

STUDIES OF BIOCHEMICAL METHODS FOR INVESTIGATING  
PROTEIN-ENERGY MALNUTRITION AND THE DIAGNOSIS OF  
MARGINAL PROTEIN DEFICIENCY

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ABSTRACT

There is a great deal of knowledge about the value of biochemical indices in the severe states of PEM (kwashiorkor or marasmus). Not very much information in this regard is available in marginal or moderate malnutrition, but from the point of view of public health, moderate malnutrition presents a far more wide-spread problem than kwashiorkor or marasmus. In experimental models of protein-energy malnutrition, animals have been subjected to rather severe deficiencies of protein or energy or both and very little is known about marginal protein malnutrition produced experimentally.

The work reported here was an attempt to assess the value of biochemical methods in marginal protein deficiency by using a more appropriate model. One other important consideration was that the measurements to be used in the field in developing countries must be simple, involving the minimum of equipment and of samples to be analysed. The measurements should also be sensitive to small changes in dietary protein intake.

The work was undertaken between October, 1971 and October, 1974. Groups of rats weighing on average 71 g were fed for five weeks diets containing per kg 80, 120, 150, 180 and 240 g of casein, all of which allowed for substantial growth. Measurements were made at weekly intervals of body weight, serum total protein, albumin, transferrin, cholinesterase, ribonuclease and creatine kinase. The sum of the three branched chain amino acids was determined by thin-layer chromatography in 5 ul of serum. The recovery was checked with radioactive valine and averaged 85%. Not all measurements were made on all groups of rats. In some experiments muscle mass was measured at the end of the five weeks' feeding period.



The biochemical tests described could be made on as little as 50 ul of serum. The most sensitive indicators apart from the body weight were serum albumin and the branched chain amino acids. With the amino acid test, two groups of rats on 12% and 18% casein diets could be distinguished. With the albumin test the groups showed differences which were significant at the level of 5%. It was clear from the results that the greater the number of samples analysed the more sensitive the two methods become in differentiating rats on the various regimes.

The ease and simplicity with which the two methods could be used and their importance in the investigation of marginal protein deficiency is discussed in relation to field studies in developing countries where such disease abounds.

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CHAPTER ONE

INTRODUCTION

## 1. INTRODUCTION

In their introduction to the problem of "Protein malnutrition in man," Waterlow, Cravioto and Stephen (1960) stated that 'in the world as a whole the most widespread and serious dietary deficiency is that of protein,' and they emphasised that protein malnutrition 'kills a large number of people.' It is still a major problem facing the world today (Simmons, 1973).

However, in the last few years it has been recognised more and more that to think in terms of specific protein deficiency is probably an over-simplification. Measurements of dietary intakes of young children in a number of countries (Naismith, 1973; Rutishauser and Flood, 1973) have shown that there is usually a deficiency of energy as well as of protein. If the energy intake is inadequate, then dietary protein cannot be utilised and protein depletion is further enhanced (Sukhatme, 1969). Because it is so difficult to make a clear-cut separation, the term protein-calorie malnutrition (PCM) or protein-energy malnutrition (PEM) is increasingly used.

The assessment of protein deficiency is therefore of great interest and importance from several points of view. Governments in countries where protein deficiency is rife would like to get clear-cut evidence of the existence and magnitude of the problem in an attempt to justify the use of their financial resources in programmes to combat malnutrition. The physician is interested from the point of view of making nutrition more effective and of treating and preventing malnutrition.

The effective control of malnutrition is dependent on the development of better methods both for initial diagnosis and for monitoring the efficiency of preventive therapies (Whitehead, MRC-Uganda reports, 1968-1970; Simmons, 1973; Shakir, 1973).

In the attempt to assess the state of protein nutrition one is faced with many problems (Waterlow and Harper, 1974): (i) the organism, within limits, can adapt to a wide range of protein intake, but where does adaptation end?; (ii) very little is known about optimum protein nutrition; (iii) distinction must be made between biochemical tests which reflect protein intake and those which define the state of protein nutrition; (iv) as mentioned above, protein deficiency is usually associated with low energy intake and is very often complicated also by infection, which may affect various biochemical indices and interfere with their interpretation.

#### 1.1 Protein-Energy Malnutrition (PEM)

Protein-energy malnutrition is the name given to a spectrum of malnutrition ranging from kwashiorkor at one end to marasmus at the other. Inbetween these two syndromes are cases of marasmic-kwashiorkor. These names refer to severe cases (Wellcome Trust Working Party, 1970).

Far more numerous are mild-moderate cases not so easily detectable as the severe ones. Such cases have been variously described as subclinical, occult, pre-kwashiorkor, early malnutrition, and marginal protein malnutrition (Whitehead, 1969). For every case of severe PEM in the hospital there may be hundreds of undetected subclinical cases in the rural areas (Waterlow et al, 1960; Simmons and Bohdal, 1970). Jelliffe and Jelliffe(1969) refer to the problem of PEM as the public health 'iceberg'; the hidden nine-tenths are the mild-moderate cases.

That PEM is a worldwide problem has been recognised by many since it was first specifically described by Williams (1933). It's scourge is largely in the developing countries (Waterlow et al, 1960; Whitehead, 1969) and is mainly seen in children under the age of five. PEM affects their general health (Graham, 1967) and mental and physical

development (Chase and Martin, 1970). Malnutrition imposed at such a critical period of a child's growth is believed to cause impairment of brain development (Dobbing, 1973). The importance of detecting these cases in their early stages cannot be over-emphasised (Waterlow et al, 1960; Schendel, Hansen and Brock, 1962; Shakir, 1973).

Kwashiorkor is a word from the language of the Ga tribe living around Accra, the capital of Ghana, which was introduced into the medical literature by Williams (1933). It derives from the observation by the local people that premature weaning of the child from the breast often led to sickening of the infant, who became restless and jealous of the mother and would attempt to stick to her all the time. Weaning was made necessary by a superstitious belief that if pregnancy occurred within a year or two of the birth of the infant the breast milk would be poisoned and therefore harm the infant.

Trowell, Davies and Dean (1954) in their description of kwashiorkor equated it with 'mehlnahrschaden' of Czerny and Keller (1906) and of Finklestein (1924) in Germany; 'nutritional dystrophy' of Choremis (1948) in Greece and Ballabriga (1950) in Spain; the 'starch edema' of Mouriquand et al in France (1934); 'nutritional edema' described by Correa in 1908; 'edematous, multiple deficiency dystrophy' in Brazil, Chile, Colombia, Peru, Uruguay and Venezuela; and with many other synonyms from virtually every country in the world. 'Obowski' was the name given to it in Uganda. It is the classical 'sugar-baby' described by Waterlow in 1948.

Kwashiorkor has as its main clinical features oedema, dermatosis, moon-face, and discoloured, thin, sparse and easily pluckable hair. It was shown many years ago by Trowell (1945) and by Waterlow (1948) that the oedema is accompanied by a reduction in plasma total protein and albumin concentration. The main pathological feature is fatty liver.

According to Smellie (1954) the term 'marasmus' was first introduced

in 1600 by Soranio to describe the condition of infants who were suffering essentially from starvation. Classical nutritional marasmus is marked by retardation of growth and gross wasting of muscle and sub-cutaneous fat. The older terms include arhrepisia, cachexia, decomposition, infantile atrophy and inanition. Marasmus can occur at all ages but especially in the first year of life, whereas kwashiorkor occurs mainly in the age group of 1-5 years, although cases occur earlier in some areas.

Marasmic-kwashiorkor represents a mixture of the clinical and pathological features of the two extreme syndromes.

## 1.2 Biochemical Measurements in PEM

Biochemical measurements have been made with two main aims:

- (i) to understand the metabolic defects in severe cases, particularly in relation to the differences between kwashiorkor and marasmus and
- (ii) to find sensitive tests for diagnosing the nature and extent of the dietary deficiency in subclinical PEM.

### 1.2.1. Biochemical differences between kwashiorkor and marasmus.

In the past the search for biochemical differences between kwashiorkor and marasmus has sometimes taken the form of trying to find a 'diagnostic test' to distinguish between them. This really does not have much meaning, as the two syndromes are, by definition, diagnosed on clinical grounds.

On the other hand, it is meaningful to try to find specific biochemical changes which distinguish between different processes such as protein deficiency and energy deficiency. So far this search has not been particularly successful. One reason for this may be that in some countries, especially in Africa, attention has been

concentrated particularly on kwashiorkor, and marasmus has been rather neglected. In other countries, e.g. Jamaica, mixed forms predominate (Garrow, 1966) so that it is very difficult to make a clear distinction between the two processes.

In general it seems that most biochemical abnormalities are more marked in kwashiorkor than in marasmus. For example, in kwashiorkor plasma albumin and cholinesterase levels are lower than in marasmus, and the ratio of non-essential to essential amino acids is higher (see below). The general explanation is that in marasmus muscle protein is being broken down to supply energy and substrates for gluconeogenesis. Some of these amino acids seem to be available for protein synthesis in the liver. As Trowell has said: 'The marasmic lives on his own meat'.

Lunn, Whitehead, Hay and Baker (1973) have given a very detailed account of the biochemical and hormonal changes which occur during the development of kwashiorkor in children followed for several weeks or months in a clinic. So far there has been no complete description of the development of biochemical changes in marasmus. Recent attempts in this direction have come from Rao (1974) and Matz (1974) but have all been hypothetical: Rao, writing on the evolution of marasmus and kwashiorkor, stated that marasmus represents a state of good adaptation to the stress of PEM and that the response of the adrenal cortex may be crucial for this adaptation; a normal increase in plasma-cortisol helps in adequate mobilisation of muscle protein for the maintenance of metabolic integrity. Rao suggested further that the failure of the adrenal cortex to respond adequately may represent the crucial step in failure to adapt or in breakdown of adaptation, resulting in the characteristic biochemical and clinical picture of kwashiorkor.

Matz, following up Rao's hypothesis and observations by Blackburn,

Flatt, Clowes, O'Donnel and Hensle (1973) and a Leader in the Lancet (1973), suggested that the abnormal metabolism in kwashiorkor might be explained by increased secretion of insulin in response to carbohydrate ingestion with suppression of lipolysis and ketosis and ultimately the consequent rebound demand for amino acids as a source of gluconeogenesis.

One difficulty in trying to identify biochemical and metabolic differences between kwashiorkor and marasmus is that conditions seem to be different in different countries. For example Table 1 shows that in five different countries plasma growth hormone levels were consistently raised in kwashiorkor, whereas there is a very great discrepancy between the different countries in the levels found in marasmus. It looks as if the different workers, when they are using the term marasmus, are not all using it in the same way.

Table 1

Fasting Growth Hormone concentration  
in plasma (umols/ml)

\*(Kroc symposium, 1973)

	<u>kwashiorkor</u>	<u>marasmus</u>
Bolivia	-	5
Chile	25	4
India	30	5
Mexico	50 - 75	6
South Africa	25	30
Thailand	34	66

\*In some cases the values are  
approximate estimates from diagrams.

Pimstone, Becker and Hansen (1971) and Godard & Zahnd (1971),



finding increased level of growth hormone in kwashiorkor, also observed that this was not due to a low serum albumin concentration, nor probably to the abnormal amino-acid relationship present in this disorder, since infusion of amino acids failed in most cases to lower concentration of growth hormone. However, there was no increase in basal levels of GH in healthy young adults starved for four days (Irie, Tsushima, Sakuma and Matsuzaki, 1971) nor in healthy young men kept for 25 days on a protein-deficient but calorie diet (Merimee and Fineberg, 1972).

The present view is that in general the normal long term stimulus to the secretion of GH is a decrease of blood somatomedin, at least in children. It is believed that GH acts mostly or entirely in the liver bringing about the release from itself of an intermediary hormone somatomedin (Salmon and Daughaday, 1957; Tanner, 1972). It is suggested that in kwashiorkor patients this conversion is blocked, hence increased levels of GH appear in their blood due to continuing stimulation of the pituitary gland to produce the hormone.

#### 1.2.2. The subclinical states

In subclinical PEM there is no clear distinction between kwashiorkor and marasmus.

