketonuria.

The presence of an asymmetric carbon atom in phenylalanine, as in all other amino acids except glycine confers the property of stereo- isomerism. D- and L- optically active forms of phenylalanine are present in dietary proteins but it is the L-form which is of major importance in protein metabolism.
Genetic Control of Protein Manufacture: The function and character of each protein molecule is dependent upon the arrangement of individual amino acids within the molecule and upon the incorporation of prosthetic substances, e.g., iron in haemoglobin. Given a supply of raw materials (namely amino acids) and a factory, a set of blueprints or code must be available if the necessary proteins are to be manufactured.

Work of recent years has thrown considerable light on the way in which the code for each protein is stored in a structural gene which is formed from deoxyribonucleic acid (DNA). The code or genetic information stored in DNA is transmitted to the protein-making machinery of the cell, so that a specific nucleotide sequence in DNA gives rise to a unique amino acid sequence in the polypeptide chain of a protein. We now known that ribonucleic acid (RNA) participates in this process as a messenger between DNA and the protein, (Jacob and Monod, 1961; Brenner et al, 1961). Protein is made on the ribosome or on groups of ribosomes linked by the chain of messenger RNA (m-RNA) to form a polysome, (Borsook et al, 1952, Warner et al, 1963; Gierer, 1963). The ribosomes as seen by electron microscopy are small dark particles attached to the cytoplasmic membrane and consist of RNA and protein.

DNA normally occurs as a double-stranded molecule resembling a ladder that has been twisted into a helix. The sides of the ladder consist of alternating units of the sugar deoxyribose and a simple
phosphate compound. The rungs of the ladder are attached to the deoxyribose units and each rung consists of a purine and a pyrimidine base, either adenine (A) linked to thymine (T), or guanine (G) linked to cytosine (C). This is the model proposed by Watson and Crick (1953).

The molecule of RNA is generally a single-stranded structure that contains the sugar ribose in place of the deoxyribose sugar found in DNA. Four bases are linked to the ribose units, three of which are identical with those in DNA, the fourth being uracil (U) which replaces the thymine found in DNA.

The relation between DNA, messenger RNA and protein is illustrated schematically in Fig. 2 in which portions of the base or nucleotide sequence and amino acid chains are shown as lengths of paper tape.

![DNA and RNA sequence](image)

**Step 1**

**DNA**

TTC TTT CAA AGA TAA AAG GGC ATA TCA AAA CAG GCG

**m-RNA**

AAG AAA GUU UCU AUU UUC GCG UAU AGU UUU GUC CCC

**Step 2**

**PROTEIN**

Glu lys cys phe tyr leu gly leu met phe arg pro

Fig. 2 - Two-step transcription of genetic code. RNA nucleotidyl transferase, which catalyzes the DNA-dependent synthesis of RNA, is the key enzyme in the transcription of the genetic message from DNA to RNA (step 1). The code read-out step (step 2) can be considered as a translation from the four character language of proteins. m-RNA stands for messenger RNA.
It takes a base sequence of three to code one amino acid, (Crick et al, 1961). Nirenberg and Matthaei (1961) demonstrated that a triplet of uridine base (UUU) in m-RNA was the probable code for the amino acid phenylalanine. More recently, the m-RNA code for each amino acid has been demonstrated to be degenerate in that there is more than one correct base sequence for an individual amino acid. Thus, (UCU) uridine with cytosine has also been found to code for phenylalanine (Ochoa, 1964).

The significance of uridine as the m-RNA code for phenylalanine will be further discussed in relation to chloramphenicol toxicity.

Before phenylalanine can be united with other amino acids on the ribosome or protein factory, it is first activated by being coupled with a source of energy, adenosine triphosphate (ATP) in the presence of a specific enzyme (Hoagland et al, 1956). The activated phenylalanine is then attached to a specific RNA called soluble RNA or transfer RNA (Hoagland, 1955). Each different amino acid in the cytoplasm of the cell is "recognized" and attached to a specific transfer RNA. The transfer RNA's carry the amino acids to the ribosome, where they are joined in proper order to form a protein. The transfer RNA's are released intact to pick up more amino acid molecules (see Fig. 3) Zubay, 1963.

It is evident that the transfer of information from the basic genetic material, DNA, through a complementary sequence of bases in a
The triplet code is read on the ribosome as it "moves along" the messenger RNA chain. Transfer RNA brings the amino acid named by the code into position on the ribosome. The amino acid forms a peptide linkage with the peptide chain which is being formed and the transfer RNA is released.
A single gene mutation with alteration of a base pair in the DNA helix should result in the replacement of one amino acid in a protein by another different amino acid. This hypothesis was verified in the case of sickle-cell anaemia in man. Glutamic acid, present in normal haemoglobin is replaced by valine which results in the sickling properties of the red blood cell (Ingram, 1961). Thirty different haemoglobin molecules, the result of genetic controlled substitutions of amino acids, have now been described (Hirschhorn, 1964).

Phenylalanine is incorporated into many different protein molecules according to the codes contained in the genetic material. If no phenylalanine is available for protein synthesis then normal protein cannot be manufactured. Should there be interference with the code for phenylalanine at the gene level or at the ribosome, then again normal protein cannot be produced by the cell.

Part of my later arguments will be to show that a dietary deficiency of phenylalanine, or a block to the incorporation of phenylalanine into protein will produce a situation identical with that produced by severe protein malnutrition.
Chloramphenicol is an antibiotic which preferentially inhibits protein synthesis in a number of organisms (Gale and Folkes, 1953; Brock, 1961; Feingold, 1963). Studies on the mechanism by which chloramphenicol inhibits protein synthesis indicate that inhibition occurs at a stage subsequent to the attachment of amino acids to soluble RNA (s-RNA) and in relation to the ribosomal assembly of amino acids on messenger RNA (m-RNA), (DeHoss and Novelli, 1956; Lacks and Gross, 1960; Nathans et al, 1962). Rendi and Ochoa (1962) suggest that chloramphenicol might interfere with the attachment of m-RNA to ribosomes. The steric configuration of chloramphenicol in solution is very similar to uridine 5' phosphate and it may be that chloramphenicol interferes with uridine coding at the ribosome, (Jardetsky, 1963; Jardetsky and Julian, 1964).

The similarity of the chemical structure of the amino acid and antibiotic is apparent (Fig. 4). Indeed, Yamada and Matsuo (1962) have used L-phenylalanine as a basis for the synthesis of chloramphenicol.

Protein synthesis in mammalian cells or mammalian cell-free systems is much more resistant to inhibition by chloramphenicol than it is in microbial systems. This difference in sensitivity is most apparent in cell-free systems where cellular permeability is not a factor. Complete inhibition of protein synthesis in E. coli cell-free systems can be obtained with 0.15 μmole chloramphenicol/ml of reaction mixture (Nirenberg and Matthei, 1961). Practically no inhibition of protein synthesis in many mammalian cell-free systems had been demonstrated with chloramphenicol concentrations up to 5-10 μmoles/ml reaction mixture (Von Ehrenstein and Lipmann, 1961). Inhibition of protein synthesis in the intact mammalian cells had been obtained only with concentrations of chloramphenicol much in excess of that required to inhibit bacteria (Allen and Schweet, 1962; Borsook et al., 1957; Britman and Webster, 1958; LePage, 1953; Rendi, 1959; Wang, 1961). Von Ehrenstein and Lipman (1961) suggest that the difference in sensitivity may be due to the presence in bacteria of an extra, chloramphenicol sensitive step, involved in the transfer of amino acids to ribosomes.

During the past few years, however, it has become apparent that under appropriate conditions chloramphenicol will inhibit mammalian protein synthesis. Ambrose and Coons (1961) were able to inhibit antibody
CHLORAMPHENICOL  PHENYLALANINE

Fig. 4
synthesis by lymph node fragments when chloramphenicol (0.15 M/mole/ml) was present in the culture medium. Svéhag (1964) found inhibition of 19S antibody formation in vitro with this concentration of antibiotic. Inhibition of protein synthesis in human bone marrow cultures was shown by Djordjevic and Szybalski (1960) at similar low concentrations of chloramphenicol. Weisberger and Wolfe (1964) demonstrated a suppression of antibody synthesis and prolonged survival of skin homografts in animals treated with chloramphenicol. They further demonstrated the sensitivity of a rabbit reticulocyte ribosome system to the inhibitory action of chloramphenicol. These observations are in accord with the suggestion that protein synthesis in intact mammalian cells may be susceptible to chloramphenicol inhibition only at a time when new m-RNA is being attached to ribosomes. More mature cells may be resistant because m-RNA already attached to ribosomes is not accessible to chloramphenicol inhibition.

Rubin et al, 1960, demonstrated increased concentration of iron and an increased saturation of iron binding globulin in the serum of patients treated with chloramphenicol probably the results of suppressed haemoglobin synthesis. Collip et al, 1965, found inhibition of glucose 6 phosphatase activity in the livers of ducks treated with chloramphenicol and have shown that this effect can be prevented by simultaneous administration of phenylalanine. Straub and Ullman (1957) report that the antibiotic inhibits synthesis of amylase by pancreatic tissue.
Certain other clinical observations indicate that chloramphenicol may inhibit protein synthesis by mammalian cells. For example, Saidi et al., 1961, found that administration of chloramphenicol to patients with pernicious anaemia in relapse prevents the reticulocyte response which is normally obtained with vitamin B₁₂. The production of vacuoles in the cytoplasm of bone marrow red cell precursors associated with anaemia and a reduced reticulocyte response during the course of chloramphenicol therapy (Saidi and Wallerstein, 1960; Saidi et al., 1961; Rosenbach et al., 1960; McCurdy, 1961) might well be a further manifestation of suppressed protein synthesis.

The ability of chloramphenicol to inhibit protein synthesis in mammalian cells may have relevance to the production by this drug of fatal aplastic anaemia. Abnormal ribosomal material and RNA accumulates in cells exposed to chloramphenicol, Gale, 1963. It may be that prolonged use or high dosage of the drug could result in absolute inhibition of normal ribosome or m-RNA formation and produce aplasia of the red and, or white cell, precursors. Inherent genetic factors may influence the drug's action on the bone marrow cells of the individual host and account for individual susceptibility to chloramphenicol toxicity. Immature infants are particularly susceptible to chloramphenicol toxicity. A clinical picture called the "Gray-baby" syndrome was produced in newborn and premature infants on chloramphenicol therapy. The syndrome has practically vanished since a lower recommended dose of the antibiotic has been employed for this age group.
It is thus evident that chloramphenicol has an effect on a number of different mammalian tissues. Part of my thesis will be to assess the effect of chloramphenicol on host amino acid metabolism and observe the interaction of the antibiotic and phenylalanine in the clinical and laboratory situation.

Noteworthy, enzyme and clinical abnormality first postulated by Garrod (1909) was clearly demonstrated for the first time in the disease phenylketonuria. Folling (1934) described 16 patients who excreted phenylpyruvic acid and were mentally deficient. Jervis (1939, 1940) proved the condition was transmitted by a single autosomal recessive gene, and showed that large amounts of phenylalanine accumulated in the blood and spinal fluid of these patients. The name phenylketonuria was introduced by Penrose and Quastel (1937) but the disease has been variously known as imbecillus phenylpyruvic, Folling's disease and phenylpyruvic oligophrenia (Nickel, 1954). Jervis (1947, 1953) pin-pointed the defect and demonstrated that the liver of these patients failed to hydroxylate phenylalanine to tyrosine. The genetic defect results in a severe deficiency of the enzyme phenylalanine hydroxylase.

Phenylalanine Hydroxylase: The main pathway of degradation of phenylalanine begins with a conversion to tyrosine, catalysed by a specific hydroxylating enzyme (Udenfriend and Cooper, 1952; Hitomi et al, 1956; Mitoma, 1956; Kaufman, 1957). This enzyme, called phenylalanine hydroxylase, catalyses a reaction involving molecular oxygen.
PHENYLALANINE METABOLISM AND PHENYLKETONURIA

The relationship between gene, enzyme and clinical abnormality first postulated by Garrod (1909) was clearly demonstrated for the first time in the disease phenylketonuria. Folling (1934) described 10 patients who excreted phenylpyruvic acid and were mentally deficient. Jervis (1939, 1940) proved the condition was transmitted by a single autosomal recessive gene, and showed that large amounts of phenylalanine accumulated in the blood and spinal fluid of these patients. The name phenylketonuria was introduced by Penrose and Quastel (1937) but the disease has been variously known as imbecillitas phenylpyruvica, Folling's disease and phenylpyruvic oligophrenia (Bickel, 1954). Jervis (1947, 1953) pin-pointed the defect and demonstrated that the liver of these patients failed to hydroxylate phenylalanine to tyrosine. The genetic defect results in a severe deficiency of the enzyme phenylalanine hydroxylase.

Phenylalanine Hydroxylase: The main pathway of degradation of phenylalanine begins with a conversion to tyrosine, catalysed by a specific hydroxylating enzyme (Udenfriend and Cooper, 1952; Mitoma et al, 1956; Mitoma, 1956; Kaufman, 1957). This enzyme, called phenylalanine hydroxylase, catalyses a reaction involving molecular oxygen which is irreversible. It occurs in the liver and catalyses hydroxylation of phenylalanine at the para position in the presence of molecular oxygen and a folic acid derivative (Kaufman and Levenberg, 1959) (Fig. 5).
which is irreversible. It occurs in the liver and catalyses hydroxylation of phenylalanine at the para position in the presence of molecular oxygen, reduced TPN (tri-phosphopyridine nucleotide) and a folic acid derivative (Kaufman and Levenberg, 1959) (Fig. 5). Phenylalanine hydroxylase consists of two fractions (Kaufman and Levenberg, 1959) a labile one found only in the liver and a stable one which is found in many body tissues; only the former is lacking in phenylketonuria.

The hydroxy group may be added to the aromatic ring of phenylalanine, either in the para- or the ortho- position. Normally, the para- position is favoured, so that tyrosine (p-hydroxyphenylalanine) is the major product of the oxidation of phenylalanine. Small amounts of ortho- hydroxy derivatives are found in the urine of normal individuals but very large amounts occur in the phenylketonuric (Armstrong and Shaw, 1955). The basic defect in phenylketonuria is then the lack of enzymatic activity for the para- oxidation of phenylalanine. Since this major pathway for the utilization of phenylalanine is blocked, phenylalanine and some of its metabolites accumulate and more phenylalanine is diverted to the ortho- hydroxy pathway, normally a minor route. High plasma concentrations of phenylalanine are characteristic of the disease (15-63 mg/100 ml plasma). The normal values for phenylalanine concentration in the plasma are 1-4 mg/100 ml. Phenylalanine also accumulates in the cerebrospinal fluid and is excreted in excess in the urine. Transamination is stimulated, and
Fig. 5

Tyrosine + TPN + H₂O

Hydroxylase

Phenylalanine + O₂

Phenylalanine + TPN + H⁺ (Pteridine)
large amounts of deaminated derivatives are found in the urine. These derivatives include phenylpyruvic acid, (Jervis, 1950), O-hydroxy-phenylacetic acid (Armstrong et al, 1955), phenylacetyl glutamine (Woolf, 1951) and phenylethylamine.