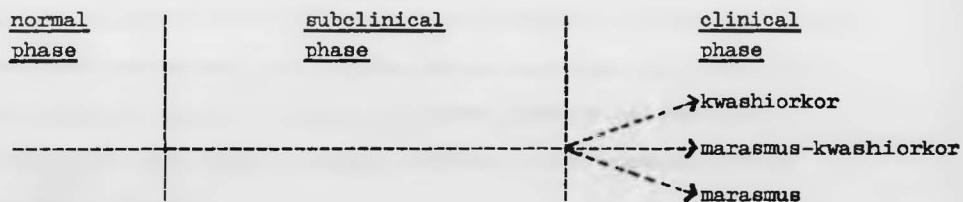


Fig. 1: A child on the way to PEM

In the subclinical stage the child is of course undernourished; he is perhaps 75% of the expected weight for age or height. Now the question is: what are the determining factors that decide which pathway the child will follow in the clinical phase? Four possible explanations have been proposed.

First is the balance of energy and protein in the diet. This is what might be termed the classical theory. It states that kwashiorkor results from a deficiency of protein with a relatively adequate supply of energy. However, in the opinion of Gopalan (1968) the capacity of the child to adapt is much more important than differences in diet in producing either marasmus or kwashiorkor. Gopalan's concept is still not very clearly defined. A third explanation involves the part played by infection. Investigations by Poskitt (1971) showed that in Uganda measles alters the nutritional state of the child by depressing plasma albumin concentration. The child is thus made more at risk to an episode of kwashiorkor. However, experience in Jamaica shows that kwashiorkor can occur without any evidence of a precipitating infection (Waterlow, personal communication). A fourth possible explanation is the relative requirements of the child for energy and protein. Presumably there is variability of requirements in children as in adults. If a child has a high energy requirements, energy will be the limiting factor in the diet and he will develop marasmus. If the child has a high requirement for protein but a low requirement for energy, on the same diet protein will be limiting and he would develop kwashiorkor. The difficulty here is that in the common subclinical state of malnutrition the child is usually getting a diet somewhat low in protein and energy.

Anthropometry has been the main method used up to the present for assessing subclinical PEM, but it is not very specific. It is

not known at this early stage whether there is any biochemical distinction between the effects of low protein and low energy. Rutishauser and Whitehead (1969) made both anthropometric and biochemical measurements in three Ugandan tribes with different dietary background, and found differences which could be related to the previous diet. More comparative studies of this kind are needed.

### 1.3. Biochemical Measurements For Assessing The Extent of Protein Depletion

There is no clear-cut method for assessing the extent of protein depletion although numerous biochemical measurements have been proposed. These may roughly be divided into two types: those which reflect the recent supply of nutrient and those which measure the actual pathological effect of deficiency. For example, urinary urea output falls in the first class and plasma albumin concentration in the second. Measurements have been made on (1) the whole body (2) organs and tissues (3) urine and (4) blood serum/plasma.

#### 1.3.1 Measurements on the whole body.

Nitrogen balance, total body water (TBW), total body potassium (TBK) and total protein turnover studies are measurements which have been made on the whole body. Nitrogen balance is a measure of the extent of utilisation of the nitrogen supplied; if nitrogen is consistently retained, it follows that the subject might have been depleted. Utilisation of protein depends also on its quality; nevertheless nitrogen balance represents the best method for monitoring the progress of the patient's state of protein nutrition (Waterlow and Harper, 1974) although it is subject to considerable error if not meticulously carried out.

Indirect estimation of cellular mass can be obtained from TBW. Lean body mass (LBM) and hence total body protein can be calculated on the assumption that 72% of LBM is water (Pace and Rathbun, 1945). This is only true if hydration is normal, but in the malnourished subject this may not hold because of oedema. A correction can be made by simultaneous measurement of extra-cellular fluid (ECF), but the methods for measuring it (e.g. thiocyanate space and inulin space) are not very satisfactory. This problem then brings into question the practicability of the use of TBW as an indicator of protein nutrition.

A still indirect but alternative method is the measurement of TBK. Here the assumption is made that the ratio of K to N in normal tissue is about 2 mEq K per g N. 2 mEq is a weighted average, since brain contains more (about 6 mEq), skin and bone less (about 1 mEq) and liver and muscle about 2 mEq (Klose, 1914; Widdowson and Dickerson, 1960; Garrow, 1967). Depending on the availability of a whole body counter, measurement of TBK is simple and has been found to relate well with creatinine output (Alleyne, Viteri and Alvarado, 1970).

The study of protein turnover in the body has been done mostly by methods based on the use of labelled amino acids. From turnover measurements the overall rates of protein synthesis and breakdown can be calculated, provided that the nitrogen intake and output are known (Picou and Taylor-Roberts, 1969). It remains to be determined how far measurements of this kind may be useful in giving, directly or indirectly, information on the state of protein nutrition.

#### 1.3.2. Measurements on organs and tissues

Parameters that have been looked at include DNA, RNA, protein and enzyme content in liver and muscle tissue obtained by biopsy; leukocytes; and hair root protein.

Waterlow and Weisz (1956) and Mendes and Waterlow (1958) measured protein and RNA content in relation to DNA in liver and muscle samples obtained by biopsy and found large increases during recovery from malnutrition. In principle such measurements should provide an accurate method of assessing protein status since they give an estimate of the protein content per cell, and indirectly of the amount of protein in the whole organ. In practice the method is limited by the difficulty of obtaining samples. It is also an open question whether the very small samples are representative of the organ mass as a whole.

Some work has been done on enzymes in tissues such as liver and muscle. A review of the results suggest that at the present time these measurements do not provide much help in assessing the state of protein nutrition (Waterlow and Stephen, 1969).

It has been found that the phagocytic response of leukocytes from malnourished children is depressed. This led Metcoff, Frenk, Yoshida, Torres-Pinedo, Kaiser and Hansen (1966) to do further work on this aspect of the leukocyte phagocytic response. In leukocytes from children with PEM they found a reduced activity of pyruvate kinase and of some of the intermediates involved in the production of energy. They obtained suggestive evidence that adenylate kinase and ATP generation were similarly depressed (Metcoff, 1967; Yoshida, Metcoff and Frenk, 1968). It seems possible that measurements in these enzymes could provide a means of assessing protein status in both children and adults.

The protein content of hair roots has been used as a measure of protein status in a hospital population (Bollet and Owens, 1973). The data suggest that it is perhaps a more sensitive index than albumin in assessing protein nutrition. Bradfield (1971) found this to be so in his earlier studies on volunteers on a protein-free diet.

### 1.3.3 Measurements on urine

The following are some of the measurements made on urine: urea nitrogen, creatinine, hydroxyproline, 3-methyl-histidine, and some other amino acids. Either the total output over 24 hours is measured, or the amount in a single sample may be related to that of creatinine, because of its relatively constant excretion (see below).

(i) Urea nitrogen At low levels of nitrogen intake the measurement of urea nitrogen gives some useful information on the state of protein nutrition. This is because the obligatory excretion of nitrogen compounds other than urea and ammonia is constant, amounting in the adult to about 1.5 g of nitrogen per day. Therefore the ratio of urea N + ammonia N to total N gives a measure of the absolute amount of nitrogen excretion and hence of the intake, if the subject is in nitrogen equilibrium (Platt and Heard, 1958; Waterlow, 1963; Simmons, 1972). The index is not sensitive at higher levels of protein intake.

Arroyave (1962) and Dugdale & Edkins (1964) proposed the ratio, urea N/creatinine, as a measure of protein status. It was claimed that the ratio could be determined on one urine sample. The method has been criticised on the grounds that in depleted or malnourished subjects the creatinine output is reduced so that the ratio is increased and thereby gives a false picture. Also it has been claimed that for accurate results water intake must be restricted which is not possible in a malnourished child.

It is important to realise that these measurements of urea output are not indices of health status but of food intake. A further note of caution is this that since ingestion of protein stimulates urea production the time at which urea is collected should be standardised in relation to meals.

The ratio of urinary inorganic sulphate to creatinine provides

information about the intake of sulphur-containing amino acids (Simmons, 1972). This is useful in areas in which the limiting factor in protein quality is deficiency of methionine or cystine.

(ii) Creatinine output. Creatine is the precursor of creatinine. Creatine is found almost exclusively in muscle and about half is combined with phosphate as creatine phosphate. Creatine phosphate cyclizes to form creatinine which is quite stable, diffuses into the blood and is excreted in the urine.

The amount of creatinine put out in the urine per day has long been supposed to depend on muscle mass. Urinary creatinine has been used as a measure of muscle mass on the basis that 50 mg of creatinine per day are produced by one kilogram of muscle (Cheek, Hill, Cordano and Graham, 1970).

It has been shown recently that the rate of synthesis of muscle protein is reduced by a deficiency of dietary protein (Waterlow and Stephen, 1968; Millward, 1974; Garlick, Millward and James, 1974). This observation therefore means that muscle mass is a good guide to the extent of repletion or depletion of body protein as a whole. Acting on this principle, Viteri and Alvarado (1970) have used creatinine/height index (creatinine output per 24 hours per cm height) as a basic measure of nutritional state. A practical difficulty with this method is that urine should be collected for 24 hours, since a shorter collection period increases the error. The diet also should be creatine-free, i.e. it should not contain meat or fish. Even when precautions are taken over these points some workers have found an unacceptably high variation in the daily output of creatinine (Scott and Hurley, 1968; Viteri, Mata and Behar, 1973).

Use of creatinine output as a measure of muscle mass under different conditions implies that the factor quoted above

(50 mg creatinine = 1 kg muscle) remains unchanged, i.e. the rate of conversion of creatine to creatinine, or creatine turnover rate, is unchanged.

Studies in rat with C-14-creatine showed that the turnover rate of creatine was indeed very much the same, regardless of age, sex, protein intake or rate of growth, provided that there was not a rapid loss of muscle as, for example, occurs in starvation or severe infection (Waterlow, Neale, Rowe and Palin, 1972). However, measurements with 15-N-creatine in children suggest that variation in creatine turnover between individuals is fairly large (Picou and Reeds, 1973).

For practical purposes the output of creatinine seems useful as a measure of muscle mass especially if serial measurements can be made from day to day to reduce variability. However, more work needs to be done on the theoretical basis of this method (Waterlow and Harper, 1974).

(iii) Hydroxyproline (OHPR) output. OHPR peptides are released and excreted in the urine in the course of collagen formation. OHPR is present only in collagen and the amount excreted is related to the rate of growth. In malnourished children who have ceased to grow, OHPR excretion is greatly reduced (Picou, Alleyne and Seakins, 1965, Whitehead, 1965). This has, therefore, been used as a test of nutritional state; its value is that it gives a measure of the rate of growth at one point in time and is therefore useful in cross-sectional surveys when serial measurements are not possible. The output of OHPR may be measured over a 24-hour period, or OHPR concentration may be related to that of creatinine in a single specimen of urine. When corrected for body weight this ratio is the hydroxyproline index (Whitehead, 1969 b). As with creatinine, there



is a good deal of day to day variability in OHPR output. The method of estimation is also tedious and on the whole this index is less popular now than it used to be a few years ago.

(iv) 3-methyl-histidine output. As far as is known this substance is present only in the myofibrillar proteins of muscle. It is formed by the methylation of histidine after it has been incorporated into peptides. Free 3-methyl-histidine is liberated in the course of muscle turnover. The amount excreted per day will therefore depend on the amount of muscle tissue and the rate of turnover of the muscle protein. Since both are reduced in protein depletion, excretion of 3-methyl-histidine should be a rather sensitive index of the state of protein nutrition.

Bagepalli, Rao and Nagabhushnan (1973) have shown that in children with PEM the output of this substance per kg body weight is reduced to about 33% of that of normal children. Young, Haverberg, Bilmazes and Munro (1973) found a decrease of about 40% in 3-methyl-histidine output in urine from adults fasted for 20 days, and showed that this decrease was much greater than the decrease in creatinine output. Though little information is available so far, this method seems very promising for the assessment of protein status.

(v) Some other amino acids. Schendel, Antonis and Hansen (1959) found a relative increase in aminoaciduria in malnourished children on their first visit to hospital compared with normal children. The following workers, quoted by Edozien (1965), confirmed the findings of Schendel: Sarrouy, Garcia, Cabannes and Clause (1957); Vis, Dubois, Loeb, Vincent and Bigwood (1958); Dubois, Vis, Loeb and Vincent (1959).

Edozien, Phillips and Collis (1960) observed an increased excretion of beta-aminoisobutyric acid (BAIBA) and of ethanolamine. Vis et al reported that in mild cases of PEM excess excretion of BAIBA and taurine

is due to tissue destruction and that in more severe cases, when the kidney itself is affected, hyperaminoaciduria extends to all urinary amino acids.

#### 1.3.4. Measurements made on serum or plasma.

There are about 24 plasma proteins which have fairly high concentrations in the blood and a host of others at low concentrations. Albumin falls in the first group. Proteins present in trace amounts include many of the protein hormones, blood clotting factors and various enzymes. The major protein fractions are albumin and globulins.

(i) Albumin is quantitatively the most significant plasma protein accounting for 50-60% of the total plasma protein. The concentration in normal serum is 3.5-4.5 g per 100 ml plasma. Two major functions of albumin are maintenance of blood osmotic pressure and transport of bile acids, fatty acids, drugs, vitamins, steroid hormones and calcium.

Reduction in plasma or serum albumin has been the classic 'test' for protein deficiency (Trowell, 1948; Brock, 1961; Weech, 1938-39; Allison, 1958). In the treatment of malnutrition it has been shown that plasma albumin concentration begins to rise (from its low level of about 2 g per 100 ml) before body weight or muscle mass are restored. It is evident therefore that a relatively normal albumin may co-exist with considerable depletion of body protein, particularly if the diet was deficient in energy as well as in protein.

From isotopic studies it has been shown that the synthesis of plasma albumin is sensitive to amino acid supply (James and Hay, 1968; Kirsch, Frith, Black and Hoffenberg, 1968). On a low protein diet the synthesis rate falls but a mechanism comes into play whereby a fall in the catabolic rate and a shift of albumin from the extra- into the intra-vascular compartment help to maintain the circulating albumin mass.

The effect of this homeostatic mechanism is that a small change in albumin concentration is accompanied by, or provokes a much greater change in albumin breakdown rate (Hoffenberg, Black and Brock, 1966). Waterlow (1963) had suggested many years ago that albumin breakdown rate is likely to be a more sensitive index of protein nutrition than the static measurement of albumin concentration. The measurement of the breakdown rate is not, however, applicable on a large scale.

Advances in technique have greatly enhanced the value of measuring plasma albumin concentration. Careful standardization of automated methods based on dye-binding techniques seems to have improved accuracy and reproducibility (Dumas, Watson and Biggs, 1971; Coward, Sawyer and Whitehead, 1971). In a most impressive study on children developing kwashiorkor, Whitehead, Froot and Poskitt (1971), Whitehead, Coward and Lunn (1973) and Lunn et al (1973) have shown that other parameters like growth hormone, insulin, cortisol and plasma amino acids change very little until albumin has fallen below 3 g per 100 ml of serum. This point can be regarded as the dividing line below which results are abnormal. Values in the zone of 3.0 and 3.5 g per 100 ml may be regarded as presumptive evidence of impending deficiency.

It is of great interest to note that in some of the measurements (Whitehead et al, 1971) the albumin concentration began to fall before there was any faltering in the weight gain.

The evidence at present seems to single out change in albumin concentration as the only biochemical change which is more sensitive than body weight.

(ii) Transferrin is a glycoprotein and is the principal beta-globulin of the plasma. It is responsible for the transport of iron and is synthesised in the liver. Studies on the isolated perfused liver have shown that the rate of synthesis of both albumin and transferrin

is reduced in livers taken from depleted animals (Morgan and Peters, 1971).

In studies on children with kwashiorkor in Nigeria, McFarlane, Ogbeide, Reddy, Adcock, Adeshina, Gurney, Cooke, Taylor and Mordie (1969) claimed that transferrin concentration in plasma is a particularly sensitive index of the severity of protein malnutrition, but Ismadi, Susheela and Rao (1971) in India were not able to confirm this. The synthesis of other glycoproteins, e.g. fibrinogen in the liver is stimulated by injury, by an unknown mechanism. It may well be that the concentration of transferrin in the plasma is not related to nutritional state.

(iii) Ceruloplasmin is a copper-containing, fast moving alpha-2-globulin. The concentration in normal serum ranges from 26-63 mg per 100 ml. It functions as an oxidase. Studies on malnourished subjects (Ismadi et al, 1971) indicate no significant changes in ceruloplasmin concentration in the plasma when compared with controls.

(iv) Pre-albumin is a plasma protein which is thought to bind tri-iodotyrosine ( $T_3$ ) and to a lesser extent thyroxine ( $T_4$ ). It is well established that vitamin A circulates as retinol bound to a specific transport protein (retinol-binding protein) and that this circulates as a protein-protein complex with plasma pre-albumin (Raz and Goodman, 1969; Raz, Shiratori and Goodman, 1970).

Ingenbleek, De Visscher and Ph. de Nayer (1972) have stated that pre-albumin appears to be a sensitive indicator of protein deficiency and that serum levels were raised by nutritional treatment. The workers have suggested that pre-albumin may allow the detection of pre-kwashiorkor and a differential diagnosis of the various forms of PEM. They claim that the radial immunodiffusion method for its estimation is simple, reproducible, accurate and inexpensive.

(v) Blood urea nitrogen (BUN). In a recent study Kumar, Chase, Hammond and O'Brien (1972) found BUN to be a useful screening test in the detection of reduced protein intake but it does not reflect nutritional status.

(vi) Enzymes in the plasma.

(a) The esterases, e.g. pseudocholinesterase, lipase, amylase, alkaline phosphatase are found to be more reduced in kwashiorkor than in marasmus (Srikantia, Jacob and Reddy, 1964; Waterlow, 1969). Results in respect of some of these enzymes have been found to be variable in the past (Dean and Schwartz, 1953; Baron, 1960; Edozien, 1961). The findings of Schendel, Hansen and Brock (1962) are of interest in that a statistically significant correlation was found between serum albumin and serum cholinesterase in a series of 127 children in various states of protein nutrition. Kumar et al (1972) have found amylase to be useful in the detection of reduced dietary intake of protein.

(b) Enzymes derived from cell destruction. McLean (1962) and Sandstead, Shukry, Prasad, Gabr, El Hifney, Mokhtar and Darby (1965) have shown that amino transferases and dehydrogenases may be increased in the plasma in severe malnutrition. McLean (1966) was able to show a clear correlation between serum alanine aminotransferases and mortality rate. Interpretation of changes in these enzymes is difficult because there are two opposing factors: damaged cells are found to increase the output of these enzymes, but there is also a reduced ability to synthesize enzyme in a damaged cell (McLean, 1966).

(c) Lysosomal enzymes. e.g. acid phosphatase, beta-glucuronidase and aryl sulphatase are present in increased concentrations in the plasma in malnutrition (Itterayah, Dumm and Bachhawat, 1967; Bengum and Itterayah, 1970). There is also an increase in the excretion of these enzymes in the urine. There is no information yet as to the importance of the level

of these enzymes in relation to the degree of protein nutrition.

(d) Ribonuclease. Two earlier works (Munro and Clark, 1960; Allison, Wannemacher, Banks, Wunner and Gomez-Brenes, 1962) led Albanese, Orto, Zavattaro and de Carlo (1971) to study the activity of this enzyme in the blood. In studies in man they found that serum ribonuclease activity was correlated with changes in nitrogen balance, the activity being higher when the balance was negative.

(e) Creatine kinase. This enzyme is found predominantly in skeletal muscle and spills over into plasma. Whitehead in 1968 found a low activity of this enzyme in the serum in patients with kwashiorkor. Balmer and Rutishauser (1968) believed that its measurement might be of value in assessing changes in muscle mass in malnourished children. In a recent study, Reindorp and Whitehead (1971) found the enzyme not to be of much value in the assessment of malnutrition because it was affected by other factors such as infection.

(vii) Plasma amino acids. Many workers have explored the possibility that plasma amino acid concentrations may provide information about protein status, mainly in subjects with PEM.

Richardson, Blaylock and Lyman (1953) suggested that much information about amino acid intake could be obtained from the study of the free amino acids of plasma. They suggested further that such information should be useful in kwashiorkor which is always accompanied by a deficiency of dietary protein. Cravioto (1958) found that the total amino acid concentration of plasma in kwashiorkor was low. Westall, Roitman, De La Pena, Rasmusser, Cravioto, Gomez and Holt (1958) confirmed this observation. Edozien, Phillips and Collis (1960) showed that the amino acid content was in fact reduced to about half of the normal value and the essential amino acids were reduced more than the non-essential ones.

Interest in the biochemistry of kwashiorkor was renewed at the beginning of the sixties by observations which point to the inability of patients to catabolise certain amino acids. Whitehead and Arnstein (1961) observed increased excretion of urocanate, an intermediate metabolite in the degradation of histidine - an observation which points to a defect in the metabolism of this amino acid. Derangement in the metabolism of phenylalanine has also been shown (Whitehead and Milburn, 1962).

In general a distorted amino acid pattern in the plasma is observed in kwashiorkor and is one of the main biochemical abnormalities. There is a striking reduction in the branched chain amino acids, leucine, isoleucine and valine (Whitehead, 1964). Such reduction has been confirmed by other workers: Snyderman, Roitman, Bayer and Holt (1964); Swendseid, Tuttle, Figueroa, Mukare, Clark and Massey (1966); Young and Scrimshaw (1968); Weller, Margen and Calloway (1969); Young, Tontisirin, Ozalp, Lakshmanan and Scrimshaw (1972); Weller, Margen, Calloway and Meissner (1973).

Whitehead (1964) devised the non-essential/essential (N/E) amino acid ratio to detect early protein deficiency (subclinical kwashiorkor). He used for field studies a simple method of one-dimensional paper chromatography. To avoid the difficulty of quantitative recovery he expressed the results as the ratio of four non-essential amino acids (glycine, serine, glutamine, and taurine) to four essential ones (valine, leucine, isoleucine, and methionine). Several workers have expressed faith in the N/E ratio for detecting protein deficiency (Swendseid et al, 1963; Ittyerah, Pareira and Dumm, 1965; Arroyave and Bowering, 1968; Simmons and Bohdal, 1970; Prasanna, Desai and Rao, 1971). Others have pointed out that the ratio gives negative results in children with uncomplicated calorie deficiency, i.e. marasmus (McLaren, Kamel and Ayyoub, 1965; Truswell, Wannenburg, Witmann and Hansen, 1966; Heard,



Kriegsman and Platt, 1969). The ratio has been found to be unreliable if dietary treatment has already been started or if there is folate deficiency (Jacob and Fleming, 1970) or administration of the drug cotrimoxazole (England and Cole, 1972).

Recently Bjornesjo, Mellander and Jagenburg (1968; 1969) have shown that a good indicator of inadequate protein intake might be the ratio of distribution of certain amino acids within the red cells and in the plasma. Azar, Sadre, Hedayat and Donoso (1972) supported this by stating that the measurement of the ratio of the distribution of glycine, alanine and valine between the erythrocytes and plasma may prove to be a more sensitive test of nutritional status.

Linblad (1970) has proposed the glycine/valine ratio as a useful measure of protein nutrition.

In their recent studies, Salem, Hegazi and Morcos (1973) found that the ratio, serine + glycine/threonine, gave a significant correlation with the severity of dietary protein deprivation whilst the N/E ratio did not.

The disadvantage of these ratios is that they may be altered by a change either in the numerator or in the denominator. The most consistent change which has been found in all the studies of plasma amino acids is a fall in the concentration of the branched chain amino acids, particularly valine, in protein deficiency. Therefore, the most logical test will be to measure the absolute concentration of the amino acids, singly or together. This was done by Lunn and co-workers (1973) in their studies of the development of kwashiorkor. They compared changes in plasma valine concentration with those in albumin and in several hormones. The measurements of valine were made by column chromatography, and this rather sophisticated equipment may not be available in many developing countries.



#### 1.4. The Purpose and Scope of the Thesis

This short summary of the biochemistry of PEM pin-points the problem: that at the present time no single biochemical determination has been found which most workers agree will pick out the minimally malnourished child (Nutrition Reviews, 1971).

Most workers who have attempted to produce animal models of PEM have used rather severe dietary restrictions of protein or energy or both (McCance and Widdowson, 1966; Widdowson and Whitehead, 1966; Heard et al, 1968; Grimble and Whitehead, 1969; Kumar et al, 1972). In many cases biochemical changes were produced, particularly a fall in serum albumin and a rise in N/E ratio. However, these experiments do not give much information about the effects of marginal protein deficiency. It was to fill this gap that the present experiments were designed.