Abnormalities of tryptophan metabolism are regularly encountered in phenylketonuria. There is diversion of tryptophan metabolism from the nicotinic acid pathway and the serotonia pathway (Pare, et al, 1958) more of the tryptophan being transaminated with the formation of indolepyruvic, indolelactic and indoleacetic acids (Armstrong and Robinson, 1954); indoxyl compounds are also formed and there is indicanuria. The cause of the abnormal tryptophan metabolism is not established. Possibly, the increased transamination of phenylalanine has a stimulating effect on other transaminations, or the abnormal metabolites of phenylalanine may inhibit the serotonia and nicotinic acid pathways.

Fig. 6 summarises the metabolic pathways discussed in this and the previous chapter. Also shown are the sites of metabolic block found in Tyrosinosis and Alcaptonuria.

Clinical Features and Management: The incidence of phenylketonuria in Britain was calculated to be between 2 and 6 per 100,000 (Munro, 1947) while in American, the incidence of the disease is about 1 in 25,000 births.
The metabolism of phenylalanine and tyrosine and the points at which metabolic impairment occurs in phenylketonuria, tyrosinosis, and alcaptonuria are summarized below.

**Fig. 6**
(Holt and Snyderman, 1964). Improved diagnostic survey methods may show higher figures than those quoted. Kennedy in a report to the New England Pediatric Society (March, 1965) described 29 cases of phenylketonuria detected in Massachusetts by the Guthrie test (Guthrie, 1962). Capillary blood samples of all infants born in Massachusetts were tested at 4 days and again at 4 weeks of age. The incidence of phenylketonuria for the period 1963-1965 was 1 in 9,000 births. All but one of the cases reported by Kennedy had elevated blood levels of phenylalanine by the fourth day of life. Sex incidence of the disease is equal.

In the untreated case, mental retardation is generally evident by the age of 6 months. Seizures and other neurologic abnormalities, lack of pigmentation of hair and skin, and eczema may occur. The majority of patients are idiots, a few are imbeciles, and rare patients have borderline intellectual development. Waisman and Harlow (1965) have produced experimental phenylketonuria in infant rhesus monkeys fed 3 G L-phenylalanine per kg of body weight per day. This intake of L-phenylalanine depresses phenylalanine hydroxylase activity and results in mental retardation with grand mal convulsions in the animals.

Pathologically, the only evidence of the disease is retardation of myelination of the brain (Alvord et al, 1950). The precise nature of the alterations induced in the developing brain is not known.

Dietary management of phenylketonuria has recently been described in detail in a Report to the Medical Research Council of the Conference
on Phenylketonuria (1963) and by Clayton et al, 1965. Low phenylalanine casein hydrolysate preparations are now commercially available and greatly facilitate the management of the diet. Adequate control of therapy depends on meeting, but not exceeding the phenylalanine requirement and, at the same time, meeting the caloric and other nutritional requirements (Paine and Hsia, 1957). Previous estimates of an adequate intake of phenylalanine for the young infant have been too low.

Brimblecombe et al (1961) reported "...that in the first year of life, while growth is rapid, the basic requirements of phenylalanine are relatively higher in mg/kg body weight than at older ages. Excessive restriction of phenylalanine intake at this stage may lead to subnormal 5-hydroxyindoleacetic acid in the urine is unchanged but the serotonin levels of the plasma phenylalanine, with loss of weight, vomiting, listlessness, and a generalised eczematous rash. Mental development is also retarded during these episodes."

Three deaths are known to have occurred in phenylketonuric children with these symptoms. At least one fatal case had megaloblastic anaemia (Royston and Parry, 1962). Sherman et al (1964) reported anaemia, with maturation arrest of red cell precursors and failure to thrive in a premature phenylketonuric infant with low plasma levels of phenylalanine following treatment.

The fine control of the disease thus becomes extremely important and the intake of phenylalanine must be carefully assessed for the individual patient. At present, this means regular estimation of the serum concentration and adjustment of the dietary phenylalanine intake.
accordingly. Proper management of phenylketonuria is not possible if some accurate method of measuring blood levels of phenylalanine is not available. Urinary phenylpyruvic acid excretion ceases when the plasma phenylalanine concentration falls below 0.6 μmoles per ml. (10 mg/100 ml). Estimation of urinary phenylpyruvic acid levels is then of little value when the concentration of phenylalanine in the plasma should be in the range 1.5 - 4.0 mg/100 ml to ensure optimum control of the disease.

All the major biochemical abnormalities of phenylketonuria are reversed by the low phenylalanine diet (Knox, 1960). The amount of 5-hydroxyindoleacetic acid in the urine is unchanged but the serotonin in the blood rises during treatment (Pare et al, 1953). Abnormal components in the B-globulin fraction of plasma in about half of the cases disappears with treatment and reappears when phenylalanine is reintroduced to the diet (Brown et al, 1955).

Clinical improvement in behaviour and attention span with an improved pattern on electroencephalogram has been reported (Hsia et al, 1958). Motor performance improves as tremors and muscular hyper-tonicity decreases. The effect on intelligence is closely related to the age at which the corrective diet is instituted. If this is done in early infancy, a reasonable mentality can be expected. The results are less satisfactory the longer the delay in starting treatment (Horner and Streamer, 1959; Knox, 1960). There is as yet no good evidence on
which to base the decision to cease therapy. It has been suggested that 4 years is a reasonable age at which to stop treatment because brain myelination is, by this age, virtually complete (Horner and Streamer, 1962).

Study 1: Effect of Phenylalanine Deficient Diet on Bone Marrow and Amino Acid Metabolism in Normal Infants.

The present study is an investigation of the effects of a phenylalanine-deficient diet* in normal infants. Specifically, an attempt is made to correlate changes in amino acid metabolism with bone marrow changes. Previous reports of normal infants receiving diets deficient in phenylalanine make no mention of bone marrow changes or anaemia (Holt, 1938). There is one report of a megaloblastic type of erythroid maturation in a child with phenylketonuria on a phenylalanine-deficient diet (Bostyn and Parry, 1962). Sherman et al. (1964) reported anaemia and red cell precursor vacuolisation in a premature infant with phenylketonuria treated with a low phenylalalanine diet.

SUBJECTS: Six healthy premature infants between 12 and 39 days old (JO, SP, JD, GA, ET, RU) were studied while they were in the hospital waiting to attain routine discharge weight. Their weights at the time of the study ranged from 1.6 to 2.4 kg. One 2.3 kg newborn infant (RU) with an extensive myelomeningocele, 2 full term newborns weighing 5.2 kg (SU) and 4.5 kg (SK), and a 6 month old infant weighing 7.3 kg (JO) were also studied.

*Lafenolac, kindly supplied by Nestle Johnson & Johnson, Inc.
PART II

CLINICAL AND EXPERIMENTAL FINDINGS

Study 1: Effect of Phenylalanine Deficient Diet on Bone Marrow and Amino Acid Metabolism in Normal Infants.

The present study is an investigation of the effects of a phenylalanine-deficient diet* in normal infants. Specifically, an attempt is made to correlate changes in amino acid metabolism with bone marrow changes. Previous reports of normal infants receiving diets deficient in phenylalanine make no mention of bone marrow changes or anaemia (Holt, 1958). There is one report of a megaloblastic type of erythroid maturation in a child with phenylketonuria on a phenylalanine-deficient diet (Royston and Parry, 1962). Sherman et al, (1964) reported anaemia and red cell precursor vacuolisation in a premature infant with phenylketonuria treated with a low phenylalanine diet.

SUBJECTS: Six healthy premature infants between 12 and 39 days old (FO, SP, JO, HA, HY, HU) were studied while they were in the hospital waiting to attain routine discharge weight. Their weights at the time of the study ranged from 1.6 to 2.4 kg. One 2.3 kg newborn infant (RU) with an extensive myelomeningocele, 2 full term newborns weighing 3.2 kg (SU) and 4.5 kg (FR), and a 4 month old infant weighing 7.5 kg (OB) were also studied.

*Lofenalac, kindly supplied by Mead Johnson Co., Evansville, Ind.
DIETS: Diet I was a proprietary cow's milk preparation modified by the manufacturer to simulate human milk.* It was reconstituted to contain 1.5% protein, 7.0% carbohydrate, and 3.7% fat. It contained adequate supplements of vitamins and minerals.

Diet III was a proprietary casein hydrolysate preparation from which the phenylalanine had been removed and to which certain amino acids had been added. Supplements of vitamins and minerals had been added as they had to Diet I. Fat and carbohydrate had been added so that the fat content of the phenylalanine-deficient feeding mixture was 2.7%, carbohydrate 8.5%, and nitrogen equivalent to protein 2.25.

The manufacturer's analysis records that each 32 ounces of the feeding mixture contains:

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vit. A</td>
<td>1500 U.S.P. units</td>
</tr>
<tr>
<td>Vit. D</td>
<td>400 &quot;</td>
</tr>
<tr>
<td>Vit. E</td>
<td>5 international units</td>
</tr>
<tr>
<td>Vit. C</td>
<td>30 mg</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.46 &quot;</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.05 &quot;</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.03 &quot;</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1.8 &quot;</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>4.0 &quot;</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5 &quot;</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>3.2 &quot;</td>
</tr>
<tr>
<td>Vit. B</td>
<td>4.5 μg</td>
</tr>
<tr>
<td>Choline Cl</td>
<td>150.0 mg</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>15.0 mg</td>
</tr>
</tbody>
</table>

Diet II was the same proprietary phenylalanine-deficient diet complemented by L-phenylalanine added in amounts of 100 mg per kg of body weight per day.

*Enfamil, Mead Johnson Co., Evansville, Ind.
DESIGN OF DIETARY SEQUENCES: Eight of the first 9 studies involved 48-hour periods of ingestion of the deficient diet. In the ninth, FR ingested the deficient diet for 96 hours. Three infants (OB, SP, FO) were studied in the sequence of control Diet I, Control Diet II, deficient Diet III, and then Diet II. Two of these infants (SP₁, FO₂), as well as 4 others (HA, HY, JO, FR), were given the deficient diet immediately after Diet I.

Studies were carried out with SU, RU, and FR as subjects, in which the infants ingested Diet II for 6 to 14 days and then Diet III for 4 to 5 days.

In various ancillary studies, supplements of other amino acids were added to the daily diets as follow:

200 mg of L-methionine to Diet I for 3 days (RU);
150 mg of L-valine and 1.0 g of L-valine to Diet II for 2 days each (FO, JO);
150 mg of L-alanine and 1.0 g of L-alanine to Diet II for 2 days each (SP, HU);
1.0 g of L-alanine to Diet III for 4 days and for 2 days (HU, JO);
0.5 g of L-valine to Diet III for 2 days (JO).

Studies were also carried out in HY when he ingested only 10% glucose in water for 24 hours.

The feeding mixtures were offered to the infants 4 to 8 times a day in ad lib volumes. The volume of intake was estimated from the
weights of the bottles before and after a feeding. During each study, the infants received no other nutritive materials.

**CLINICAL AND LABORATORY PROCEDURES:** Infants were weighed at the beginning of each 24-hour period. Urinary collections were made with constant urinary drainage, using external devices. Venous blood specimens for serum amino acid measurements and bone marrow material for examination were obtained at the end of diet periods. The last few amino acid studies, as noted below, utilized plasma from heparinized venous blood. Bone marrow was aspirated from the iliac crests and the tibias. Bone marrow specimens were also examined by an independent assessor who was unaware of the clinical or experimental state. All bone marrow specimens were stained with Wright-Giemsa stain with Prussian Blue.

Microhematocrit and reticulocyte counts were performed by standard methods every 2 days during the period of study. Free amino acids in serum or plasma were measured by the Technicon Amino Acid Analyzer adaptation of the technique of Spackman, Stein and Moore (1958). Measurements were made of proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. Total urinary free amino acids excreted by FO, FR, SU, RU, HU were determined by ion exchange on Dowex-50 columns using the ninhydrin end point of Moore and Stein (1954), and the individual amino acids...
excreted by all patients (except JO) were identified by 2 dimensional paper chromatography using the butanol-acetic acid-water system and the phenol-ammonia-water system (Smith, 1960 and Morrison, 1953). Estimates of change in individual amino acid excretion were made from the area and intensity of color of the spots on one dimensional paper chromatography. Urinary creatinines were measured, and all aliquots applied to paper represented a volume containing 4 \( \mu g \) of creatinine (Peters, 1962).

**RESULTS:** Infants took the diets well. The mean daily intake was 245 ml/kg, and no significant difference was noted between the various diets. There were no changes in body weight, hematocrit, or reticulocyte count related to dietary changes. However, diarrhea, with excoriation of buttocks, was frequently noted when the children received either Diet II or III, both of which contained the casein hydrolysate preparation. All the infants thrived save for the child with the myelomeningocele whose study had to be interrupted when meningitis due to *P. aeruginosa* supervened. Concentrations of individual amino acids in the diets are recorded in Table III.

The serum amino acid concentrations for the first 9 studies are shown in Table IV. The weight and age of the infants are also indicated. It may be seen that the initial ingestion of the casein hydrolysate mixture in either Diet II or Diet III was accompanied by an increased serum concentration of acids with the exception of phenylalanine. The tyrosine concentrations varied unpredictably. Urinary
### TABLE III

Concentrations of Individual Amino Acids in Diets I and III.
(Modified cow's milk feeding mixture and phenylalanine-deficient casein hydrolysate feeding mixture.)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Diet I Modified cow's milk* (mg/100 ml formula)</th>
<th>Diet III Phenylalanine-deficient† (mg/100 ml formula)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>202</td>
<td>280</td>
</tr>
<tr>
<td>Proline</td>
<td>89</td>
<td>98</td>
</tr>
<tr>
<td>Glycine</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Alanine</td>
<td>43</td>
<td>105</td>
</tr>
<tr>
<td>Valine</td>
<td>38</td>
<td>179</td>
</tr>
<tr>
<td>Methionine</td>
<td>16</td>
<td>71</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>26</td>
<td>99</td>
</tr>
<tr>
<td>Leucine</td>
<td>87</td>
<td>277</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>50†</td>
<td>110</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>36</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>67</td>
<td>415</td>
</tr>
<tr>
<td>Histidine</td>
<td>22</td>
<td>51</td>
</tr>
<tr>
<td>Threonine + Tryptophane</td>
<td>Manufacturer's analyses 78</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

* EnfamilR.
† LofenalacR.
† Acid hydrolysis of Diet I resulted in marked decomposition of tyrosine and the value given was extrapolated to zero time. No correction has been made for decomposition of glutamic acid and proline (Hirs, 1954).
## TABLE IV

Effect of 48 Hour Dietary Periods on Morphologic Appearance of Bone Marrow and Concentration of Amino Acids in Serum or Plasma.