To investigate this gap in our knowledge of marginal protein deficiency two requirements had to be fulfilled. Firstly, the development of methods and modifications to existing ones with a view to making them simple but sensitive and reproducible for use manually and on a micro-scale. The ideal biochemical test should require only a small (capillary) sample of blood or 'spot' sample of urine and a minimum of equipment. Secondly, the application of such methods to biological samples from apparently healthy animal populations living on diets differing only marginally in the levels of protein content. The idea was to find out if biochemical differences existed between such groups. If biochemical differences did exist then a pertinent question that follows will be, 'does it matter physiologically?'. This aspect of the problem could not be pursued in the present work but a study by Stewart (1973) on rats maintained on marginally deficient diets revealed marked behavioural disturbances.

The need is really pressing to distinguish between normal subjects on the one hand and individuals with mild-moderate malnutrition on the other in community studies.

CHAPTER TWO  
EXPERIMENTAL AND METHODS

## 2.1. CHEMICALS, ANIMALS, DIETS AND GENERAL PROCEDURE

Chemicals. All chemicals were obtained from British Drug House (Poole, Dorset, England) unless otherwise stated.

Animals. Litter-mate black and white hooded male rats were used. They were about 21 days old, average weight 47 g, when weaned. On arrival from the supplier the rats were weighed, earmarked and housed <sup>4</sup> to a cage in a room with a temperature of 25° and controlled humidity. Sheets of filter paper over saw dust served to collect faeces and spilled food and food intake was measured in all the experiments. During the first seven days the rats were put on a stock diet, 'Oxoid' cubes containing about 24 g protein (Herbert C. Slykes, Bewdley Ltd., Worcestershire), to accustom them to the new surroundings. They were then reweighed (49-92 g, mean 71 g) and allocated to groups in such a way that the mean weight in each group was about the same. The rats were weighed three times a week throughout each experiment.

Diets and Food intakes. Five dietary regimes, differing only in the protein content (8%, 12%, 15%, 18%, 24%) were fed ad lib. The diets were based on casein, maize starch, potato starch, arachis oil, dextrose, vitamins and salt mixtures (see appendix I for the composition of diets). Variations in protein content were achieved by altering the proportions of casein and starch.

At the beginning of each experiment, which lasted a period of five weeks, weighed quantities of food were put in weighed pots fitted with a wire cover to reduce scattering. The apparent food consumption by the four rats in each cage was measured. Material collected on filter paper in each tray was sieved to separate the spilt food powder from the faeces. The sieved food was weighed and

this weight was subtracted from the apparent food intake for the group. These measurements were made twice a week and the average weekly food intake calculated.

Blood sampling, killing and dissecting of animals. Blood (50-100 ul) was removed once a week by suction of the tip of the tail and collected in plain capillary tubes. One end of the tube was sealed with a flame and the serum separated by centrifugation at 3000 rpm (2500 g) for 15 minutes. Serum was deep-frozen until analysed. In general blood samples were taken in the morning, the rat having had access to food during the night. When fasting blood samples were needed food was removed overnight. Rats were injected with 2-4 uCi <sup>14</sup>C-creatine intraperitoneally one week before killed if radioactivity measurements were to be made on muscle.

At the end of the fifth week each rat was anaesthetized with ether, after a sample of blood from the tail had been obtained, and 1-2 ml of blood removed by syringe from the heart. The animal was then killed in ether; muscle samples were dissected out and deep-frozen until analysed.

Analyses. The components analysed in serum included total protein, albumin, transferrin, creatine kinase, cholinesterase, ribonuclease and branched chain amino acids. In muscle creatine kinase, creatine and nitrogen content were measured. The analytical techniques are given in the sections below. Some clearly routine methods (e.g. preparation of muscle digest for nitrogen determination, preparation of Nessler reagent, creatinine determination on muscle extract, measurement of serum ribonuclease) have been given in the appendix.

Where possible results have been expressed as mean and SEM or

SD for each dietary group and comparisons of means have been made by Student's 't' test. The 't' test also was applied to results obtained when two different methods were used for particular estimation.

Five animal experiments are reported here. In each experiment there were two or three dietary groups and four animals in each group. Altogether 52 rats were studied. Not all the biochemical measurements cited above were made in each experiment because methods were being developed while experiments were already in progress.

## 2.2. CHEMICALS METHODS

### 2.2.1 MEASUREMENT OF TOTAL SERUM PROTEIN

Wu (1922) first proposed the use of the Folin phenol reagent for the measurement of protein. Since then there have been a number of modified analytical procedures utilizing this reagent. The method commonly employed is that of Lowry, Rosebrough, Farr and Randall (1951). The assay time has been reduced to about 15 minutes (Schacterle and Pollack, 1973) but this involved heating for 5 minutes in a water-bath at 55°. In the method described below there is no need for heating but the assay time is 40 minutes. There are two distinct steps in this method: the first is the reaction between copper and protein in alkaline solution and the second is the reduction of phosphomolybdic-phosphotungstic reagent by copper-treated protein.

The method has been modified to be applied to amount of protein between 5-25 ug per final volume of 3.3 ml. The optical density was read at 750nm. At a protein concentration of more than 25 ug per tube the optical density reading was made at 500nm.

#### Reagents.

- A: 50 ml of 2% Na<sub>2</sub>CO<sub>3</sub> in 0.1N NaOH mixed with 1 ml of 0.5% CuSO<sub>4</sub> in 1% Sodium/potassium tartrate.
- B: Folin-Ciocalteu reagent. Stock was diluted 1:1 with water.

#### Method.

To 100 ul of test sample (containing 1.5-24 ug protein) 3 ml of reagent A were added and mixed. The mixture was allowed to stand for 10 minutes. Then 200 ul of reagent B was added and the absorbance read at 750nm

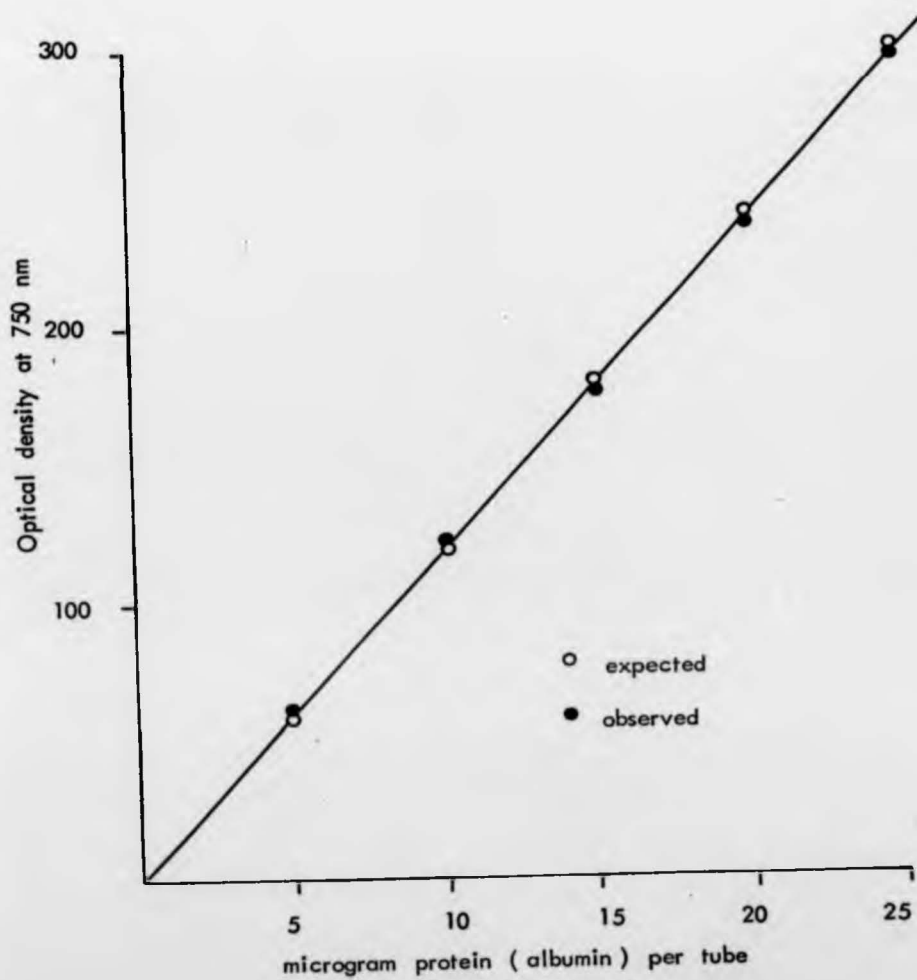
after 30 minutes. Note: The optimum pH of the reaction is 10.

At this pH the F-C reagent is active for a short time only so it is important to syringe reagent into the tube to effect complete mixing.

Standard Curve. The relationship between the absorbance and the protein concentration up to 25 ug per tube is shown in Fig. 2.

Albumins were used for constructing the standard curve.

FIG. 2 TOTAL PROTEIN DETERMINATION:  
ALBUMIN STANDARD CURVE





### 2.2.2. DETERMINATION OF ALBUMIN LEVELS IN SERUM

Salt fractionation, electrophoresis and dye-binding are the methods commonly used for routine measurement of serum albumin. Among the advantages of dye-binding are that it is rapid, involves few manipulations and does not require separate determination of total protein as in electrophoresis. Dye-binding techniques are also readily suitable for automation (Pemberton and DeJong, 1971; Doumas, Watson and Biggs, 1971; Coward, Sawyer and Whitehead, 1971; Westgard and Poquette, 1972).

Many of the disadvantages of dye-binding methods have been overcome by the use of bromocresol green, BCG (McPherson and Everard, 1972). Since Rodney first used BCG in 1965 it has been shown to be superior to 2-(4'-hydroxybenzeneazo)-benzoic acid (HABA) because of its specificity for albumin and freedom from interfering substances such as bilirubin, abnormal globulins and lipoproteins (Rodney, 1964; Bartholomew and Delany, 1966). Reilly, Sterling and Wallberg (1970) evaluated the BCG, bromocresol purple, and HABA dye methods on a Beckman Discrete-sample-analyser and found that the results of both bromocresol dye methods compared better than HABA with those of electrophoretic analysis. Good comparison with electrophoresis was also reported when BCG method was used with the DuPont Automatic-clinical-analyser (Westgard and Lahmeyer, 1972). Miyada, Baysinger, Notrica and Nakamura (1972) evaluated the "AlbuStrate" BCG method and found that icterus, lipemia and haemolysis interfered less than in the case of HABA dye. Doumas et al (1971) found that results with BCG agreed with those obtained by electrophoresis and salt fractionation.

In comparing abnormal serum albumin levels, Webster, Bignell and Atwood (1974) have pointed out that there was a discrepancy between

the results obtained by the automatic BCG method and by cellulose acetate electrophoresis, and that the latter agreed with two immunoprecipitation methods whereas the automated BCG method overestimated the albumin levels. Webster (1974) explained that the overestimation by the BCG method was due to the fact that alpha- and beta-globulins of the serum fraction also combined with the dye. He concluded by stating that BCG dye might not be specific for albumin in abnormal serum. However, the evidence suggests that the BCG method is valid for estimating albumin in normal serum.

The method used here is a slight modification of the BCG dye-binding method as used by Dumas and co-workers. The use of surfactant (Brij-35) reduces the absorbance of the blank, prevents turbidity and provides linearity up to 8 g per 100 ml. At low levels of albumin in normal rat serum (e.g. serum diluted 21 times) the binding capacity of the dye was not affected in any adverse manner.

#### Reagents.

Brij-35 solution. 30% solution of Brij-35 (polyoxyethylene lauryl ether) was made with warm water.

Albumin standards (2-8 g per 100 ml). These were prepared from solid Bovine albumin, fraction v (B.D.H.) or albumin stock standard solution (KABI). When solid albumin was used for preparing standards a correction was made for moisture content.

Succinate buffer, pH 4, 0.1M

Bromocresol green (BCG) solution. (a) Stock. 0.60mM. This was prepared by dissolving 419 mg BCG in 1 litre 0.001M NaOH. It was stored at 4°C (it keeps indefinitely).

(b) working dye solution. To 100 ml of (a) was added 300 ml of succinate buffer, followed by 1.6 ml of 30% Brij-35 (or 3.2 ml to

reduce blank O.D. to its minimum).

Method.

Measurements were made either with 25 ul undiluted serum or albumin standards, or with 200 ul of sample diluted 21 times (25 ul sample plus 500 ul water). The reason for this dilution was to enable duplicate measurements to be made on a sample of 25 ul, since micropipettes of smaller volume may not be accurate. 5 ml of working dye solution were added to each test sample in a test tube and mixed. The optical density was read at 628nm after 10 minutes.

The standard curves are shown in Fig.3. The optical densities of albumin standards with BCG over a period of five weeks showed remarkable consistency, the standard error of the mean being at the most 3% (Table 2).

Table 2  
Net OD of albumin standards (undiluted) over  
a period of five weeks

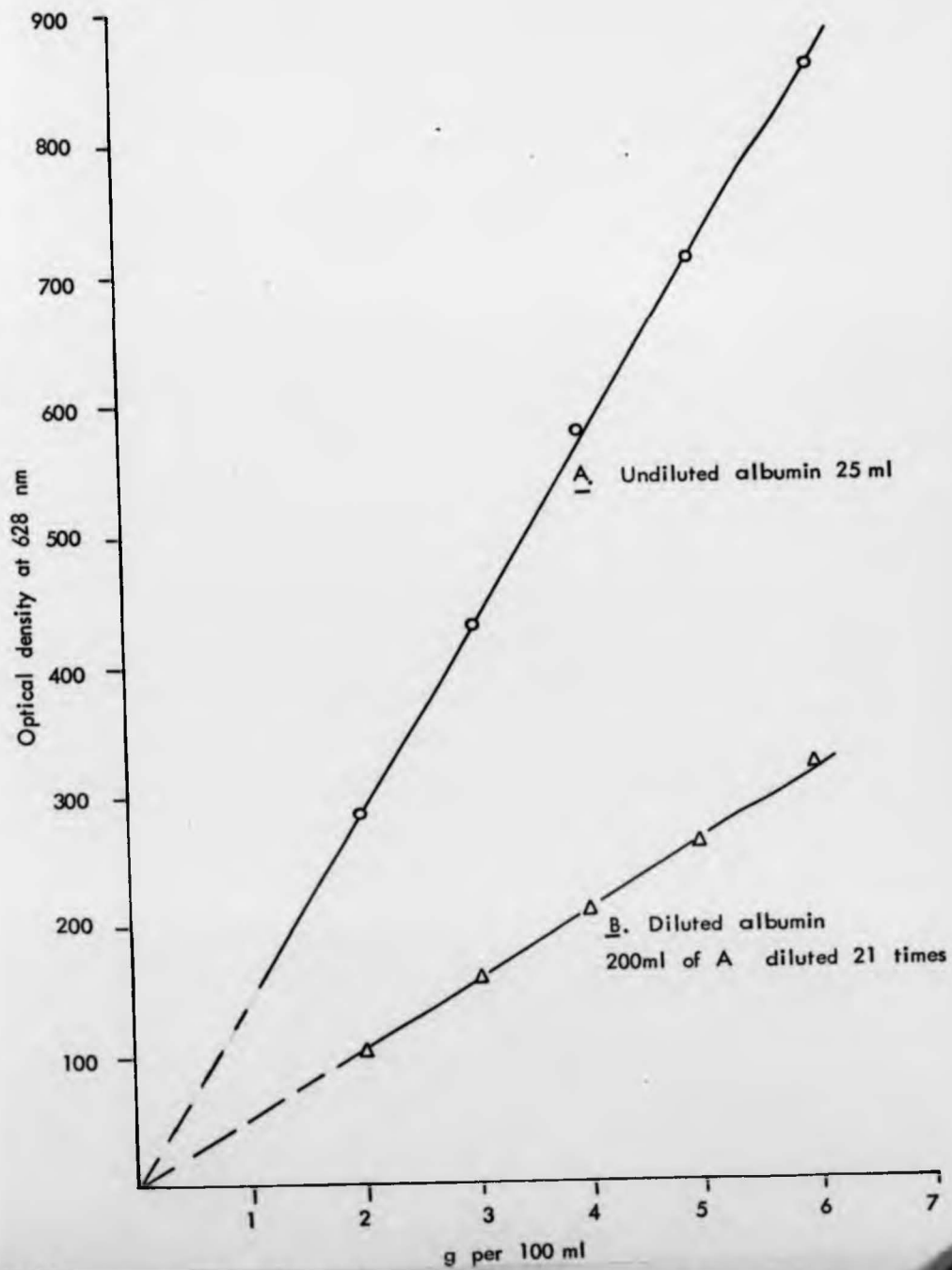
	<u>25/5/72</u>	<u>31/5</u>	<u>6/6</u>	<u>14/6</u>	<u>20/6</u>	<u>Mean</u>	<u>SD</u>	<u>% Error</u>
2 g/100 ml	285	282	286	272	286	282	5.9	2.0
3 "	424	431	429	405	428	423	10.6	2.5
4 "	550	569	572	544	575	562	14.0	2.5
5 "	682	697	715	680	715	697	17.0	2.4
6 "	804	831	858	816	859	833	24.1	3.0

(OD multiplied by 1000)

Effect of dilution. The ratio of albumin concentration to the OD in diluted and undiluted albumin standard was the same. This indicates that dilution does not affect the binding capacity of albumin to BCG.

For example, with standard 2 g per 100 ml, the following results were found:

FIG. 3 ALBUMIN STANDARD CURVE:  
NET ABSORBANCE / CONCENTRATION RELATIONSHIP



A.	25 ul of undiluted stand = 0.5 mg albumin.	Mean OD = 282
B.	200 of diluted standard = 0.19 mg albumin.	Mean OD = 105
<u>A/B</u>		<u>2.62</u> <u>2.69</u>

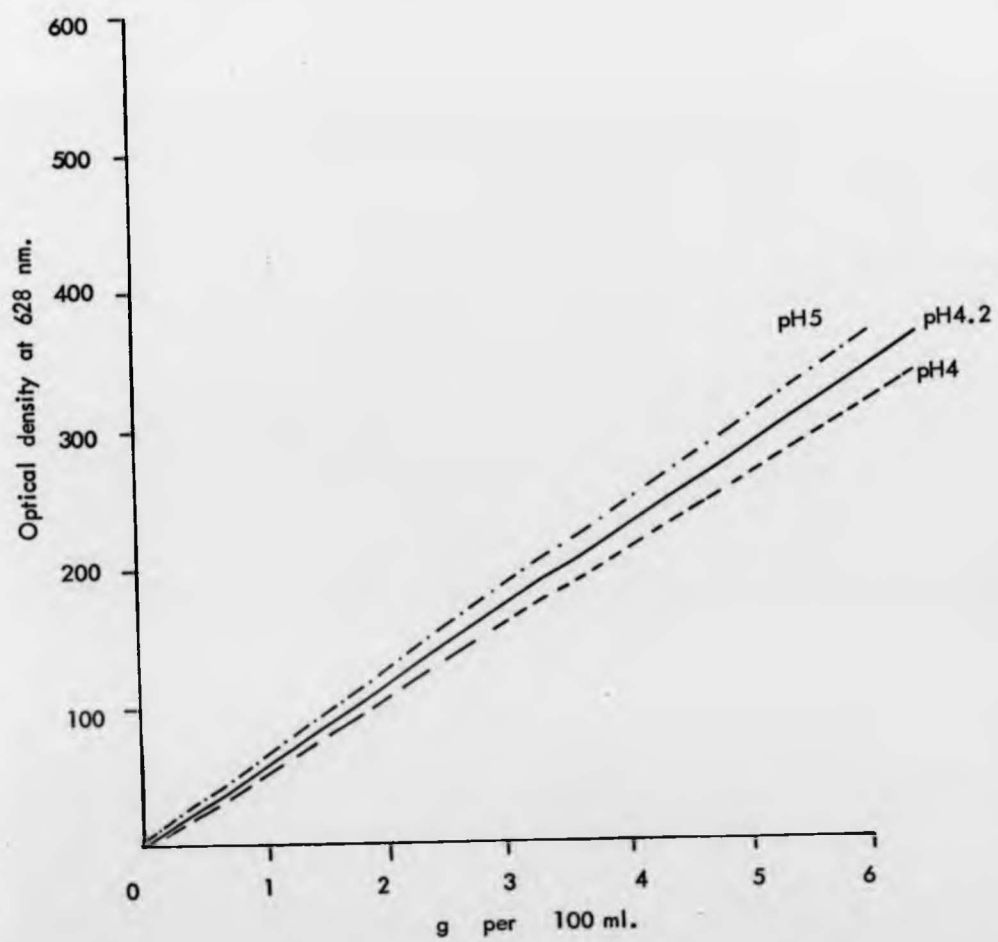
The two ratios are not significantly different.

The optical density of the blank at 628nm varies with the pH of the working dye solution and the amount of the Brij-35 solution added. Depending upon the source of BCG dye used for stock solution the OD of the blank has a range of 0.065-0.100 at pH 4. In order to fix the OD of the blank at its minimum around 0.065, double the recommended volume of the Brij-35 solution should be added to the BCG working dye solution, i.e. 3.2 ml instead of 1.6 ml.

The effect of pH on optical density is shown in Fig.4. It appears that at higher pH there is a slight increase in dye binding.

FIG. 4 ALBUMIN STANDARDS

CONCENTRATION / ABSORBANCE RELATIONSHIP AT 3 DIFFERENT pHs



### 2.2.3. MEASUREMENT OF SERUM TRANSFERRIN.

Transferrin is the iron binding protein found in human serum. Until quite recently transferrin levels in the serum have been determined primarily on the basis of serum iron levels. This is usually calculated by measuring the amount of iron which the serum can bind, and expressed as total iron binding capacity (TIBC) ( $\mu\text{g}$  of Fe/100 ml). Measurement of TIBC generally involves oversaturation of the serum with iron in vitro with subsequent removal of the excess unbound iron by means of magnesium carbonate (Ramsay, 1957; Brozovich, 1968), an ion exchange resin (Peters, Giovanniello, Apt and Ross, 1956; Charlton, Hardie and Bothwell, 1965), charcoal coated with haemoglobin (Herbert, Gottlieb, Lau, Fisher, Gevitz and Wasserman, 1966) or sephadex G25 after treatment of the iron-enriched serum with 1,10-phenanthroline and  $\text{Na}_2\text{S}_2\text{O}_4$  (Nielsen, 1968).

The TIBC method of determining transferrin may be misleading since iron is not only bound to transferrin but also distributed over other serum components. It is therefore likely that TIBC is not identical with transferrin content immunochemically determined (Van der Heul, Van Eijk, Wiltink and Leijnse, 1972).

The immunochemical measurement of transferrin has been found to be simple, reliable and specific. The principle of the method is that the sample containing transferrin is placed in a well in agar gel containing specific antibody to transferrin. The antigen diffuses into the agar and forms a precipitin ring. The diameter of this ring is directly related to the concentration of the testing antigen. The method described here is a modification of the one adopted by Mancini, Carbonara and Heremans (1965).

### Reagents.

Barbiturate buffer pH 8.6 containing 0.05% sodium azide.  
Special noble agar (Difco). Rat transferrin and antibody to rat transferrin (kindly supplied by Dr. A.H. Gordon of the National Institute of Medical Research, Mill Hill, London, U.K.)

### Method.

Preparation of the agar. 3 g of the agar were dissolved in 100 ml of the buffer. The suspension in a beaker was placed in boiling water with continuous mixing until all the agar dissolved.

Agar-antibody mixture. The gel and the antibody were mixed in the proportion, 14 ml : 0.16 ml respectively. It was essential that the gel be cooled to a temperature of about 45° before the antiserum was added. Higher temperature tended to denature the antiserum.

Preparation of antibody-agar plate. About 3 ml of the warm gel were pipetted into a plastic plate (2.5cm x 7.5cm by 1mm deep) and the gel spread itself across the plate. It was allowed to cool down to room temperature. Circular wells were punched out in the gel using a capillary tube of 2 mm bore. The plate was covered with polythene film and stored in a refrigerator at 4° until used.