Diet I = modified cow’s milk feeding mixture. Diet II = Base III plus 100 mg per kg per day of L-phenylalanine.
Diet III = phenylalanine-deficient casein hydrolysate feeding mixture.

<table>
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<tr>
<th>Subject Wt, kg Day Diet</th>
<th>No. of vacuoles in bone marrow</th>
<th>Amino Acid, Micromoles/L</th>
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<tr>
<td></td>
<td>Vacuoles</td>
<td>Glycine</td>
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<td>HY 1.6 2 I</td>
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<tr>
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</tr>
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<tr>
<td>34 days 2.4 I</td>
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<td>37 days 2.2 I</td>
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<tr>
<td>3 days 1.8 II</td>
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<td>4 mos. 7.5 4 III+++</td>
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<td>7.5 6 II</td>
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<th>Glycine</th>
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<th>Valine</th>
<th>Methionine</th>
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<th>Leucine</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
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<th>Histidine</th>
<th>Arginine</th>
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<tr>
<td>Mean</td>
<td>I</td>
<td>178 ± 13.3</td>
<td>196 ± 11.2</td>
<td>317 ± 28.5</td>
<td>171 ± 7.2</td>
<td>27 ± 1.11</td>
<td>46 ± 2.6</td>
<td>110 ± 7.0</td>
<td>99 ± 12.1</td>
<td>45 ± 2.7</td>
<td>121 ± 7.4</td>
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<td>194</td>
<td>62</td>
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<td>III</td>
<td>258 ± 14.7</td>
<td>238 ± 27.2</td>
<td>520 ± 41.2</td>
<td>332 ± 32.2</td>
<td>166 ± 25.8</td>
<td>67 ± 4.4</td>
<td>169 ± 20.9</td>
<td>70 ± 12.4</td>
<td>10 ± 2.2</td>
<td>356 ± 38.7</td>
<td>92 ± 9.9</td>
<td>91 ± 7.5</td>
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*P value = III-III < 0.01 NS 0.01 < 0.01 0.01 0.01 NS 0.01 NS

* 150 mg per day L-alamine added to Diet II in last period.
* 150 mg per day L-valine added to Diet II in last period.

* Plasma utilized for amino acid determinations.
amino acid excretion increased within 8 hours of beginning these diets and continued throughout the period, paralleling the increase in serum concentrations. The increase in total amino acid excretion was accounted for largely by increases in urinary valine, alanine, glycine, methionine, and histidine. No further increase in either serum or urinary amino acids was seen when the diet was changed from Diet II to Diet III. Indeed, there were some decreases in serum concentrations.

At the end of the period of dietary deficiency, serum phenylalanine concentrations had fallen significantly in each patient. Final values were less in the smaller children (4-9μmols/l vs. 18 and 22μmols/l). Phenylalanine concentrations after 48 hours of Diet III were slightly lower in SP and RO when Diet III was preceded by Diet I (6 and 4 micromoles per liter) than when Diet III was preceded by Diet II (9 and 8 micromoles per liter). (Fig. 7).

Two days on Diet III (phenylalanine-deficient) were sufficient to produce significant bone marrow erythroid vacuolizations in the 5 premature subjects when this diet was preceded by Diet I (modified cow's milk). The number of erythroid precursors with vacuolizations and the number of vacuoles in each immature red cell varied from subject to subject, with the bone marrows of SP2 and FO2 being the least involved (Fig. 8). The Wright-Giemsa stained bone marrow specimens revealed similar patterns regardless of the site from which the marrow was aspirated. In general, the cellularity of the specimens was considered normal throughout the period of study. The most striking findings were
EFFECT OF PHENYLALANINE POOR DIET

CONTROL DIET
○ MOD. MILK
○ DEF. DIET + P.A.

SERUM PHENYLALANINE

50
40
30
20
10

MICROMOLS /L

MOD. MILK
DEF. DIET
DEF. DIET
DEF. DIET

○ ○ ○ ○ ○ ○ ●
○ ○ ○ ○ ○ ○ ○ ○

Fig. 7

Subject ○ with normal white cell precursors
○ with normal white cell precursors
● with no white cell precursors
× with no white cell precursors

52.
Fig. 8

Subjects • with vacuoles in Red and White cell precursors
○ with vacuoles in White cell precursors
× with no vacuoles
the presence of vacuolizations in the erythroid elements, most numerous in the erythrogonies and normoblasts A and less numerous in normoblasts B. The vacuoles were cytoplasmic, round, and of variable size and number in a given cell. They were never seen in the more mature erythroid precursors (normoblasts B) when absent in the erythrogonies and normoblasts A. The maturation of the erythroid series was always normoblastic; megaloblasts were not seen, and there was no evidence of a maturation arrest. Maturation of the myeloid series was also normal without maturation arrest, and the megakaryocytes were present in normal numbers and appeared to be making platelets. Myeloblasts, promyelocytes, myelocytes, and metamyelocytes also contained cytoplasmic vacuoles. These vacuoles were round and of variable size within a cell. In general, they were slightly larger and less numerous in any given cell than were the vacuoles in the erythroid precursors. The largest vacuoles were noted in the eosinophilic myelocytes and metamyelocytes. Vacuoles were not found in the megakaryocytes, reticuloendothelial cells, and lymphocytes. Free iron was demonstrated in the bone marrow by the Prussian Blue stain. Fig. 9 depicts the typical cytoplasmic erythroid vacuolizations produced by feeding Diet III for 2 days after the infant had been receiving Diet I.

Erythroid vacuolization was not seen when FO and SP received Diet II for 2 to 4 days and then Diet III for 2 days. Nor were erythroid vacuoles seen in the bone marrow of the 2 full term infants FR and SU.
Control

Deficient Diet

Deficient Diet + Phenylalanine
and the older child OB after they had ingested the deficient diet for 2 to 4 days in various experiments. Definite vacuoles morphologically indistinguishable from those in Fig. 9 did appear in the cytoplasm of the myeloblasts, myelocytes, and metamyelocytes under these experimental conditions in PO, SP, OB, and FR.

Bone marrow and blood examinations were also carried out after only 24 hours on Diet III following Diet I in infant HA. The findings were the same as seen in all 5 premature infants after 48 hours of deficiency although they were less marked. Two days of Diet II following the deficient diet sufficed to revert the bone marrow to normal morphologic appearance in all cases although the serum phenylalanine concentrations had not fully returned to control values in each instances.

Three infants (SU, RU, FR) were fed Diet II for 6 to 14 days and then Diet III for 4 to 5 days in order to show more clearly the effect of the ingested load of amino acids upon serum and urinary concentrations and differentiate it from the dietary deficiency of phenylalanine per se. Serum and urine concentrations of amino acids for these 3 infants are given in Table V. The increases in amino acid concentrations in serum and urine which were found in all infants receiving Diet II occurred as expected in these studies. When the diets fed these infants were changed from Diet II to Diet III, nonsignificant effect on amino acid concentrations other than serum phenylalanine was demonstrable. Vacuoles in bone marrow red
TABLE V
Concentrations of Amino Acids in Plasma During Prolonged Periods of Ingestion of the Deficient Diet Adequately Complemented with Phenylalanine and of Subsequent Ingestion of Deficient Diet Alone. Urinary excretion of total free amino acids are also recorded.

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<th>Subject</th>
<th>Weight (kg)</th>
<th>Day</th>
<th>Diet</th>
<th>Prolin</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Valine</th>
<th>Methionine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Tyrosine</th>
<th>Fallylaude</th>
<th>Lysine</th>
<th>Histidine</th>
<th>Arginine</th>
<th>Urinary free amino acids (100 mg)</th>
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* Meningitis diagnosed.
cell progenitors of RU were seen after 5 days of Diet III but not after 2 days in this sequence. SU developed no marrow changes, and FR developed vacuoles in only the myeloid cells.

Three infants (SP, RU, JO) were given supplemental amounts of 150 mg, 1g, and 1g of alanine per day respectively and two infants (FO, JO) 150 mg and 0.5 g of valine per day during control periods of Diets I or II and during periods on the deficient diet to determine whether there was any toxic effect on bone marrow. No vacuoles or other toxic effects of the amino acids were demonstrable nor were any seen in RU's bone marrow when L-methionine, 200 mg per day, was added for three days to Diet I. Serum levels of the administered amino acid were increased after these supplements.

No significant changes in bone marrow specimens or amino acid concentrations in infant HY were demonstrated following a 24-hour period in which he ingested only carbohydrate and water.

**COMMENT:** The striking features of this study were the rapidity with which the premature infants showed evidence of phenylalanine deficiency with depression of serum phenylalanine concentration and the rapid development of vacuoles in the cytoplasm of the red cell precursors in the bone marrow. The bone marrow lesions were as rapidly reversed when phenylalanine was added to the diet.
In none of these infants have anaemia, change in circulating reticulocytes, or untoward symptoms been seen in the short periods in which they received the deficient diet nor in an observation period of several months thereafter.

Diet I contains less than optimal quantities of phenylalanine for the needs of premature infants. On the other hand, the greater tyrosine content of Diet II might have made the infants more resistant to phenylalanine deficiency since an excess of tyrosine can accentuate the increased intake of other amino acids in the deficient diet. A load effect was clearly demonstrated with marked increase of serum concentrations of methionine, valine, alanine, and lysine and lesser increases of other amino acids when infants ingested either Diet II or III. This was accompanied by increased urinary excretion of these amino acids. It is likely that much of the excess amino acid in the urine was of the D-form (Schendel et al., 1959) but this was not determined. The effect of the increased intake of amino acids was sufficient to obscure any possible effect of phenylalanine deficiency per se on amino acid concentrations.

Supplemental ingestion of methionine, alanine and valine produced no apparent bone marrow or haematologic effects. Therefore, it is unlikely that the bone marrow changes noted can reasonably be attributed to other than phenylalanine deficiency.

Premature infants, RU, FO, and SP did not develop cytoplasmic vacuoles in cells of the erythroid series after 48 hours of the deficient diet when the preceding control diet had been Diet II. RU continued to be fed Diet III for another 3 days and then had eryth-
roid vacuoles in his bone marrow. This inhibition of vacuole formation may have been the result of greater phenylalanine intake with Diet II when with Diet I (125 mg per kg per day vs. 90 mg per kg per day).

If this were so, it would suggest that cow's milk modified as in Diet I contains less than optimal quantities of phenylalanine for the needs of premature infants. On the other hand, the greater tyrosine content of Diet II might have made the infants more resistant to phenylalanine deficiency since an excess of tyrosine can reduce the daily requirements for phenylalanine of the older individual. The age of these premature infants prevents acceptance of this speculation although the increased tyrosine intake might have been important in the resistance of the full term and older infants to the effects of the phenylalanine-deficient diet.

All subjects except SU developed vacuoles in their white cell precursors after 48 hours of the deficient diet. The presence of myeloid vacuoles in the absence of erythroid vacuoles may reflect the more rapid rate of turnover of white cells. On the other hand, vacuolization of mature granulocytes is seen in a variety of bacterial infections (Ponder and Ponder, 1942). Some support for the hypothesis that longer periods of deficiency are required for development of erythroid vacuoles is derived from the observation that erythroid vacuoles never were seen in these studies in the absence of myeloid vacuoles.
The bone marrow changes may be reversed before the serum phenylalanine concentrations are fully returned to control values when the infants are given phenylalanine supplements. Although the concentrations of amino acids tended to be greater when serum rather than plasma was used, the changes noted were comparable. This was not unexpected since it has previously been reported that the concentration of total amino acids is greater in serum than in the comparable plasma (MacFayden, 1942). This same relationship has been found to hold for individual amino acids (Salii et al., 1961; Rosenbach et al., 1950; and McCurdy, 1961). The relation of these early, reversible lesions to later, more serious bone marrow depression is unknown. Similar cytoplasmic vacuoles in the erythrocytes have been reported in erythroblastoid myelosis (Guadino, 1961), acute alcoholic (McCary et al., 1962), kwashiorkor (Tey and Leidi, 1964), and in an infant with phenylketonuria receiving a phenylalanine deficient diet (Sherman et al., 1964). The lesions in the infant receiving this diet were reversed by the addition of phenylalanine. An attempt is made to study the effect of the administration of phenylalanine upon the vacuolization of the erythrocytes produced by chloramphenicol. The study was also designed to correlate the amino acid content of blood and urine with the bone marrow changes.

**SUBJECT:** Chloramphenicol was administered to 9 children for treatment of a variety of infections. These included meningitis,
Study 2:

a) Effect of Chloramphenicol on Bone Marrow and Amino Acids in Infants.

b) Effect of Ingestion of Phenylalanine on the Toxic Action of Chloramphenicol on Bone Marrow.

The major toxic effect of chloramphenicol is the production of bone marrow arrest and aplastic anaemia. Early in the development of chloramphenicol toxicity, vacuoles appear in the erythroid and myeloid precursors in the bone marrow (Saidi and Wallerstein, 1960; Saidi et al, 1961; Rosenbach et al, 1960; and McCurdy, 1961). The relation of these early, reversible lesions to later, more serious bone marrow depression is unknown. Similar cytoplasmic vacuoles in the erythrogones have been reported in erythraemic myelosis (Di Guglielmo, 1962), acute alcoholism (McCurdy et al, 1962), kwashiorkor (Foy and Kondi, 1964), and in an infant with phenylketonuria receiving a phenylalanine deficient diet (Sherman et al, 1964). The lesions in the infant receiving this diet were reversed by the addition of phenylalanine. An attempt is made to study the effect of the administration of phenylalanine upon the vacuolization of the erythrogones produced by chloramphenicol. The study was also designed to correlate the amino acid content of blood and amino acid excretion in the urine with the bone marrow changes.

SUBJECTS: Chloramphenicol was administered to 9 children for treatment of a variety of infections. These included shigellosis with diarrhea, septicemia and urinary infection due to Escherichia in a parenteral or oral dose of 50 to 100 mg. per kilogram of body weight per day.
with diarrhea, septicaemia and urinary infection due to Escherichia coli, meningitis due to Haemophilus influenzae Type B and salmonella in a patient with sickle-cell disease. The physician responsible for the patients' care decided to institute and to discontinue the antibacterial therapy. Chloramphenicol was administered in doses of 50 to 100 mg. per kilogram of body weight per day parenterally or by mouth. Patients ranged in age from a newborn infant, three days old, to an eight year old girl and in size from 1.7 to 21.0 kg. Table VI lists the illnesses, ages, weights and sex. During the last six days of chloramphenicol treatment, B.A. ingested, in addition to his diet, 1 gm. of L-alanine per day for two days and then 100 gm. of L-phenylalanine per kilogram of body weight for the last four days. Similarly, the diets of B.R., S.I. and L.O. were supplemented with the same dose of phenylalanine for the last two, three and six days of chloramphenicol treatment respectively.

The newborn infants were given a diet consisting solely of a proprietary milk mixture modified by the manufacturer to simulate human milk.* The older infants and children took a mixed diet well except for S.I., who was sick enough that this intake was poor for the first four days of treatment, and B.A., the patient with meningitis due to H. influenzae Type B, who was maintained on intravenously administered fluids for the first week of therapy and thereafter received only cow's milk by mouth.

* In the form of Enfamil, Mead Johnson Company, Evansville, Indiana
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<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Weight (kg.)</th>
<th>Diagnosis</th>
<th>Day of Chloramphenicol Treatment</th>
<th>Day of Phenylalanine Ingestion</th>
<th>No. of vacuoles in Bone Marrow cells*</th>
<th>Erythroid series</th>
<th>Myeloid series</th>
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<td>S.I.</td>
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<td>M</td>
<td>15.0</td>
<td>Salmonella osteomyelitis; sickle-cell anemia.</td>
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* Roughly quantitated 0 to ++++.  
† 1 gm. of L-alanine given by mouth on 9th & 10th days of chloramphenicol treatment.  
‡ No chloramphenicol or phenylalanine for 3 days.
**TABLE VI (Concluded)**

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<tr>
<th>Patient</th>
<th>Day of Chloramphenicol Treatment</th>
<th>Day of Phenylalanine Ingestion</th>
<th>Proline</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Valine</th>
<th>Methionine</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Tyrosine</th>
<th>Phenylalanine</th>
<th>Lysine</th>
<th>Histidine</th>
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METHODS: Bone marrow specimens were obtained from the iliac crests or tibias before the beginning of chloramphenicol therapy, after two or three days and six to nine days of treatment with chloramphenicol and at intervals of two or three days thereafter. Bone marrows were examined after staining with Wright-Giemsa medium. An independent assessment was also made by a haematologist (J.D.S.) who was unaware of the patient's disease and of the treatment administered. At the time of bone marrow aspiration, venous blood was obtained for measurements of amino acid concentrations in serum or heparinized plasma. The Technicon Auto-Amino Acid-Analyzer modification of the method of Moore and Stein was employed. Measurements of proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine were made. Twenty-four hour collections of urine were obtained from 3 children (B.U., B.R. and T.A.). Total urinary free amino acid excretions were determined by ion exchange on Dowex-50 columns by means of the ninhydrin end point of Moore and Stein. Identification was carried out by two-dimensional paper chromatography with the use of the phenol-ammonia-water and the butanol-acetic acid-water systems. Aliquots for paper chromatography were adjusted to contain 4 microgm. of creatinine as determined by the method of Peters. Estimations of quantitative changes of individual amino acids were made from one-dimensional chromatography, the size and intensity of the stained area being used. Chloramphenicol concentrations in the blood of 4 of
the patients were measured by Dr. C. F. Weiss and Dr. C. E. Moyer, of Parke, Davis and Company, Detroit, Michigan. Microhaematocrit and reticulocyte determinations were carried out by standard methods.

**RESULTS:** All the children recovered from the infections for which they were being treated with chloramphenicol. No relation between the therapy and slight anaemia that developed in several children could be established. In S.I., the patient with sickle-cell disease and salmonella osteomyelitis, a hypoplastic crisis developed during therapy; the haematocrit fell from 28 to 14 per cent, and he was transfused with packed red cells, which returned the haematocrit to 27 per cent. At the time of the hypoplastic crisis the reticulocyte count was only 1 per cent. Three days after the end of therapy, his count rose to 5 per cent, and the bone marrow showed erythroid hyperplasia. The reticulocyte counts and appearance of smears of peripheral blood in the other patients did not alter during the study.

Table VI presents the values for amino acid concentrations in serum or plasma of the 9 subjects during the course of therapy with chloramphenicol. Also given are the times when venous-blood specimens were obtained and the bone marrow appearance at these times. In 5 of the patients (B.R., T.A., S.I., B.A. and L.O.) numerous vacuoles developed in the erythroid precursors of the bone marrow. In R.O., vacuoles developed only in rare erythrocytes. Numerous vacuoles are
occurred in the myeloid precursors of these patients. These changes were morphologically identical with those seen in the infants receiving a phenylalanine-deficient diet. B.U. and M.O., in whom erythroid vacuoles did not develop, had vacuoles in the myeloid precursors. There were no changes in the bone marrow of F.L. after nine days of chloramphenicol therapy. The appearance of the bone marrow specimen was similar regardless of the site of aspiration. In general, the cellularity of the specimens was considered normal except in the patient with sickle-cell disease mentioned above. The maturation of the erythroid series in the other patients was always normoblastic, without evidence of any arrest. The maturation of the myeloid series was normal in all specimens without maturation arrest. Megakaryocytes were present in normal numbers, and platelet production appeared normal.

The most striking findings were the presence of vacuolizations in the erythroid elements. The vacuoles were most numerous in erythrogones and normoblasts A and less numerous in normoblasts B. The vacuoles were cytoplasmic, round and of variable size. The numbers of vacuoles in each immature red cell varied from subject to subject. They were never seen in the more mature normoblasts B when absent in the erythrogones and normoblasts A.

Myeloblasts, promyelocytes, myelocytes and metamyelocytes contained similar cytoplasmic vacuoles. They were round and of varying size and, in general, were slightly less numerous in a given cell
than the vacuoles in the erythroid precursors. Vacuoles were not found in megakaryocytes, reticuloendothelial cells and lymphocytes.

The administration of chloramphenicol was discontinued by the physicians guiding the clinical therapy in B.U., R.O., M.O., F.L. and T.A. after seven to nine days of treatment. Chloramphenicol administration was continued for six more days from the time of the appearance of the vacuoles in B.A. and L.O., two more days in B.R. and three more days in S.I. These four patients were the sickest of the group. B.A. had meningitis, and S.I., osteomyelitis, and B.R. and L.O. were newborn infants with septicemia due to Esch. coli. The bone marrow changes in B.A. were not affected by the administration of alanine for two days, but the administration of phenylalanine was associated with a return of the bone marrow to normal in B.R., L.O. and B.A. and a reversal of the changes in S.I. to the point that only rare erythrogonones were seen to contain a few very small vacuoles.

Fig. 10 demonstrates photomicrographs of bone marrow specimens obtained from B.R. before chloramphenicol administration, after eight days of chloramphenicol treatment and after two days of phenylalanine administration in addition to chloramphenicol. The typical cytoplasmic erythroid vacuoles are readily seen after eight days of chloramphenicol administration but are no longer present after two days of additional chloramphenicol therapy coupled with the ingestion of L-phenylalanine, 100 mg. per kilogram of body weight per day.
Fig. 10

Control

Chloramphenicol

Chloramphenicol plus Phenylalanine
After seven and eight days of chloramphenicol therapy, the bone marrow specimens from L.O. and B.A., respectively, revealed similar vacuoles in the cytoplasm of both the red-cell and white-cell series.

After four more days (B.A.) and six more days (L.O.) of chloramphenicol therapy combined with the ingestion of 100 mg. of phenylalanine per kilogram per day, the bone marrow specimens were completely normal.

A specimen (from B.A.) obtained two days after the beginning of phenylalanine supplementation still contained cytoplasmic vacuoles of the erythrocytes, but the myeloid vacuolization was no longer present. L.O. showed rare erythroid vacuoles after four days of phenylalanine with no myeloid vacuoles. Only a few erythrocytes contained cytoplasmic vacuoles after the addition of 100 mg. of L-phenylalanine per kilogram per day to the diet of S.I. even though the administration of chloramphenicol was continued. In addition, the vacuoles still present not only were fewer in number per involved cell but also were much smaller in size. At this time, no vacuoles were found in the myeloid series.

Table VI shows that the measured serum and plasma amino acid concentrations increased when chloramphenicol was administered. This increase was generalized, but only alanine and lysine concentrations rose significantly. The increase in circulating amino acid concentrations was greatest in the youngest infants (B.R., M.O., B.U., L.O. and R.O.). When the bone marrow changes improved after supplementation of the diet with phenylalanine the concentration of most of the amino acids decreased or remained the same whether or not supplementation continued to increase significantly.
acids decreased or remained the same whereas alanine concentrations continued to increase significantly.

Urinary amino acid excretion increased rapidly with chloramphenicol therapy and then tended to reach a plateau paralleling serum or plasma levels. This increased excretion was made up in large part of alanine, glycine, proline and histidine. A perceptible increase of phenylalanine excretion was also associated with chloramphenicol administration. Urinary amino acids were examined in only 1 patient treated with phenylalanine after the bone marrow lesion had been demonstrated. In this case, the administration of phenylalanine did not seem to make any difference in the urinary pattern of total free amino acid or individual amino acid excretion.

Chloramphenicol concentrations in serum varied widely from patient to patient and to a lesser extent from time to time in the same patient. There was no correlation between the average or individual concentrations of chloramphenicol and the morphologic appearance of the bone marrow.

COMMENT: It seems reasonable to assume that the vacuoles seen in the erythroid precursors of the bone marrow were indeed a toxic manifestation of chloramphenicol since the other conditions in which such vacuolization has been observed - erythremic myelosis, acute alcoholism, dietary phenylalanine deficiency and kwashiorkor - need not be considered in these patients. It is possible that the vacuoles seen in the
cytoplasm of myeloblasts, promyelocytes, myelocytes and metamyelocytes were also produced by chloramphenicol. However, vacuoles may be seen in circulating mature granulocytes in infections. The correlation in time of appearance of the vacuoles in the myeloid series with that of erythroid vacuoles suggests similar pathogeneses. The myeloid vacuoles appeared sooner and, at times, disappeared more rapidly on recovery than the erythroid vacuoles, as might be expected from the more rapid rate of myeloid-cell turnover. In patients with phenylalanine deficiency the erythroid vacuoles were never seen in the absence of myeloid vacuoles.

The changes in circulating free amino acid concentrations suggest a block in the utilization of amino acids. Increased urinary excretion of these amino acids rules out the possibility that the elevations in serum or plasma concentrations were due to renal dysfunction produced by chloramphenicol. The greatest increases in circulating free amino acids were noted in the youngest children.
Study 3: Chloramphenicol and L-phenylalanine Therapy in Experimental Klebsiella Pneumoniae Infection in Mice.

The ingestion of L-phenylalanine has been shown to reverse one of the early toxic effects of chloramphenicol on human bone marrow, namely, the cytoplasmic vacuolization of erythrogones. The relationship of these vacuoles to the aplastic anaemia associated with chloramphenicol treatment is not clear. Before investigating the possibility that prophylactic administration of phenylalanine might prevent the later more serious toxic effects of chloramphenicol, it is necessary to evaluate the effect of administration of the amino acid upon the antibacterial efficacy of chloramphenicol.

The need for the study was also suggested by the possibility that the cytotoxic effect of chloramphenicol in clinical situations and the antibiotic activity of the drug might involve a common mechanism. In addition, Woolley (1950) reported that phenylalanine inhibits the antibacterial action of chloramphenicol in vitro.

MATERIALS AND METHODS: The following materials in varying combinations were injected into Swiss White male mice (25 ± 2 gm initial weight) in each test. A strain of Klebsiella pneumoniae isolated from the blood culture of a patient was used throughout. The organism was maintained and diluted in brain-heart infusion broth. Mice were infected intraperitoneally with 0.5 ml of an overnight culture diluted 10^7 for all but one experiment which used 10^6 organisms/ml with an ID_{50} of 10^3.0. The initial concentration of chloramphenicol for the test strain was 6.3 mg/ml.
culture diluted $10^{-2}$ in all but two experiments which used $10^{-4}$ dilutions. An overnight culture resulted in approximately $10^{10}$ organisms/ml with an $LD_{50}$ of $10^{-3.6}$. The minimum inhibiting concentration of chloramphenicol for the infecting strain was 6.2 $\mu$g/ml.

L-phenylalanine was injected subcutaneously in a volume of 0.4 ml suspended in 0.89 per cent sodium chloride acidified to pH 4 with 1 N HCl. Acidified saline, 0.4 ml, was used as a control solution.

In other words, the higher dose of phenylalanine is detrimental.

Chloramphenicol succinate was given subcutaneously in a volume of 0.2 ml at a site distant to that employed for the phenylalanine.

Table VII presents the data from these experiments. The results include those of the experiments with pre-loading of phenylalanine or there was no difference in host response between this group and all others where the animals were given phenylalanine simultaneously with the infecting organisms and chloramphenicol. Fig. 12 represents the antimicrobial efficacy of chloramphenicol.

Mice under light ether anaesthesia were given the intraperitoneal injection of organisms followed in rapid sequence by the subcutaneous chloramphenicol and phenylalanine or control solution in all but one test. In the latter, the phenylalanine or control solution was injected four hours before the other injections. Cages were inspected twice daily at which time the numbers of dead mice were recorded. The animals were observed for ten days, but the final mortality was reached in all experiments by 72 hours, most deaths occurring in the first 36 hours. In two experiments, the dose of chloramphenicol was varied between 10 and 70 mg/kg; in all others, the dose was 25 to 40 mg/kg.

**RESULTS:** All the infected animals that did not receive chloramphenicol died whether or not they received phenylalanine in doses
from 50 to 400 mg/kg. All non-infected animals given phenylalanine or saline with or without chloramphenicol survived.

Fig. 11 demonstrates the effect on mortality of phenylalanine in two dose ranges, 50-100 mg/kg and 300-400 mg/kg, when the dose of chloramphenicol is between 25-50 mg/kg. There is a decrease in mortality at the lower dose which is not statistically significant. At the higher range, there is a significant increase in mortality. In other words, the higher dose of phenylalanine is detrimental to the antibiotic efficacy of chloramphenicol.

Table VII presents the data from these experiments. The results include those of the experiments with pre-loading of phenylalanine as there was no difference in host response between this group and all the others where the animals were given phenylalanine simultaneously with the infecting organisms and chloramphenicol. Fig. 12 represents the same results graphically. The results are grouped in the table both by dose of chloramphenicol and by the ratio of phenylalanine to chloramphenicol dosage. The ratio is given both as mg/mg and mmol/mmol. The significance of the differences was calculated by the fourfold table, and the values of both $x^2$ and $p$ are given.