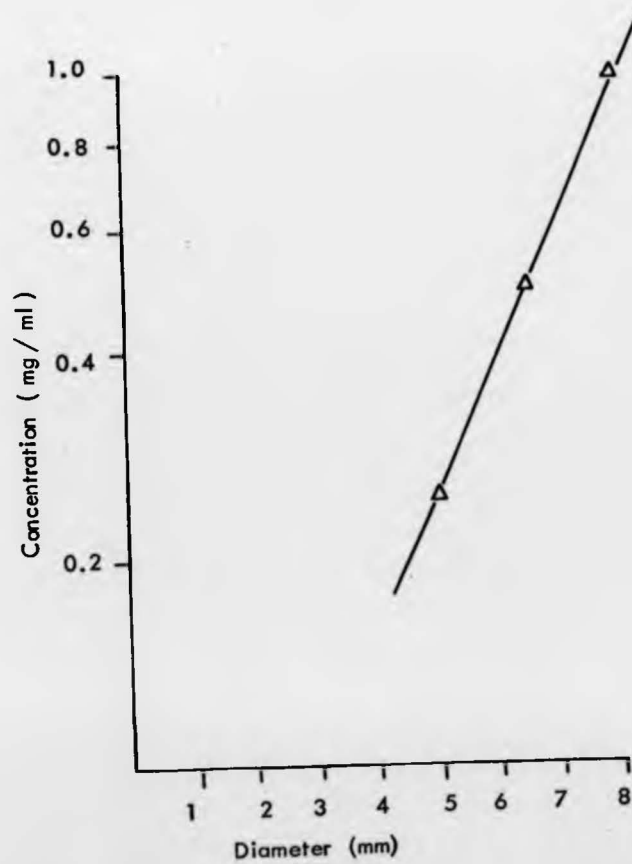
Application of serum sample. Each plate had three wells for standards (in three different concentrations) in addition to the test specimens of serum. The plate was either kept at room temperature for 8 hours or incubated in an oven at 37° for 4 hours for the antibody/antigen reaction to go to completion.

Measurement of ring size. The diameter of the precipitin ring was measured with hand magnifier with an attached measuring device. The clarity of the rather indistinct precipitin rings was improved by dipping the plate in 7.5% acetic acid for 2 minutes, and rinsing in distilled water.



Standard Curve. The diameters of the precipitin rings of the three standards were plotted on a 2-cycle semilogarithm graph paper on the horizontal (arithmetic) scale and the concentrations of the reference standards on the vertical (logarithmic) scale. A straight line of best fit was drawn through the points. The concentrations of the unknown specimens were determined by reference to the standard curve (Fig.5).

FIG. 5 TRANSFERRIN IMMUNOASSAY:  
CONCENTRATION OF TRANSFERRIN vs. DIAMETER OF  
PRECIPITIN RING



#### 2.2.4. DETERMINATION OF AMINO-NITROGEN

The method is based on the reaction between ninhydrin and amino-nitrogen as given by Jagenburg (1959), modified for use on micro-scale.

##### Reagents.

0.2M citrate buffer, pH 5.0

KCN-Ninhydrin. 50 ml 0.002M KCN in methyl cellosolve mixed with 10 ml of 5% (w/v) ninhydrin in methyl cellosolve, just before use.

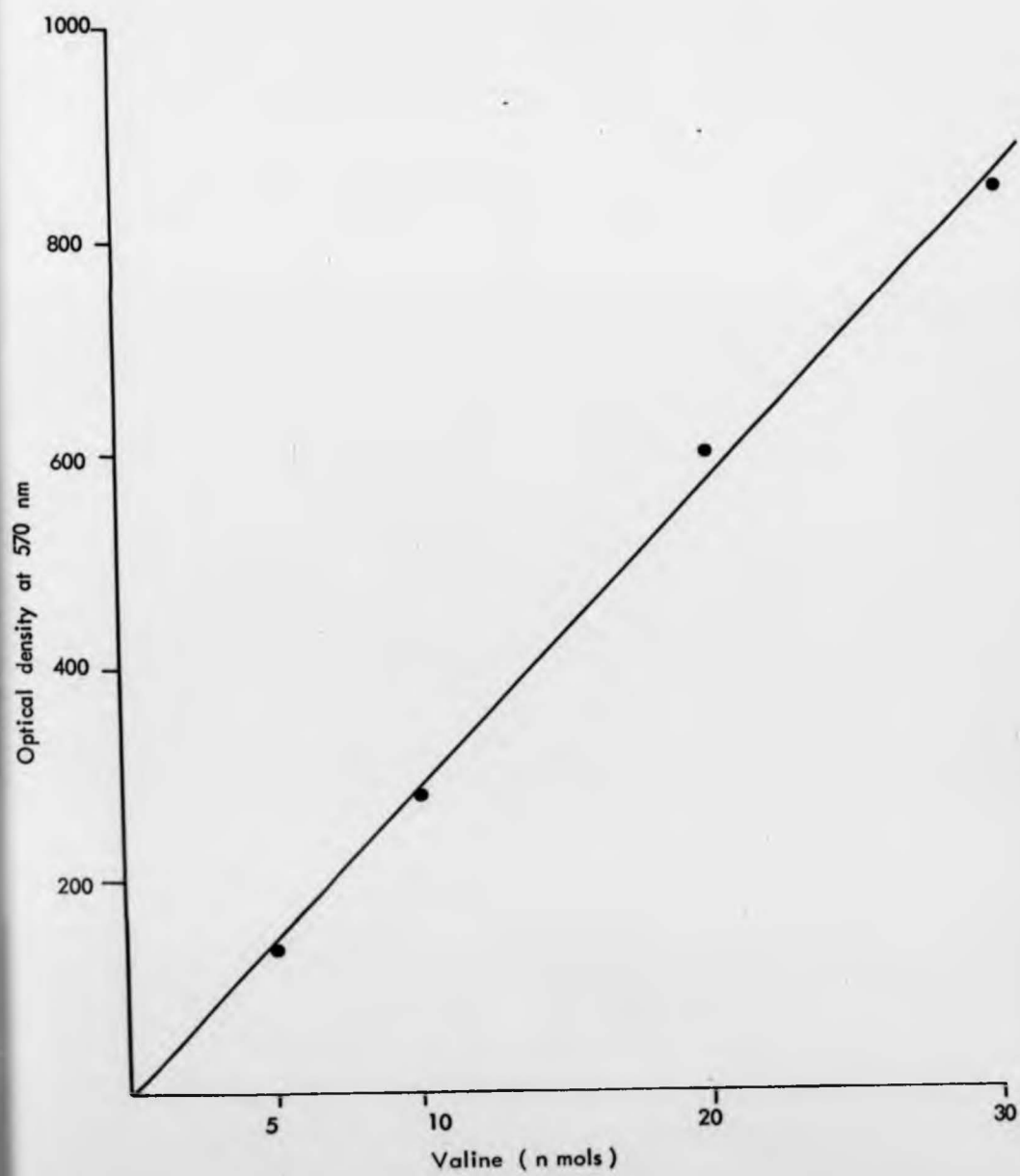
Ethanol-water mixture. Equal volumes of 98% ethanol and distilled water.

##### Method.

To a sample of up to 100 ul volume, containing 2-30 nmols amino-N, were added 100 ul buffer followed by 100 ul KCN-Ninhydrin. The mixture was heated in a boiling water bath for 15 minutes, the tubes being capped or covered with tin foil. The tubes were cooled under running tap water and then centrifuged at 3000 rpm for 5 minutes to bring down any drop of liquid which had condensed on the walls. 500 ul of ethanol-water mixture were added and the absorbance read at 570nm in microcuvettes.

A standard curve made with valine is shown in Fig.6.

FIG. 6 AMINO-N DETERMINATION



#### 2.2.5. DETERMINATION OF SERUM CHOLINESTERASE

Serum cholinesterase was measured by a modification of the method of Garry and Routh (1964) and of Uete, Miyamoto, Ohnishi and Shimano (1972). The cholinesterase acts on acetylthiocholine to release thiocholine, which reacts with 5:5-dithiobis-(2-nitrobenzoic acid) to produce a yellow colour. The activity was measured after incubation at 37° for 3 minutes. The reaction was stopped by the addition of guanidine sulphate. The difference in absorbance between the blank and the test sample was measured at 412nm and compared with a glutathione standard. A volume of 5-10 ul of serum was sufficient for the determination.

##### Reagent.

DTNB (Ellman's reagent) (5:5-dithiobis-2nitrobenzoic acid) buffer,  
pH 7.4 : 25 g DTNB were dissolved in 62.5 ml of 0.2 M Tris solution. 1.66 g NaCl were added, followed by 100 ml of 0.1 N HCl. The volume was made to 250 ml with distilled water.

Acetylthiocholine iodide. 5.2 mg/ml i.e. 0.018 M. This constituted the substrate and was stable for one week if kept at 4°.

Quinidine sulphate, 0.5%. This was the inhibitor.

Glutathione standard. The stock solution contained 0.25 mg/ml (8.13 x 10<sup>-4</sup>M).

##### Method.

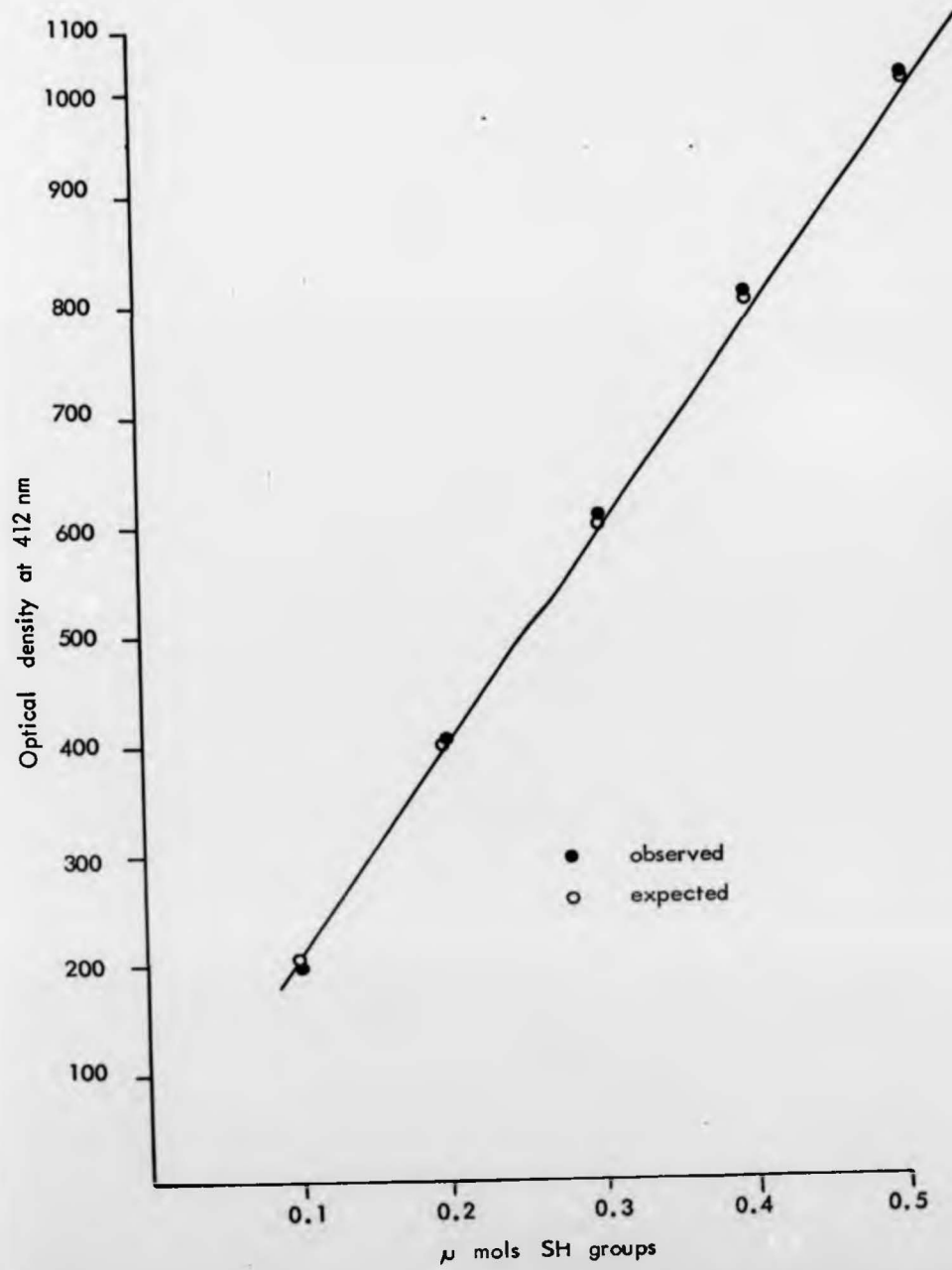
Into each of three tubes, two for the test sample and one for the blank, were put 4 ml of buffer. The solution was allowed to equilibrate for two minutes in a water-bath at 37°. 10 ul of serum were added to each tube followed by 1 ml of quinidine sulphate (inhibitor) to the blank only. 0.5 ml acetylthiocholine was added to each tube and mixed. Three minutes after the addition of the substrate the reaction in

the test samples was stopped by the addition of 1 ml of the inhibitor. The tubes were cooled to room temperature and absorbance read at 412nm. It was absolutely essential that the volumes of the reagents and samples were measured accurately.