It may be seen that the addition of phenylalanine in doses approximately equimolar to the doses of chloramphenicol (0.6 - 1.3 mmol/mmol) reduced mortality significantly from that of the animals which received the same amount of chloramphenicol without supplemental phenylalanine.
PHENYLALANINE EFFECT ON KLEBSIELLA INFECTION
IN MICE RX WITH CHLORAMPHENICOL (25-50 MG/KG)

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SIG. Δ

P > .1
P < .01

Fig. 11
TABLE VII

Mouse Mortality from Klebsiella Pneumoniae Infection Treated with Chloramphenicol and L-Phenylalanine

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### PHENYLALANINE EFFECT ON KLEBSIELLA INFECTION IN MICE TREATED WITH CHLORAMPHENICOL

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Fig. 12
Doses of phenylalanine which on a molar bases were 2 to 5 times those of chloramphenicol had no significant effect on overall mortality. When only those animals which received 25 to 40 mg/kg of chloramphenicol are considered, however, these doses of phenylalanine had an adverse effect which was statistically significant ($p < .05$).

Larger amounts of phenylalanine (molar ratio to chloramphenicol of 8-20/1) markedly increased mortality.

Thus, equimolar doses of phenylalanine augmented the beneficial effects of chloramphenicol while 8 to 20 times as much phenylalanine inhibited the antibiotic effect. Intermediate doses of phenylalanine (2-5 times those of chloramphenicol) probably inhibited antibiotic effect.

The inhibition of chloramphenicol's effectiveness by the larger doses of phenylalanine is consistent with the in vitro studies of Woolley. Phenylalanine is known to directly inhibit antibody response.

COMMENT: The interaction of phenylalanine and chloramphenicol in relation to mammalian protein synthesis and bacterial growth is one of great interest. Its significance in therapeutic situations remains to be proven. Woolley (1950) proposed that the antibiotic could be regarded as a metabolic analogue of phenylalanine. Jardetsky (1962, 1964) and others have shown that chloramphenicol will block the incorporation of amino acids into protein at the ribosome and it may be that phenylalanine counteracts the chloramphenicol affect at this level. Whatever the final mechanism, the present study suggests that parenteral L-phenylalanine given in a dose equivalent to that
of the chloramphenicol will not inhibit the drug's antibacterial activity in the experimentally infected mouse. Indeed, there is a potentiation of the host resistance to Klebsiella infection. This is not a direct effect of the amino acid since phenylalanine by itself had no demonstrable antibiotic activity. A preferential uptake of phenylalanine by the host cell might block the site of activity of chloramphenicol on the host cell and yet allow a more effective antibacterial concentration of chloramphenicol outside the host cells. Another possible explanation is that this dose of phenylalanine might prevent the inhibitory effect which chloramphenicol has on some protein dependent body defence mechanisms such as antibody formation. This possibility will be considered later in the thesis.

The inhibition of chloramphenicol's effectiveness by the larger doses of phenylalanine is consistent with the in vitro studies of Woolley. L-phenylalanine is known to directly inhibit antibody response (Ryan, 1965), so this may be another factor in the diminished host response to infection.

The relevancy of these findings to infections with other organisms or to human infections remains to be established. In the previous study (2b), phenylalanine was given to patients only after three to eight days of effective chloramphenicol therapy. The results presented, however, suggest that further investigation of the combined use of the phenylalanine and chloramphenicol in clinical situations would be both worthwhile and appropriate.
Study 4: Effect of Phenylalanine Deficient Diet and Chloramphenicol on the Bone-marrow of young Rats.

In an attempt to identify the nature of the cytoplasmic vacuoles, a variety of staining techniques for fat, tyrosine, desoxyribonucleic acid and ribonucleic acid were tried on the bone marrow slides from phenylalanine deficient infants and infants treated with chloramphenicol. The vacuoles failed to take any of the stains tried. It was felt that the vacuoles might contain biosynthetic precursors of protein and nucleoprotein so an attempt was made to introduce tritiated amino acids and nucleic acid and see if these substances would localise in the vacuoles.

METHODS: Male and female rats of the Sprague-Dawley strain, 1 month old and weighing 70 G ± 5 G, were used. The low phenylalanine diet employed was Cymogram*, which contains only 10 mg. phenylalanine per 100 G of preparation. The phenylalanine requirement of the rat is only 10 mg./kg. body weight/day. A control diet was the same casein hydrolysate with added L-phenylalanine. Rats were force-fed by plastic gastric tube twice daily and had free access to water.

Chloramphenicol was administered as chloromycetin succinate by subcutaneous injection at a dose equivalent to 400 mg./kg. body weight/day. Control animals were injected with a like volume of N saline. Rats were weighed daily and at the eighth day when the experiment was ended.

Twenty-four hours before the animals were sacrificed, tritiated alanine, phenylalanine or uridine was injected by the tail vein.

In all experiments, several groups of animals, each of the same sex, age and weight, were used.

DIETS: Diet A was the casein-hydrolysate low phenylalanine diet. Diet B the same with a complement of L-phenylalanine.

The animals were sacrificed at the eighth day, having been anaesthetised with intraperitoneal pentobarbitone, then exsanguinated by collecting blood from the abdominal aorta. Bone-marrow smears were obtained from the split femora.

RESULTS: There was a very high mortality from the force-feeding of the deficient diet and the groups of animals were combined so that the results are given for 15 survivors in each group in Table VIII. Haemoglobin, haematocrit and reticulocyte values showed no significant differences in the 4 groups. Chloramphenicol blood concentrations were zero 3 hours after injection of the drug.
### TABLE VIII

<table>
<thead>
<tr>
<th></th>
<th>Diet A</th>
<th>Diet A &amp; Chloramphenicol</th>
<th>Diet B</th>
<th>Diet B &amp; Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Mortality at 8 days</td>
<td>67</td>
<td>53</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Marrow</td>
<td>Hypocellular fatty</td>
<td>Some increase in fat Normal cellularity</td>
<td>Normal Normal</td>
<td></td>
</tr>
<tr>
<td>% age weight change per day</td>
<td>21% decrease</td>
<td>18% decrease</td>
<td>32% increase</td>
<td>20% increase</td>
</tr>
</tbody>
</table>
It can be seen (Table I) that there was mortality when diet A was fed alone and when combined with chloramphenicol. Fig. 13 compares the appearance of an animal from Diet A group with one from Diet B. In spite of the similar appearance, poor hair and general appearance,

unfortunately there was no infiltrate of the red cells present. Reticuloendothelial cell localization of the radioactive cells and there was no localization of the reticuloendothelial cells. The control diet was an isocaloric but nutritionally complete diet (Diet B) for 8 days.

Fig. 13

Animal on the Left was force-fed a phenylalanine deficient diet (Diet A) for 8 days. Control animal on the Right was a litter-mate, force-fed an isocaloric but nutritionally complete diet (Diet B) for 8 days.
It can be seen (Table VIII) that there was a mortality when Diet A was fed alone and was somewhat lower when combined with chloramphenicol. Fig. 13 compares the appearance of an animal from Diet A group with one from Diet B. It shows the runted appearance, poor hair and general wasting.

Unfortunately, vacuoles were not produced in the cytoplasm of the red cell precursors in any of the marrows examined. Reticuloendothelial cells in both the group A diet and group A diet with chloramphenicol did show vacuoles. No particular localisation of the radioactive amino-acids or uridine could be determined and there was no localisation to the few vacuoles present in some cells of the reticuloendothelial system.

COMMENT: The high mortality of the force-fed deficient diet group is in keeping with the findings of Sidransky and Verney (1964). Failure to produce vacuoles with chloramphenicol in the rat is probably the result of the very rapid excretion of this drug by the rat. The choice of animal for this experiment was unfortunate. Monkeys of the Rhesus type have produced vacuoles following chloramphenicol therapy and would probably be the animal of choice for this experiment.
Study 5: Management and Progress of Two Phenylketonuric Infants Diagnosed Early.

The application of the Guthrie (1961) screening test in the neonatal period has greatly facilitated the early diagnosis of phenylketonuria. Two such infants, where the diagnosis was made by this technique, had the diagnosis confirmed at the eighteenth and twenty-third days of life respectively. The following is a description of their response to a restricted dietary intake of phenylalanine.

SUBJECTS: J.M. was diagnosed as having phenylketonuria at eighteen days of age. He is a white male infant born normally at term with a birth weight of 2.8 kg. His history and family history are given in Appendix I.

B.G. was diagnosed as having phenylketonuria at twenty-three days of age. He is a white male infant born prematurely at seven months gestation with a birth weight of 1.75 kg. His history and family history are given in Appendix I.

METHODS: The initial dietary management with a low phenylalanine diet (Lofenalac - Mead Johnson) was that generally recommended (Hsia et al, 1958). Sufficient of this formula for caloric and
fluid requirements was given and the phenylalanine intake adjusted to 20 mg/kg body weight per day.

Blood amino acid concentrations were determined on 0.2 ml. samples of capillary whole blood by column chromatography. See Appendix II for a full description of the method.

Where low blood concentrations of phenylalanine indicated that an increase of dietary phenylalanine was required, this was achieved by adding small increments of pure L-phenylalanine to the Lofenalac diet. When the required amount of additional L-phenylalanine, which maintained a blood concentration of 1-2 mg per 100 ml had been determined, the volume of whole cow’s milk which would supply this amount of amino acid was estimated and the diet so complemented.

Bone marrow was aspirated from the iliac crest and stained with Wright-Giemsa stain. These specimens were also examined by a haematologist (J.D.S.), an independent assessor, who was unaware of the patient’s clinical state.

Microhaematocrit and reticulocyte counts were performed by standard methods at regular intervals throughout the periods of study.

J.M. - Management and Progress: The blood phenylalanine concentration at 18 days of life was 26.7 mg per 100 ml and the
decision to start therapy was made after full assessment. A low phenylalanine diet which allowed a phenylalanine intake of 20 mg/kg/day was commenced. After two days on this regimen, the blood phenylalanine was 9.2 mg/100 ml, and after five days, values of less than 0.1 mg/100 ml were reached. At this point in time, vacuolization of the cytoplasm of the red cell precursors in the bone marrow became apparent (Fig. 14). These changes persisted while blood levels of phenylalanine were below 0.1 mg/100 ml (6-9 μmol/litre). As with the normal infants on this diet described in Study 1, there were elevated blood levels of alanine, lysine, valine and methionine. While the phenylalanine concentration remained at a low level, L-alanine, 1 G per day, was fed in addition to the low phenylalanine diet for four days. The blood levels of alanine increased as a result but the phenylalanine concentration remained low and the marrow vacuoles persisted. Lysine, 400 mg per day, Valine, 400 mg/day and Methionine, 200 mg/day, fed each in turn for 3 days similarly failed to eradicate the vacuoles present in the red and white cell precursors.

Increments of pure L-phenylalanine were gradually added to the diet and it finally required a phenylalanine intake of 50 mg/kg per day to maintain the blood phenylalanine concentration between 1-2 mg per 100 ml. at which time the bone marrow reverted to normal and there was a marked reticulocyte response. The quantity of whole cow's milk required to supply the additional phenylalanine was
calculated and these show a rise in the haemoglobin in place of the pure phenylalanine diet.

The loss of red blood cells and capillary leakage of whole blood are shown in Fig. 14. The rise in the intake of 40-50 mg. phenylalanine per day maintain an adequate adult intake of iron, riboflavin, and vitamin A. Within 4-5 days, the increase in haemoglobin is noticeable, and after a week, the increase is prominent and the red blood cells are increased. During the fourth day, red blood cells are counted in the body, and the increase is evident.

Fig. 14

A psychological evaluation of the patient was done using tests.
calculated and this volume added to the low phenylalanine diet in place of the pure L-phenylalanine.

The weight chart, dietary intake of phenylalanine and capillary whole blood phenylalanine concentrations for the first year of life are shown in Fig. 15. It can be seen that a dietary intake of 40-50 mg. phenylalanine/kg per day is necessary to maintain an adequate blood level of the amino acid.

Haemoglobin values were slow to rise in spite of additional iron, riboflavin, vitamin B₁₂, folic acid, vitamin C and vitamin E supplements. At two months of age, his haemoglobin was 7.0 G/100 ml. blood. When the additional dietary phenylalanine was introduced, the marrow vacuoles resolved and within a period of 19 days, the haemoglobin had risen to 8.4 G/100 ml. There is little doubt that during the period from the twenty-third day of life until the sixty-fourth day, the blood phenylalanine concentrations were somewhat below the optimum value.

Between the tenth and eleventh month of life, the mother relaxed the diet and it proved impossible to estimate the intake of phenylalanine. When strict dietary control was re-introduced, blood phenylalanine concentrations quickly returned to the normal range. Blood tyrosine and other amino acid concentrations were never outside the accepted range of normal.

A psychological evaluation at six months of age, using Gesell
PHENYLKETONURIA

DIETARY MANAGEMENT

Dietary intake Phenylalanine
mg/kg/day

0 10 20 30 40 50 60

PHENYLALANINE (mg/100ml Blood)

0 2 4 6 8 10 12

AGE (months)

Percentile

% loss of diet control

Fig. 15
Developmental Schedules gave a developmental level of approximately
20 weeks and the electroencephalograph at this time was normal.

A repeat assessment at 12 months of age showed a developmental
level of about 10 months while his weight was approaching the 50th
percentile for his age and the haemoglobin was 11.5 G/100 ml. The
electroencephalograph was again normal.

B.C. - Management and Progress: At twenty-three days of age,
the patient weighed 1927 G and had a blood phenylalanine concentration
of 42 mg. per 100 ml. He, at this time, started a low phenylalanine
(Lofenalac) diet which allowed a daily intake of 50 mg. phenylala-
nine per day. For three weeks, there was a reasonable weight gain
but then his weight gain slowed. The skin rash which had been
present from about the 6th day of life became suddenly much worse
with intense erythema and desquamation. He became extremely
irritable and increasingly pale. His haemoglobin was 8.2 G with a
haematocrit of 28% and the red cells showed hypochromia and slightly
macrocytosis. The white blood cell count was 10,500 per cu. mm.
with a normal differential and normal platelet count. The total
red cell count was 3.2 million per cu. mm. and the reticulocyte
count was 2%. A negative Coombs test and less than 1 mg./100 ml.
bilirubin concentration, a normal serum folic acid (8.1 mG/ml.)
and serum vitamin B₁₂ (441 μG/ml.) were found at this time. The
total serum protein was 5.0 G/100 ml. with albumin 45.8%, alpha₁
globulin 9.7%, alpha₂ globulin 8.3%, beta globulin 23.6% and gamma globulin 12.5%. Serum electrophoresis and immunoelectrophoresis were normal, serum iron was 50 μG/100 ml. and serum unsaturated iron-binding capacity was 300 μG/100 ml.

The serum phenylalanine concentration at this time was less than 0.1 mg % and a bone marrow smear showed that the red cell precursors had marked vacuolization of their cytoplasm. Occasional myeloid cells, reticuloendothelial cells and plasma cells, as well as megakaryocytes, contained vacuoles. The maturation of the erythroid series was normoblastic; megaloblasts were not seen, and there was a mild increase in erythrogones and normoblasts A and B suggestive of a slight maturation arrest in this series. The maturation of the myeloid series was normal without maturation arrest and the megakaryocytes were present in normal numbers and appeared to be making platelets.

Twenty-five ml. of packed red cells was given and 150 mg phenylalanine/day was added to the diet. The infant showed a sustained weight gain from 2238 G to 2550 G in the following 9 days; the skin rash cleared and the infant was much less irritable. He was discharged home at this point with a haemoglobin of 9.4 G/100 ml. and a normal bone marrow. His mother put him on to a whole milk diet and when he returned after one week, his blood phenylalanine was 60 mg./100 ml. Strict dietary control was re-instituted with
a daily intake of phenylalanine of 200 mg. Bone-marrow vacuoles were again apparent after 5 days but resolved when the intake of phenylalanine was increased to 380 mg./day. It was found that 150 mg./kg/day phenylalanine maintained a blood level of 1-2 mg/per 100 ml. and a normal bone-marrow.

The subsequent control of dietary phenylalanine was managed by frequent estimations of blood phenylalanine concentrations. At six months of age, 100 mg/kg/day, and at 12 months, 50 mg/kg/day of phenylalanine was the required dietary intake to maintain a normal blood phenylalanine concentration.

At 16 months, he weighed 8.0 kg. (50th percentile for 7 months), his length was 70 cm. (50th percentile for 9 months) with a head circumference of 46 cm. (50th percentile for 9 months). His haemoglobin was 11 G per 100 ml. with a haematocrit of 32%.

A psychological evaluation at six months of age, using Gesell Developmental Schedules, gave a developmental level of approximately 15 weeks and the EEG at this time was normal. The repeat assessment at 12 months showed a developmental level of about 28 weeks and when examined at 16 months of age, he had reached a 50 week developmental stage. Again, the electroencephalographs were normal.

COMMENT: These two cases illustrate well the difficulties involved in controlling the dietary intake of phenylalanine. The
red cell precursor vacuoles appear directly related to low blood phenylalanine concentrations, as these changes could not be reversed by increased dietary intake of alanine, valine, lysine or methionine in J.M. They did resolve promptly when the phenylalanine intake was increased and blood phenylalanine concentrations approached normality. The anaemia similarly appears to be a direct consequence of the phenylalanine deficiency and responded dramatically to the increased dietary intake of phenylalanine.

The quantities of phenylalanine required by these infants is much in excess of that generally recommended.

Knock (1958) suggested that the small amount of tyrosine formed may be attributed to other unknown reactions or to residual activity of the genetically altered tyrosine protein. Direct assays of phenylalanine hydroxylase in the liver of seven older phenylketonuric patients, however, failed to show any enzyme activity.

Jacobsen (1961) suggested that if there were any enzyme activity in the young infants with phenylketonuria, it might be possible to enhance this activity by supplying increased amounts of the enzymic cofactor described by Kaufman (1958), Fig. 15. If this proved effective, then optimum dietary control of the infant might be more readily achieved.

Some children with phenylketonuria were studied. N.J. and L.C. are brother and sister, J.M. and J.E. were not related to each other or to N.J. Case histories are described in Appendix I and Table II summarizes the salient features.
Study 6: Folinic Acid - Its Effect on the Phenylalanine Tolerance Test in the Phenylketonuric Child.

The deficiency of the enzyme phenylalanine hydroxylase in the infant with phenylketonuria may not be absolute. Udenfriend (1953) found that some tyrosine was manufactured from C\textsuperscript{14} labelled phenylalanine fed to phenylketonuric patients. Knox (1958) suggested that the small amount of tyrosine formed may be attributed to other unknown reactions or to residual activity of the genetically distorted enzyme protein. Direct assay of phenylalanine hydroxylase in the liver of seven older phenylketonuric patients, however, failed to show any enzyme activity.

Jacobson (1964) suggested that if there were any enzyme activity in the young infants with phenylketonuria, it might be possible to enhance this activity by supplying increased amounts of the pteridine cofactor described by Kaufman (1958), Fig. 16. If this proved effective, then optimum dietary control of the infant might be more readily achieved.

**SUBJECTS:** Four children with phenylketonuria were studied. B.G. and T.G. were brother and sister, J.M. and C.B. were not related to each other or to B.G. Case histories are described in Appendix I and Table IX summarises the salient features.
Phenylalanine

phenylalanine hydroxylase

(1) PTERIDINE

p-Tyrosine

oxidase

(2) PTERIDINE

3,4-Dihydroxyphenylalanine
DOPA

Melanin
Nor-adrenaline
Adrenaline

p-Hydroxyphenylpyruvic acid

oxidase

(3) PTERIDINE

Homogentisic acid

Fig. 16
<table>
<thead>
<tr>
<th>AGE</th>
<th>AGE DIAGNOSED</th>
<th>BIRTH WEIGHT</th>
<th>PRESENT WEIGHT</th>
<th>PHENYLALANINE DAILY INTAKE (mg/kg body wt)</th>
<th>DIETARY CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.M.</td>
<td>6 months</td>
<td>18 days</td>
<td>2.8 kg.</td>
<td>6.6 kg.</td>
<td>47</td>
</tr>
<tr>
<td>B.G.</td>
<td>16 months</td>
<td>23 days</td>
<td>1.75 kg.</td>
<td>8.0 kg.</td>
<td>24</td>
</tr>
<tr>
<td>T.G.</td>
<td>33 months</td>
<td>17 months</td>
<td>2.4 kg</td>
<td>12.0 kg.</td>
<td>17</td>
</tr>
<tr>
<td>C.B.</td>
<td>45 months</td>
<td>9 months</td>
<td>3.0 kg</td>
<td>15.8 kg.</td>
<td>24</td>
</tr>
</tbody>
</table>
METHODS: The four subjects were maintained on their prescribed diet with the phenylalanine intake indicated in Table IX. Capillary whole blood was obtained for fasting blood amino acid content and again at 4, 8, 12, 16 and 24 hours after ingestion of 100 mg/kg of L-phenylalanine. The test was repeated after 2-3 days interval when 24 mg. folinic acid (given as 60 mg. Leucovorin) was injected subcutaneously before the load of phenylalanine was given. B.G. was given his tolerance test with folinic acid 4 days before his control phenylalanine tolerance test.

Free amino acids in whole blood were measured on 0.2 ml. of sample by the Technicon Micro-Column Amino Acid Analyzer adaptation of the technique of Spackman, Stein and Moore (1958). By this technique, there are differences between whole blood and plasma levels of a number of amino acids. Tyrosine and Phenylalanine concentration in the capillary whole blood samples and capillary plasma samples withdrawn at the same time were, however, virtually identical. (See Appendix II for a fuller description of the method).

RESULTS: The patients tolerated the tests well and showed no change in their general behaviour. Blood phenylalanine and tyrosine concentrations are recorded in Tables X-XIII, and the phenylalanine tolerance curves in Figs. 17-20.

There is no obvious pattern of change in blood tyrosine after a phenylalanine load in any of the children whether given folinic
### TABLE X

<table>
<thead>
<tr>
<th>J. M.</th>
<th>PHENYLALANINE /μ moles/litre</th>
<th>TYROSINE /μ moles/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Folinic</td>
</tr>
<tr>
<td>0 hr.</td>
<td>77</td>
<td>164</td>
</tr>
<tr>
<td>4 hr.</td>
<td>1013</td>
<td>825</td>
</tr>
<tr>
<td>8 hr.</td>
<td>1232</td>
<td>784</td>
</tr>
<tr>
<td>12 hr.</td>
<td>1630</td>
<td>517</td>
</tr>
<tr>
<td>16 hr.</td>
<td>713</td>
<td>324</td>
</tr>
<tr>
<td>24 hr.</td>
<td>103</td>
<td>329</td>
</tr>
</tbody>
</table>

### TABLE XI

<table>
<thead>
<tr>
<th>B.G.</th>
<th>PHENYLALANINE /μ moles/litre</th>
<th>TYROSINE /μ moles/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Folinic</td>
</tr>
<tr>
<td>0 hr.</td>
<td>418</td>
<td>442</td>
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<tr>
<td>4 hr.</td>
<td>1178</td>
<td>951</td>
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<tr>
<td>8 hr.</td>
<td>2040</td>
<td>914</td>
</tr>
<tr>
<td>12 hr.</td>
<td>1285</td>
<td>825</td>
</tr>
<tr>
<td>16 hr.</td>
<td>1099</td>
<td>648</td>
</tr>
<tr>
<td>24 hr.</td>
<td>966</td>
<td>380</td>
</tr>
</tbody>
</table>
### TABLE XII

<table>
<thead>
<tr>
<th>T.G.</th>
<th>PHENYLALANINE (µ moles/litre)</th>
<th>TYROSINE (µ moles/litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Folinic</td>
</tr>
<tr>
<td>0 hr.</td>
<td>648</td>
<td>800</td>
</tr>
<tr>
<td>4 hr.</td>
<td>1301</td>
<td>1500</td>
</tr>
<tr>
<td>8 hr.</td>
<td>1940</td>
<td>1525</td>
</tr>
<tr>
<td>12 hr.</td>
<td>1382</td>
<td>1469</td>
</tr>
<tr>
<td>16 hr.</td>
<td>946</td>
<td>1074</td>
</tr>
<tr>
<td>24 hr.</td>
<td>1793</td>
<td>1256</td>
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</table>

### TABLE XIII

<table>
<thead>
<tr>
<th>C.B.</th>
<th>PHENYLALANINE (µ moles/litre)</th>
<th>TYROSINE (µ moles/litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Folinic</td>
</tr>
<tr>
<td>0 hr.</td>
<td>1314</td>
<td>966</td>
</tr>
<tr>
<td>4 hr.</td>
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<td>2570</td>
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<tr>
<td>8 hr.</td>
<td>1268</td>
<td>1681</td>
</tr>
<tr>
<td>12 hr.</td>
<td>1270</td>
<td>1320</td>
</tr>
<tr>
<td>16 hr.</td>
<td>1350</td>
<td>1215</td>
</tr>
<tr>
<td>24 hr.</td>
<td>893</td>
<td>1958</td>
</tr>
</tbody>
</table>
PHENYLKETONURIA
PHENYLALANINE TOLERANCE TEST

--- Control

--- Folinic

**Fig. 17**
PHENYLKETONURIA

PHENYLALANINE TOLERANCE TEST

Fig. 10
PHENYLKETONURIA

PHENYLALANINE TOLERANCE TEST

---

**Fig. 19**
PHENYLKetonuria

Phenylalanine Tolerance Test

- Control
- Folinic C.B.

Blood Phenylalanine (µMoles / litre) vs. Time (hours)

Fig. 20
acid or not. The striking feature of the phenylalanine loading test is the prolonged elevation of blood phenylalanine which results. In the cases studied, it is evident that it requires a period of 24 hours after the administration of phenylalanine for blood concentrations to return to control values.

The two smaller infants, J.M. and B.G., show a significant reduction in their blood concentration of phenylalanine in response to the folinic acid. J.M. had previously shown a similar response during a trial assessment of the procedure. Folinic acid has no obvious effect on the tolerance of the older children, T.G. and C.B., to ingested phenylalanine. These latter children had high blood concentrations of phenylalanine during the control periods.

COMMENTS: The apparent ability of folinic acid to prevent the usual high and prolonged elevation of blood phenylalanine in the test situation suggests that there is present in the two infants some active system for the metabolism of phenylalanine. It may be simply an effect on absorption of phenylalanine from the gut or an enhancement of excretion by the renal tubule. Unfortunately, the available data cannot decide this. Jacobson's postulate that folinic acid might increase the efficiency of reduced quantities of liver phenylalanine hydroxylase would require the demonstration of the active enzyme in the livers of young phenylketonuric infants. The lack of response in the two older children may be due to the relatively
smaller dose of folinic acid they received. Alternatively, their high blood concentrations of phenylalanine may have produced substrate inhibition or complete loss of any enzyme activity they might at one time have possessed.

Menkes and Avery (1963) report the effect of folinic acid in premature infants unable to metabolise tyrosine and phenylalanine normally. They showed a dramatic fall in blood concentrations of tyrosine following administration of the folinic acid but do not report any corresponding changes in phenylalanine. Their findings are consistent with the report of Zannoni et al. (1963) who found that reduced forms of folic acid were required for proper function of parahydroxy phenylpyruvic acid oxidase, the enzyme deficient in Tyrosinosis (Fig. 16, Step 3). The enzyme system which converts Tyrosine to DOPA also requires a reduced pteridine compound for its normal function (Negatsu et al., 1964). Thus, there are at least three oxidative enzyme systems involving the aromatic amino acids which require a reduced pteridine cofactor (Fig. 16). The improved overall efficiency of these liver enzyme systems could explain the more efficient handling of a phenylalanine load by the phenylketonuric infant and by the premature infant.

The structure of the naturally-occurring hydroxylation cofactor has recently been established as dihydrobioterin (Kaufman, 1963). Synthetic tetrahydropteridine compounds more active than folinic acid are known but at present are not available in sufficient quantity to
test their effect in the phenylketonuric infant. When these compounds are available, it would certainly be worthwhile to repeat these observations and, if possible, assess their effect on phenylalanine metabolism in phenylketonuria over a longer period of time.
PART III

DISCUSSION:

The developing foetus and newborn infant requires amino acids in specific quantities for the synthesis of structural proteins, enzymes and protein hormones, all of which are vital to the growth process. Proteins and amino acids involve only one facet of nutrition and during all phases of growth and development equal attention must be given to the carbohydrate, fat, water, mineral and vitamin content of the diet. Bearing this in mind, the discussion will deal with some aspects of phenylalanine metabolism in relation to protein synthesis, haematopoiesis and the growth process.

The introduction reviews the way in which the concept of protein nutrition developed from the recognition of the fact that nitrogen containing compounds were essential for tissue and body growth. Subsequent discovery of the amino acids and protein structure allowed a rational approach to the assessment of diets and their relative nutritive values.