Glutathione standard curve. 100-500 ul of the stock glutathione solution were added to tubes containing 4 ml of DTNB buffer. Each tube was diluted to a final volume of 5.5 ml and mixed by inversion. The absorbance was read at 412nm. A standard curve is shown in Fig.7. The colour developed by the standard was very constant from one run to another. Over a period of 5 weeks the OD per umole glutathione ranged from 1.9 to 2.0.

Calculation of enzyme activity. The enzyme activity was expressed as umols SH groups liberated in 3 minutes by 1 ml of serum. The difference in absorbance between the test and the blank was compared with the standard curve to obtain the concentration of the SH groups liberated from the acetylthiocholine substrate by the test sample.

FIG. 7 GLUTATHIONE STANDARD CURVE



### 2.2.6 MEASUREMENT OF MUSCLE MASS

Muscle mass was measured in two ways, both of which depend on the assumption that all the creatine in the body is in muscle. Waterlow et al (1972) found that in the rat 95% of the total body creatine was in the carcass, after removal of visceral organs and skin. In the first method,  $^{14}\text{C}$ -creatine was injected, and after several days had been allowed for equilibration with muscle creatine, measurements were made of the total radioactivity in the carcass and of radioactivity per gram muscle. In the second method muscle mass was calculated from the creatine content of muscle and the total amount of creatine in the carcass.

#### Procedure.

Black and white hooded rats, about 7 weeks old and weighing between 150-200 g, were injected intraperitoneally with 2-4  $\mu\text{Ci}$   $^{14}\text{C}$ -creatine (obtained from Radiochemical Centre, Amersham, England) one week before they were sacrificed.

Each rat was weighed and killed with ether. The skin and the viscera were removed and discarded and the remaining carcass weighed. Two samples (A and B) of wet muscle, about 1 g each, were cut out of the hind leg, and put into weighed vials. About 5 ml of cold 5% TCA was added to A. B was kept deep-frozen until used for analysis (by the enzymatic method) of creatine and creatine kinase (see below). The rest of the carcass was put into a weighed 500-ml beaker, and about 200 ml of cold 5% TCA added.

#### Preparation of muscle and of carcass extract for colorimetric estimation of creatine and of creatinine.

Muscle sample A was cut up with a pair of scissors followed by



complete mashing with a glass rod, and the homogenate centrifuged. The supernatant was poured off into a 10-ml graduated tube and the residue further washed with 5 ml of cold 5% TCA and the washings added to the first supernatant. The final volume was made up to 10 ml with distilled water and used for the analysis. The muscle residue was used for determination of non-collagen nitrogen (see appendix II).

The rest of the carcass was mashed with a mixer and the final volume of the homogenate measured by weighing. It was centrifuged and an aliquot of the supernatant used for analysis.

Fat and trichloroacetic acid were removed from the supernatant of both muscle and carcass by extracting with ether. The ether-extracted supernatants were used for measuring counts and for chemical determination of creatine and creatinine. For measurement of creatinine (appendix III) 100 ul of the extract was used and for creatine 100-200 ul of the extract diluted ten times with water.

By keeping the muscle samples cold during the preparation of the extracts conversion of creatine to creatinine was avoided. However, with the much larger carcass it was impossible to maintain a low enough temperature during the process of homogenisation (since no cold room was available). Some conversion of creatine to creatinine occurred, and therefore both had to be estimated to obtain the original total creatine content.

Preparation of muscle extract for creatine and creatine kinase assay (enzymatic method)

This was based on the method of Kuby, Noda and Lardy (1954), slightly modified to be used for about 1 g of muscle for extraction of creatine kinase.

The back and leg muscles of the rat were excised immediately after it had been anaesthetized with diethyl ether, and the muscle was put on ice. About 1 g of the muscle was weighed and put into a 10-ml tube containing 4 ml of tris-buffer pH 7.6. The muscle was cut up into pieces with a pair of scissors and carefully mashed with a glass rod. The precipitate was centrifuged down and the supernatant poured off. The precipitate was washed with 2-ml lots of the tris-buffer and the supernatant on centrifuging added to the first supernatant until a volume of 10 ml was obtained. Fat was removed by extraction with ether. The supernatant was found to have creatine kinase activity of about 30  $\mu\text{moles}/\text{min}/\text{g}$  (see below)

#### Radioactivity.

200  $\mu\text{l}$  of muscle extract and of carcass extract were counted. The dry weight of muscle extract on the planchette ranged from 8-10.4 mg and that of carcass from 14.6-16 mg. Count-rate of 1025-2185 cpm per ml were obtained with muscle extract, and between 2700 and 5000 cpm per ml with carcass extract.

#### Calculation of muscle mass.

##### (a) From radioactivity.

$$M = \frac{T_c \times W}{T_m} \quad \text{where } M = \text{muscle mass (g)}$$

W = weight of muscle sample (g)

T<sub>c</sub> = total counts in carcass

T<sub>m</sub> = total counts in muscle sample

##### (b) From creatine + creatinine

$$M = \frac{C_c \times V_c \times W}{C_m \times V_m} \quad \text{where } C_c, C_m = \text{creatinine + creatine content (}\mu\text{moles/ml) in solutions from carcass and muscle; } V_c, V_m = \text{volume of carcass and muscle solutions.}$$

### 2.2.7. MEASUREMENT OF CREATINE IN SERUM AND IN MUSCLE EXTRACT

Two methods for measuring creatine were used: (i) direct colorimetric assay - a modification to the method of Ennor and Stocken (1948) and (ii) an enzymatic method according to Tanzer and Gilvarg (1959).

#### Creatine (colorimetric assay).

##### Reagents.

- A: Alkali solution: 30 g of NaOH and 64 g of  $\text{Na}_2\text{CO}_3$  in 1 litre.  
B: 0.3 g alpha-naphtol in 50 ml of alkali solution A.  
C: 1% diacetyl diluted with water 1 in 100 before use.  
D: Mixed reagent. This was made up by adding equal parts of reagents B and C.

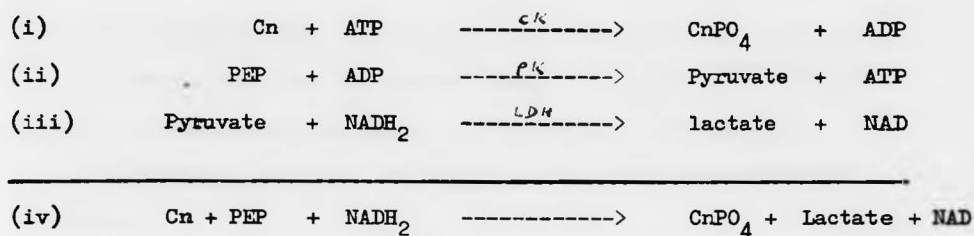
##### Method.

3 ml of reagent D were added to 100 ul of sample (containing up to 0.05 umols creatine) and optical density measured at 520 nm after 30 minutes. The pink colour is stable and the calibration curve is linear with amount of creatine up to at least 0.05 umols.

#### Creatine (enzymatic assay)

The enzymatic method for creatine determination is more specific than the colorimetric method, and its sensitivity is about the same. A modification of Tanzer and Gilvarg's method was used to measure both creatine and creatine kinase in serum and in muscle extract.

Principle of method. By coupling three-enzyme catalysed reactions (i), (ii), (iii), a summed reaction (iv) is obtained:



The conversion of Cn to CnPO<sub>4</sub> is accompanied by the oxidation of an equimolar amount of NADH<sub>2</sub>. NADH<sub>2</sub> becomes the indicator of the reaction and the change of its absorbancy at 340nm is the direct measure of the amount of Cn in the assay. To start the reaction creatine kinase was added in a highly purified form to the system which already contained the reagents below. The reaction was allowed to go to completion, the OD being measured every 2 minutes. The reaction was finally finished in about 20 minutes.

The conditions for the reaction included a temperature of 25°, pH 9 and solutions kept in icebath until used.

Reagents.

Clycine buffer, pH 9	(0.5 M)	5 ml
MgSO <sub>4</sub>	(0.1 M)	1 ml
ATP	(14 mg/ml)	1 ml
PEP	(5 mg/ml )	1 ml
NADH <sub>2</sub>	(2 mg/ml )	1 ml
LDH	(5 mg/ml )	200 ul
PK	(10 mg/ml)	50 ul

A common medium (CM) was made of the reagents by mixing the volumes indicated. Into a 4-ml silica cell, light path 1 cm, were put 1 ml each of the CM and of distilled water. As a standard, creatine

substrate was added in a volume of 50 ul. The cuvette was allowed to stand until there was no further change in absorbancy at 340nm. 0.3 mg creatine kinase was added and the change in OD measured.

The amount of creatine in the sample was calculated from the relation:

$$\text{creatine (umols)} = \frac{\text{OD}}{E_{\text{NADH}}} \times \text{volume in cuvette.}$$

$$E_{\text{NADH}} = 6.22 \times 10^6 \text{ cm}^2/\text{mole}$$

(Umbreit, Burris and Stauffer, 1964).

A typical example of creatine estimation

Into each of four silica cells C1, C2, C3, and C4 were put

1 ml common medium

1 ml distilled water

50 ul Cn standard (0.1 umols)

The reagents were mixed with exception of creatine kinase (CK). Each cuvette had a final volume of 2.1 ml. By waiting until there was no further change in absorbancy at 340nm any ADP or pyruvate in the system would be consumed. The CK was then added to the system and the OD changes measured (every 2 minutes) until they stopped or became linear with respect to time. The blank C1 was always set at OD of 0.600 before each reading of C2, C3 and C4.

Results: (0.1 umols Cn in each cuvette)

<u>Time</u>	<u>C1</u>	<u>C2</u>	<u>C3</u>	<u>C4</u>
initial OD	0.600	0.600	0.600	0.600
18 min.	0.600	0.293	0.293	0.349
<u>ΔOD</u>	<u>0.0</u>	<u>0.307</u>	<u>0.307</u>	<u>0.296</u>

The change in absorbancy of the reaction is shown in Fig.8. The reaction was complete after 18 minutes. The mean observed change in OD was 0.303. The theoretical change in OD with 0.1 umol creatine is :  $\Delta OD = \frac{0.1 \times 6.22}{2.1} = 0.296$

There was thus satisfactory agreement between the observed and the expected change in optical density.

Typical example of creatine kinase measurement.

The same assay system was used to measure creatine kinase activity, except that excess of substrate, 0.4-0.16 umols creatine, was added. The reaction was started by adding the sample to the common medium in the cuvette. The change in OD was measured in a Unicam SP 800 ultraviolet recording spectrophotometer, and the activity of the enzyme calculated from the slope over the first two or three minutes.

Establishment of optimum conditions for measurement is shown in Fig.9 (A, B, C, and D). The reaction could be made to go to completion in about three minutes if 0.2 umols Cn (i.e. 25 ul of 8 umols/ml Cn) and 0.15 mg of CK (i.e. 25 ul of 6 mg/ml CK) were added to the common medium irrespective of order, CK or Cn first (A and B). The conditions in A and B give a suitable time period for measurement of the slope and hence the activity of the enzyme.