Nutrition and Haematopoiesis: The striking findings in Study I were the rapidity with which the premature infant showed evidence of phenylalanine deficiency, a lowered serum concentration of phenylalanine
and the development of vacuoles in the cytoplasm of bone marrow red cell precursors. Bone marrow lesions were rapidly reversed when phenylalanine was added to the diet. The severity of the phenylalanine deficiency may have been accentuated by the increased intake of other amino acids in the deficient diet. A load effect from the diet was clearly demonstrated with marked increases in serum concentrations of methionine, valine, alanine and lysine and lesser increases of other amino acids when infants were fed the phenylalanine deficient diet or the deficient diet complemented with phenylalanine. This was accompanied by increased urinary excretion of these amino acids. The increased intake of amino acids was sufficient to obscure any possible effect of phenylalanine deficiency per se on amino acid concentrations. Supplemental ingestion of methionine, alanine and valine produced no apparent bone marrow or haematologic change. It is unlikely, therefore, that the bone marrow changes noted can be attributed to other than phenylalanine deficiency.

Three premature infants did not develop marrow changes after 48 hours of the deficient diet when the preceding control diet had been Diet II, which allowed 125 mg/kg/day of phenylalanine. One of these infants continued with the deficient diet for a further 3 days and did then develop erythroid vacuoles in his bone marrow. The failure of these three infants to develop marrow changes as quickly as those fed modified cow's milk as the control diet would suggest that cow's milk modified as in Diet I (allowing 90 mg/kg/day of phenylalanine)
contains less than optimal quantities of phenylalanine for the needs of some premature infants. On the other hand, the greater tyrosine content of Diet II might have made the infants more resistant to phenylalanine deficiency since tyrosine can replace about 75% of the daily requirements for phenylalanine of the older individual. The age of these infants prevents acceptance of this speculation, although the increased tyrosine intake might have been important in the resistance of the full term and older infants to the effects of the phenylalanine deficient diet. Studies by Holt and Snyderman (1964) on the dietary requirements of premature infants were so designed that the infants were several weeks old before the requirements could be evaluated and again the measurement of the optimum diet was based on weight gain, which, as pointed out in the introduction, may not be the best method of assessment. Now that a micro technique has been evolved (Appendix II) for the measurement of blood amino acid concentrations, it would be worthwhile assessing the various diets fed to the premature infant on this basis. It is known, for instance, that many premature infants are unable to metabolise tyrosine during the second week of life (Menkes and Avery, 1963) and that this is associated with later mental retardation if, indeed, it is not the cause. Following the reports that mentally retarded infants have been born to mothers with phenylketonuria, although the infants themselves do not have the disease (Denniston, 1963, and Mabry et al, 1963) and the fact that monkeys fed diets with a high phenylalanine content are mentally retarded (Waisman, 1965), it is incumbent on paediatricians
to ensure a "normal" milieu of amino acids for the rapidly developing nervous system of the newborn and premature infant. The effect of force feeding an imbalanced amino acid diet to the infant rat is clearly seen from the mortality figures and illustrated in Study 4. Paediatricians must be sure that they are not replicating this experiment in a greater or lesser degree when they feed premature infants by gastric tube or gastrostomy.

All the infants in Study 1, except SU, developed vacuoles in their white cell precursors after 48 hours of the deficient diet. The presence of myeloid vacuoles in the absence of erythroid vacuoles may reflect the more rapid rate of turnover of white blood cells. Some support for the hypothesis that longer periods of deficiency are required for development of erythroid vacuoles is derived from the observation that erythroid vacuoles never were seen in the absence of myeloid vacuoles. The bone marrow changes may be reversed before the serum phenylalanine concentrations are fully returned to control values when the infants are given phenylalanine complements.

**Chloramphenicol Toxicity:** Toxic reactions to chloramphenicol, of main concern, are the production of clinical changes resembling shock in the premature and newborn infant and bone marrow depression occurring in the infant and older child. Some newborn infants receiving chloramphenicol within 48 hours of birth and in doses greater than 100 mg/kg/day developed a clinical picture called the
"Gray-baby" syndrome (Burns et al, 1959; Sutherland, 1959; Lambdin et al, 1960; Lischner et al, 1961). The first evidence of toxicity was vomiting followed by refusal to suck and abdominal distention. Respiratory difficulties, flaccidity and an ashen-gray colour followed. Deaths occurred in the seriously affected babies from 24-48 hours after the onset of the first symptoms. In the infants studied, unusually high blood concentrations of chloramphenicol were found. This was probably the result of an inability of the immature liver to conjugate the chloramphenicol to the biologically inactive glucuronide. The syndrome has been produced in animals using very high doses of the drug (Gruhzit et al, 1949; Kent et al, 1960; Michael and Sutherland, 1961). In none of the studies quoted were the blood or urinary concentrations of amino acids measured.

Although none of the infants observed in Study 2 developed the Gray-baby syndrome, it is of interest that the smaller infants developed very high serum concentrations of circulating amino acids with alanine particularly elevated. It is conceivable that the interference with amino acid metabolism is aetio logically related to the Gray-baby syndrome.

There is little doubt that the vacuoles seen in the bone marrow red cell precursors of the infants treated with chloramphenicol were the result of chloramphenicol toxicity. The vacuoles seen in the cytoplasm of myeloblasts, promyelocytes, myelocytes and metamyelocytes
were probably due to chloramphenicol but some reservations to this interpretation must be held in view of the presence of infections in these infants (Ponder and Ponder, 1942). Myeloid vacuoles appeared sooner and, at times, disappeared more rapidly on recovery than the erythroid vacuoles, as might be expected from the more rapid rate of myeloid cell turnover.

The changes in circulating free amino acid concentrations suggest a block in the utilization of amino acids. Increased urinary excretion of these amino acids rules out the possibility that the elevations in plasma concentrations were due to primary renal dysfunction produced by chloramphenicol.

It was only shortly after the introduction of chloramphenicol for clinical use that scattered reports associated therapy with severe depression of the bone marrow resulting in aplastic anaemia or pancytopenia (Welch et al, 1954). There is now no question that chloramphenicol can cause severe bone marrow depression with fatal outcome (Dameshek, 1960; DeNosagno, 1960; Krakoff, 1955). Of 169 deaths listed by the Registrar-General as being due to therapeutic misadventure in England and Wales during 1962, twelve deaths from blood dyscrasia were caused by chloramphenicol. Although the incidence of this complication is reported as 1 in 20,000 to 1 in 50,000 patients treated with chloramphenicol, the incidence would probably be higher if a child population were analysed. This same report argues that chloramphenicol should be reserved solely for the treatment
of Typhoid. Many arguments have been raised about the efficacy of penicillin alone in the treatment of Haemophilus meningitis but the majority of paediatricians would still employ the well-tried and effective chloramphenicol with penicillin therapy. There is, likewise, a good argument for the use of chloramphenicol in Gram negative septicaemias in the neonatal period. Whatever the final outcome of such arguments might be, there is no doubt that the antibiotic is a very effective one. If it were possible to remove the cellular toxicity to the host and yet maintain an effective antibiotic action, there would be great advantage.

**Chloramphenicol and Phenylalanine:** Study 2b demonstrates the reversal of marrow vacuolization in four chloramphenicol treated infants given L-phenylalanine, 100 mg/kg/day, orally while chloramphenicol therapy continued. Phenylalanine was not given to these infants until at least three days of effective chloramphenicol treatment had been completed. The doses of L-phenylalanine were approximately equal on a molar basis with those of chloramphenicol and the duration on this regimen perforce short. Nonetheless, the effects were dramatic in these four infants.

The interaction of phenylalanine and chloramphenicol in relation to mammalian protein synthesis and bacterial growth is one of great interest but its significance in therapeutic situations remains to be proven. Woolley (1950) proposed that the antibiotic could be regarded as a metabolic analogue of phenylalanine and the similarity
of chemical structure is apparent in Fig. 4, p. 31. Yamada et al, 1963, have used L-phenylalanine as a basis for the synthesis of chloramphenicol.

More recently, Jardetsky (1962 and 1964) has shown that the steric configuration of chloramphenicol in solution is very similar to uridine 5' phosphate. He suggests that the site of action of chloramphenicol is on the ribosome and that it acts by interfering with uridine coding. The genetic code for phenylalanine, as carried by messenger RNA to the ribosome, is a triplet of uridylic acid [UUU] or two uridylic residues with one cytidylic [UCU] (Bernfield and Nirenberg, 1965). Thus, any interference with uridine should, in theory, preferentially inhibit the incorporation of phenylalanine into protein.

Gale, 1958, has demonstrated that chloramphenicol interferes with synthesis of both protein and nucleic acid in vitro. His experiments showed that this interference leads to the accumulation of glutamic and adenine-labelled substances which, he thought, represented a piling up of biosynthetic precursors whose utilization had been blocked by chloramphenicol. These substances have not been identified but possibly represent an amino-acyl nucleotide complex, a precursor of nucleoprotein synthesis. When chloramphenicol is removed from an inhibited bacterial culture, the material which accumulates in the presence of chloramphenicol will then be incorporated in more complex
molecules. It is interesting to speculate that the red cell precursor vacuoles described in patients treated with chloramphenicol might contain substances analogous to those demonstrated by Gale in bacterial culture. An analysis of the vacuole "content" in the phenylalanine deficient and chloramphenicol treated red cell precursor by radio-autographic or other techniques is an obvious step in the further elucidation of the blocking mechanism involved. The main limitation to this study is the lack of a suitable experimental animal which will readily show vacuoles in the red cell precursors. Rhesus monkeys will show changes in their marrow similar to that of the human infant when given large doses of chloramphenicol (Weiss, 1965), and would be an obvious choice of animal for such a study.

Whatever the final mechanism, study 3 suggests that parenteral L-phenylalanine given in a dose equivalent to that of the chloramphenicol will not inhibit the drug's antibacterial activity in the experimentally infected mouse treated with a single injection of the antibiotic. Indeed, there is a potentiation of the host resistance to Klebsiella infection. This is not a direct effect of the amino acid since phenylalanine by itself had no demonstrable antibiotic activity. A preferential uptake of phenylalanine by the host cells might block the site of activity of chloramphenicol on the host cells and yet allow a more effective antibacterial concentration of chloramphenicol outside the host cells. Another possible explanation is that this dose of phenylalanine might prevent the inhibitory effect
which chloramphenicol has on some protein dependent body defence mechanism such as antibody formation (Ambrose and Coons, 1963; Weisberger and Wolfe, 1964; Daniel et al, 1965). The relevance of the findings in Study 3 to infections with organisms other than Klebsiella pneumonielae or to human infections remains to be established.

**Chloramphenicol Toxicity and Diet:** In early studies by Reutner et al, 1955, on the toxic effects of chloramphenicol on the bone marrow of dogs, it was thought that the effect was in large part due to an appetite depressant action of the drug. It is known that in the protein deficiency state found in kwashiorkor bone marrow depression and cytoplasmic vacuolization of red cell precursors will occur. The chloramphenicol toxicity in the dogs was prevented by feeding extra quantities of meat. There is evidence, apart from these early dog studies, that chloramphenicol has a greater depressant effect on bone marrow in protein deficient states. A study is at present being conducted in Thailand where there is an unusually high incidence of aplastic anaemia in children, and where combined chloramphenicol therapy and protein deficiency are thought to be involved (Weiss, 1965).

Vacuoles were present in the cytoplasm of the red cell precursors of a fourteen year old girl who had the nephrotic syndrome and who had been treated for four weeks with a small dose (25 mg/kg/day) of chloramphenicol. She was found to have a low total protein and an altered albumin/globulin ratio. L-phenylalanine reversed her bone
marrow vacuolization. Elderly people tend to be relatively protein deficient and Scott et al, 1965, report that in the group they studied for evidence of chloramphenicol block in their ferrokinetic studies, it was probable that a dietary protein deficiency was the aetiologic factor in the greater sensitivity of some of their older patients to this toxic action of chloramphenicol. The findings presented indicate that chloramphenicol should not be used in any situation where there is severe malnutrition or protein deficiency states.

Testosterone has been widely used in the treatment of aplastic anaemia and the reason for its occasional beneficial activity is unknown. It may be that it has a direct effect on phenylalanine incorporation into protein (Silverman et al, 1963) or it may be that its anabolic activity increases the intake and utilization of protein. There is a good argument for trying the effect of combined L-phenylalanine, testosterone and high protein diet in any instances of aplastic anaemia caused by chloramphenicol. It may be that by the time the aplastic stage has been reached, an irreversible change has occurred which is a cogent argument for restricting the use of chloramphenicol and for ensuring a very close patient follow-up with bone marrow and serial serum iron studies.

Phenylketonuria: Much has been written about this inherited disorder and the description in Study 5 of two very young infants with the condition serves to underline the importance of early diagnosis
and the need for strict control of the diet. The morbidity and mortality from therapy, as well as from the disease itself, can be overcome by good dietary control. Pteridine derivatives may prove of value in the management of the young infant with the disease as described in Study 6, but attention must first be directed to ensuring adequate facilities for diagnosis and dietary management.

The future will pose major problems in the management of the pregnancy of the girl with phenylketonuria who has reached maturity with no physical or mental stigmata of the disease because of careful dietary management during her early formative years. It remains to be shown that we can effectively reduce blood concentrations of phenylalanine in the pregnant woman with the disease and, at the same time, ensure adequate nutrition for the developing foetus. This is a problem which requires urgent consideration if we wish to be in a position to offer effective advice and care to mother and foetus.

Phenylalanine: Foy and Kondi (1964) noted bone marrow changes and anaemia in infants with the clinical picture of kwashiorkor which are identical with those found in infants made deficient in phenylalanine. It may be that the vacuoles produced in kwashiorkor are the result of phenylalanine deficiency per se but are more likely to be due to a failure of protein synthesis. This inability to manufacture new protein because of a deficiency of one or of many essential amino-acids is true of the situation found with chloramphenicol toxicity,
where the machinery for making normal new protein is blocked. All the clinical situations where cytoplasmic vacuoles in the erythrocytes are described have in common a relative insufficiency of new protein formation. In DiGuglielmo's syndrome, it is probable that the extreme rapidity of red cell precursor proliferation outstrips the available supplies of amino acid nucleic acid.

Of course, it is not only a deficiency of an individual amino acid which can cause a block to protein synthesis. An excess of phenylalanine, for example, can inhibit new protein synthesis in the infant with phenylketonuria just as it can inhibit antibody formation in the experimental animal (Kyan, 1965) or leukaemic white cell proliferation in children (Allan et al, 1965).

Protein synthesis can thus be limited in the normal and the abnormal blood cell by an increase or a decrease of one dietary amino acid, or by a drug such as chloramphenicol which prevents the incorporation of one or more amino acids into new protein. The influence of diet on the activity of the bone marrow and cells of the reticuloendothelial system requires close attention as does the interaction of diet and drugs on the developing foetus and newborn infant.

Disturbance of protein formation through interference with the amino acid phenylalanine can produce profound changes in tissue function. Only a few aspects of such changes have been explored in this thesis.
Phenylalanine is an essential amino acid and as such is a necessary dietary constituent for the human infant. A short term dietary deficiency of phenylalanine in the normal infant is shown to lower the blood phenylalanine concentration to very low values within a period of forty-eight hours and at the same time, produce vacuoles in the cytoplasm of red and white cell precursors in the bone marrow. These changes are rapidly reversed by reintroducing phenylalanine to the diet.

Chloramphenicol is an antibiotic which inhibits bacterial growth by blocking protein synthesis. It is shown to have a marked effect on the blood amino acid concentrations of young infants treated with chloramphenicol for a variety of infections. A possible relationship between the hyperaminoacidaemia with increased aminoaciduria and the "Gray-baby" syndrome is discussed. Bone-marrow changes identical with those caused by phenylalanine deficiency were produced in the infants by chloramphenicol and in four infants studied, these changes were reversed by increasing the dietary intake of L-phenylalanine in spite of continuing chloramphenicol therapy.
When mice are experimentally infected with Klebsiella pneumoniae then given equimolar quantities of chloramphenicol and L-phenylalanine simultaneously, there is a potentiation of the antibiotic effect, but when the concentration of phenylalanine greatly exceeds that of the chloramphenicol, the antibiotic efficacy is markedly reduced.

Two infants with phenylketonuria diagnosed in the first weeks of life are described. Their dietary management and the effect of a too severe dietary restriction on haematopoiesis and growth are described in detail. These two infants had cytoplasmic vacuolisation of their erythrocytes when their blood phenylalanine levels were lowered below normal concentrations.

Rats force-fed a phenylalanine deficient diet or given parenteral chloramphenicol did not produce cytoplasmic vacuoles in their marrow red cell precursors but there was some reduction in marrow cellularity. Tritium labelled alanine, phenylalanine and uridine failed to show any particular localisation in bone-marrow radioautographs from these animals.

Phenylalanine tolerance tests were performed on four children with phenylketonuria. Folinic acid administered with the oral phenylalanine load had no demonstrable effect on the two older children so treated. Two infants, however, showed a marked reduction in blood phenylalanine concentrations when given folinic acid.
The possible value of a tetrahydrofolic acid derivative in the management of the very young infant with phenylketonuria is discussed in relation to the difficulties earlier described in the dietary management of the disease.

The final discussion reviews the role played by phenylalanine in infant nutrition and disease.
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APPENDIX I

Case Histories of Four Patients with Phenylketonuria.

J.M. - This infant, an 18 day old Caucasian male, was admitted to the Pediatric Clinical Research Center in Boston when an elevated blood phenylalanine concentration (15.5 mg. %) was detected at the four-day routine Guthrie screening test. He was born at term by spontaneous vertex delivery following a normal pregnancy to a 35 year old, gravida 3, para 3, white female. Birth weight was 2.8 kg. and there were no abnormalities detected during his four day stay in the Newborn Nursery. He was discharged home on the 5th day, feeding well on a modified milk preparation (Enfamil).

The mother is well and gave no history of phenylketonuria, mental retardation or epilepsy in her family. This child's father is unknown as is his family history. There are two siblings; a girl aged 15 years and a boy aged 14 years, to a different father from J.M. Both children are doing well at school. The mother and two children all had negative urine ferric chloride tests and blood Guthrie tests.

At the time of his admission to the ward for phenylalanine tolerance tests with and without folinic acid, he was six months old.
and weighed 6.6 kg. (3rd percentile). His crown-heel length was 64 cm. (10th percentile) and the head circumference measured 42 cm. (3rd percentile). He was, at this time, fair-haired and blue-eyed with no gross neurological abnormalities.

His haemoglobin was 11.5 G %, the haematocrit, 36% and the white blood cell count was 13,600 per cu. mm., 75% polymorph cells and 25% lymphocytes. Red cells and platelets were normal.

A full description of the management and progress of this child is given on p. 37.
B.G. - This male infant was born at 31 weeks gestation to a 25 year old Caucasian, gravida 6, para 4, mother who had an uneventful prenatal course until the spontaneous rupture of membranes and premature delivery. Birth weight was 1.76 kg. and there were no physical abnormalities detected at routine examination. He was fed a modified cow's milk preparation (Enfamil) from 24 hours. On the fourth day of life, mild clinical icterus, consistent with "physiologic icterus neonatorum," developed. At 6 days, a monilial infection involving mouth and napkin region developed and he was treated for 14 days with 200,000 units of nystatin daily by mouth. The oral lesions resolved in 4 days but the rash persisted after therapy was discontinued.

At 23 days of age, a positive Guthrie bacterial-inhibition assay test for phenylketonuria was reported by the Diagnostic Laboratories of the Massachusetts Department of Public Health. A subsequent urinary phenistix test was positive as was the ferric chloride test for phenylketonuria.

The serum phenylalanine concentration at this time was 42 mg/100 ml. as determined by the LaDu method (LaDu, 1963). Phenylalanine loading tests were carried out on both parents who were found to be heterozygous for the phenylketonuria trait. The patient's siblings were tested by the Guthrie and Phenistix tests, and the two older children were negative, but a 17 month old sister, J.G., had a
positive Guthrie test and a blood phenylalanine level of 37 mg/100 ml.

Two maternal cousins had died in the first two years of life but there was no other relevant family history.

At the time of his admission to the ward for phenylalanine tolerance tests with and without folinic acid, he was 16 months old and weighed 8.0 kg. (50th percentile for 7 months). His length was 70 cm. (50th percentile for 9 months) with a head circumference of 46 cm. (50th percentile for 9 months). He was fair-haired and blue-eyed with no major neurological abnormalities apart from developmental retardation. He was able to walk with support. His haemoglobin was 11 G per 100 ml. with a haematocrit of 32%, and a white cell count of 8,350 per cu.mm. with 60% polymorphs, 33% lymphocytes and 2% monocytes. Red cells were normocytic and normochromic and platelets were plentiful. The tuberculin skin test was negative.

A full description of the management and progress of this child is given on p. 92.
T.G. - This infant was diagnosed at the age of 17 months as having phenylketonuria at routine family screening when her brother (B.G.) was found in the newborn period to have phenylketonuria.

She was a normal full term baby with a birth weight of 2.4 kg. For family history, see B.G. During the first year of life, her weight gain was satisfactory but was described as a very irritable infant. At one year, she was unable to roll over and was unable to sit unsupported. At 17 months, a low phenylalanine diet was started and there was, according to her mother, a marked improvement in her disposition. By 24 months, she was walking and saying single words. Her dietary intake of phenylalanine is controlled by frequent estimations of blood phenylalanine concentrations.

At the time of admission to the ward for phenylalanine tolerance tests with and without folinic acid, she was 33 months old and her weight was 12.2 kg., height 87.3 cm. (normal for 2 year old) and head circumference 47.3 cm. (normal for 15 months). Her complexion was fair with blond hair and blue eyes. The anterior fontanelle was not palpable. Neurological examination showed normal motor and sensory function with good coordination. Vocabulary was very limited for her age.

Urinary ferric chloride tests at this time were negative and her blood phenylalanine concentration was 8 mg/100 ml. blood.
Her haematocrit was 36% with a white blood cell count of 7500/cu. mm with 56% polymorphs, 40% lymphocytes and 4% monocytes. Red cells and platelets were normal. The tuberculin skin test was negative.

Her blood phenylalanine concentration was found to be 12.0 mg. I and her urinary isovaleric acid concentration test for phenylketonuria was positive. Although treatment with a low phenylalanine diet was instituted, control has never been satisfactory due to poor cooperation on the part of her father.

She was born at term following a normal pregnancy and delivery at a 36-year-old, gravida 4, para 4, white woman. Weight was 3.0 kg, and there were no additional abnormalities noted during the antepartum period when she fell on a paved road while pregnant (hence).

Both parents are well with no abnormalities. She has three older siblings not in good health with negative urinary isovaleric acid and blood phenylalanine tests. There is no history of any genetic family history.

At the time of admission to the ward she appeared thin and wasted without fat folds and, she was 1 year old. She cried with every movement. Her weight was 11.7 kg, and her height was 50.0 cm, both within normal limits for her age. But her lack of development assessed 40.3 cm, which is normal for 15 months but in the 15th percentile for a term baby. Anthropological examination showed a moderately...
C.B. - This child, a Caucasian female, was first admitted to hospital at the age of nine months because of failure to thrive normally. At that time, she smiled and lifted her head but had progressed no further in her development. Her blood phenylalanine concentration was found to be 32.3 mg. % and her urinary ferric chloride test for phenylketonuria was positive. Although treatment with a low phenylalanine diet was instituted, control has never been satisfactory due to poor cooperation on the part of her mother.

She was born at term following a normal pregnancy and delivery to a 30 year old, gravida 4, para 4, white female. Birth weight was 3.0 kg and there were no clinical abnormalities noted during the newborn period when she fed on a modified milk preparation (Enfamil).

Both parents are well with no consanguinity. The three siblings are in good health with negative urinary ferric chloride and blood Guthrie tests. There is no other positive relevant family history.

At the time of admission to the ward for phenylalanine tolerance tests with and without folinic acid, she was 3 years and 9 months old. Her weight was 15.8 kg. and her height was 100.5 cm., both within normal limits for her age, but her head circumference measured 47.5 cm. which is normal for 18 months and in the 10th percentile for a 3 year old. Neurological examination showed a moderately
cooperative child who babbled and would speak two word 'sentences' to her mother. There was good muscle strength and tone with good coordination, normal tendon reflexes, flexor plantar responses and normal sensation.

Urinary ferric chloride tests were positive on admission but after 7 days on her prescribed diet containing 375 mg. of phenylalanine per day these became negative. Her haemoglobin was 11.8 G % with a haematocrit of 35%, and a white cell count of 8,650 per cu. mm. with 73% polymorphs, 26% lymphocytes, 1% monocytes and 1% basophil cells. Red cells and platelets were normal. Tuberculin skin tests were negative.
APPENDIX II

In studies I and II, determinations of the amino-acid content of plasma, serum or milk preparations were carried out using the "Technicon" Amino Acid Analyzer Adaptation of the technique described by Spackman et al, 1958. The column diameter for these estimations was 6 mm. and a 1.0 ml. sample of serum or plasma was required to ensure a single accurate analysis. For the subsequent studies which required frequent analyses of blood amino acid content in small infants the following micro-technique was employed. This technique allowed the analysis of whole blood amino acids to be performed on 0.2 ml. of blood obtained by heel or finger prick.

METHOD: The details of the apparatus and ion-exchange resin used have been previously described by Hamilton, 1963. "Technicon" Corporation supplied two jacketed columns of 1/8 th inch internal diameter. The two columns were operated simultaneously and at a temperature of 60° C. The gradient of sodium citrate buffer from pH 2.875 to pH 5.0 was supplied from separate 'Autograds' and pumped by separate 'Milroy' micro pumps. Effluent buffer and amino acids from each column were mixed with a solution of ninhydrin.
and hydridantin in methyl cellusolve, segmented with nitrogen and colour developed in an oil bath at 95°C. After cooling, the final effluent was monitored through separate photo-electric cells filtered at 570 wavelength. At this wavelength proline and other amino acids which develop a yellow colour with ninhydrin could not be accurately determined. For this particular group of analyses this proved no inconvenience but the addition of a second colorimeter with a 460 filter would allow a complete amino acid analysis. The intensity of colour developed by each amino acid with the ninhydrin automatically recorded as an optical density on logarithmic ruled paper. Fig. 21 is part of an actual tracing obtained by this micro technique from 0.2 ml. samples of whole blood. The tracing obtained from a capillary whole blood sample of a phenylketonuric infant is compared with the blood amino acid profile of a normal infant. A complete analysis of two samples takes twenty-two hours to completion. Quantitative determination of the individual amino acid content is readily obtained by measurement of the area of each peak and comparing it with the peak area of a known amount of standard pure amino acids. The total error for the method is ±5%.

Heparinised capillary whole blood and venous plasma samples were obtained from five children and analysed by this method.
Fig. 21

Part of the Amino Acid profile obtained from the blood of a phenylketonuric infant (Orange) and a normal infant (Maroon).
COLLECTION OF SAMPLES: Approximately 1.0 ml of venous blood from each of five children was collected into sodium heparin containing tubes. After careful skin preparation and immediately after collection of the venous sample free-flowing finger or heel prick, capillary blood was collected into a capillary tube containing dried sodium heparin.

PREPARATION OF SAMPLES FOR ANALYSIS: The venous blood was centrifuged for ten minutes. 0.3 ml supernatent plasma was added to 0.3 ml ion-free distilled water and 0.3 ml of 0.6 M Sulphosalicylic acid was then added, mixed thoroughly and allowed to stand for 15 minutes. Recentrifugation packed the precipitated proteins and 0.6 ml of the supernatant was applied directly to one column for analysis.

A volume of 0.2 ml heparinised capillary whole blood was measured accurately immediately after collection and mixed with 4.0 ml of 0.05% Saponin solution. After standing for 10 minutes, 1.0 ml of 15.6% Trichloracetic Acid (TCA) was added (final concentration of TCA = 3%) mixed thoroughly and allowed to stand for 15 minutes. After centrifugation, 4.0 ml of the supernatent was transferred to a clean test-tube and completely dried in an air stream. The sample could then be stored dry at -68°C or reconstituted with 0.6 ml ion-free distilled water. 0.5 ml of this solution was applied to the second column.
RESULTS: Table XIV shows the amino acid concentrations in capillary whole blood and plasma of five children. The mean values agree fairly well with a few exceptions though the small number does not allow a good statistical analysis.

COMMENTS: A good review of the techniques of handling blood for amino acid analysis is given by Dickinson et al, 1965. They found that plasma obtained by heel prick had a higher concentration of taurine, glutamic acid, glycine, alanine and ornithine than femoral vein plasma obtained at the same time.

Some possible sources of difference between the values given in Table XIV are the increased quantities of glycine, alanine and ornithine present on skin surfaces (Hamilton, 1965). The higher concentrations of some amino acids in red and white blood cells and tissue fluids from the heel punctures will also contribute to a different concentration of amino acids in whole blood when compared to the plasma of the same individual (Soupart, 1962, McMenamy et al, 1960).

For practical purposes, capillary whole blood concentrations of amino acids are adequate for the detection of hyperaminoacidaemia and for the regulation of dietary management in the phenylketonuric infant. At present, this technique would prove too expensive and
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**TABLE XIV**

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<th>Subject (5)</th>
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<td>Whole Blood</td>
<td>Plasma</td>
<td>Whole Blood</td>
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<td>331</td>
<td>279</td>
<td>337</td>
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<td>276</td>
<td>301</td>
<td>292</td>
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<td>264</td>
<td>474</td>
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time consuming for routine screening of newborn infants. The method is ideal, however, as a research tool and can give accurate amino acid analyses on 0.5-1.0 ml samples of saliva, sweat or cerebrospinal fluid.