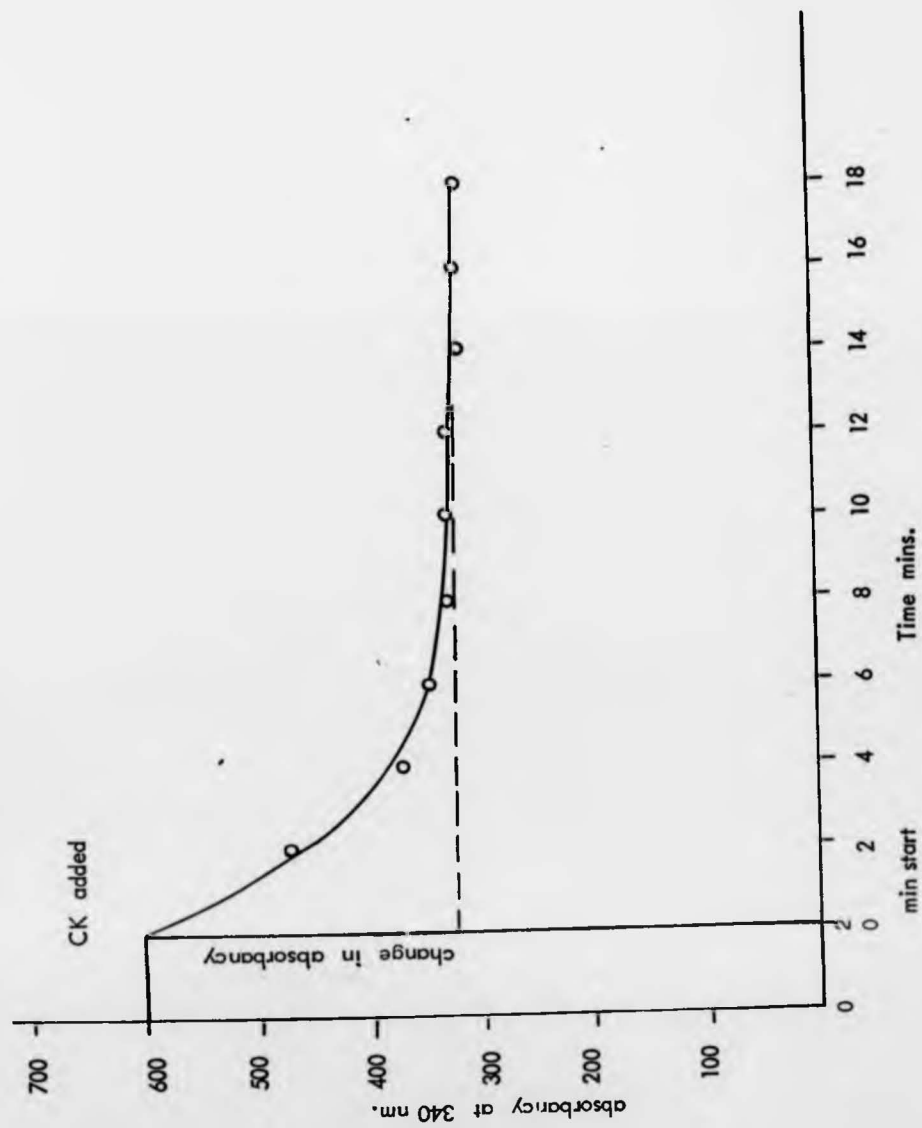
Higher concentrations of CK (0.3 mg) and of Cn (0.4 umols) sped up the reaction rate (C and D). In D the reaction was completed in just over a minute, and the slope was so steep that the rate and therefore the activity of the enzyme could not be calculated with any great accuracy. One and half minutes was the completion time in C.

Examples of rat serum creatine kinase activity are shown in E and F (Fig.10). 25 ul of rat serum (native) was added to a common medium containing 0.4 umols creatine. In one case there was a higher absorbancy

recorded immediately the serum was added to the common medium (F). This might be due to some chromogens present in the serum in F but not in E. However, it did not affect reaction rate. The change in OD per minute was found to be about 0.051 giving an activity of 0.07  $\mu\text{mols}/\text{min}/\text{ml}$  (E and F).

Graph G in Fig.10 illustrates the creatine kinase activity in rat muscle extract (diluted and undiluted: see above for preparation). The common medium contained 50  $\mu\text{l}$  of 0.16  $\mu\text{mols}/\text{ml}$  creatine. The changes in OD per minute were 0.210 and 0.110 for the undiluted and diluted extracts respectively (i.e. 2.8 and 1.48  $\mu\text{mols}/\text{ml}/\text{min}$ ). The activity per gram muscle was calculated as 30  $\mu\text{mols Cn}/\text{min}/\text{g}$ .

FIG. 8 CREATINE ESTIMATION





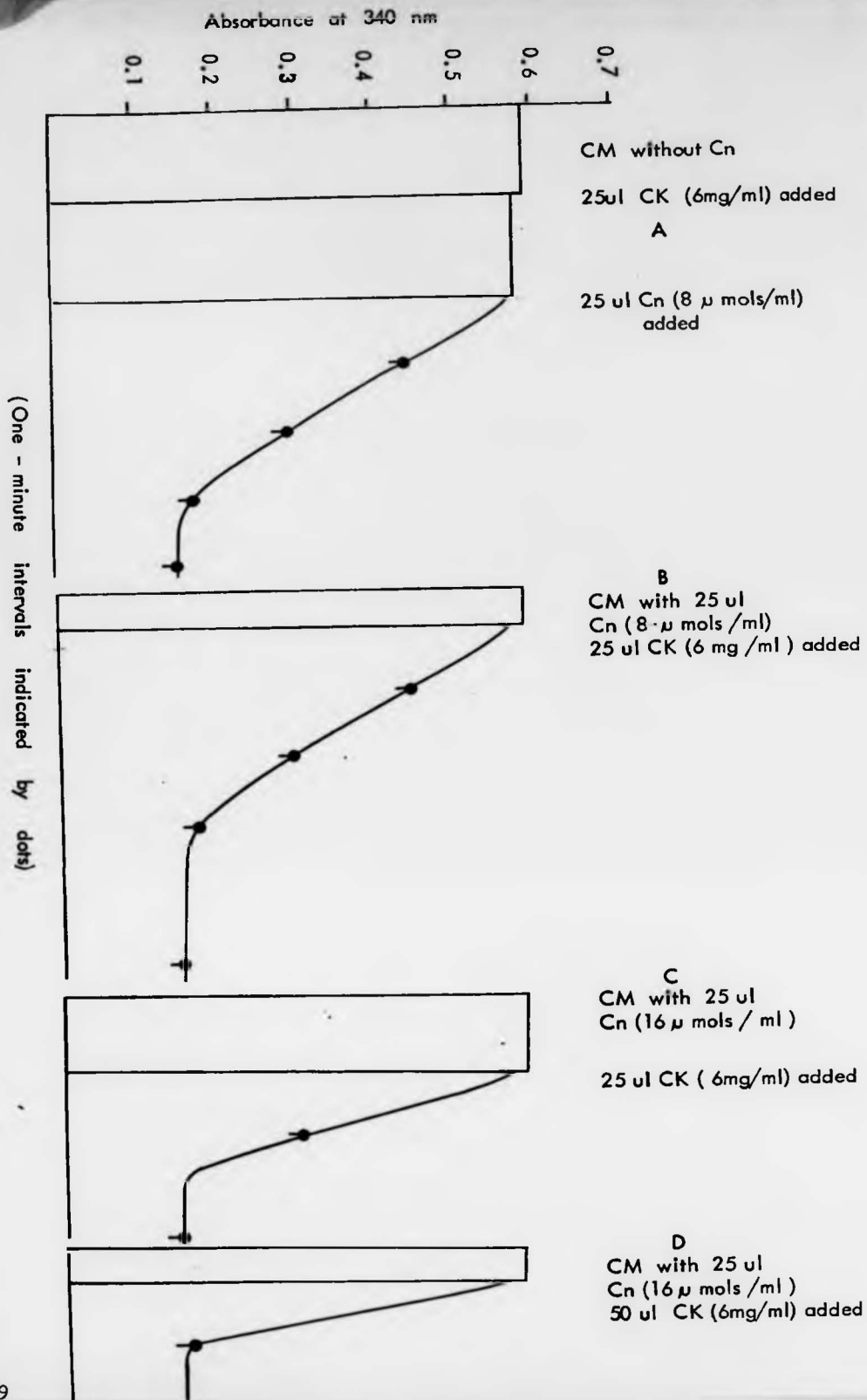
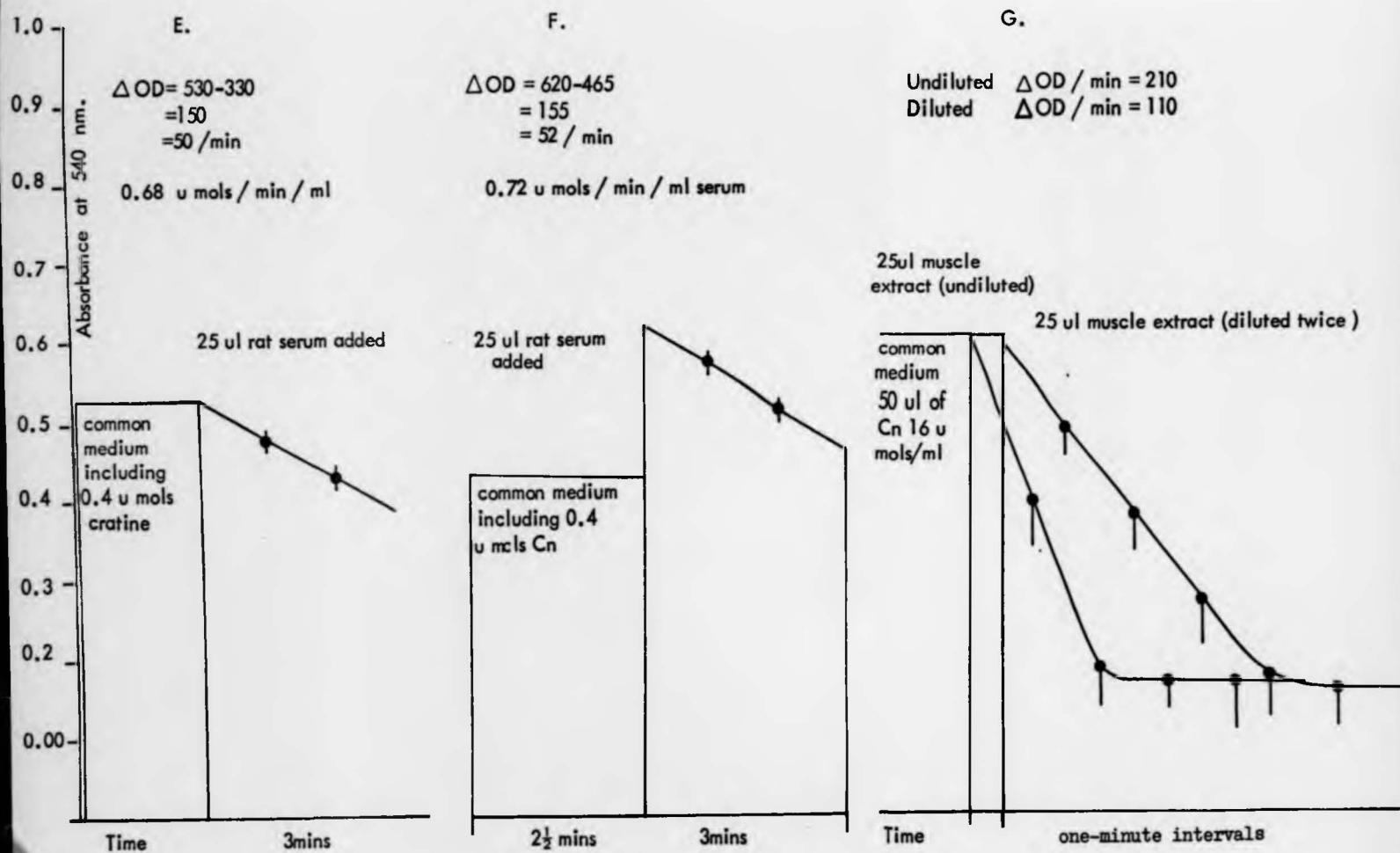


FIG. 9 MEASUREMENT ( ENZYMATIC METHOD ) OF CREATINE AND CREATINE KINASE STANDARDS ( Establishment of Volumes )

FIG. 10 RAT SERUM CREATINE KINASE ACTIVITY



C H A P T E R   T H R E E

DEVELOPMENT OF METHOD FOR THIN LAYER CHROMATOGRAPHY OF  
BRANCHED CHAIN AMINO ACIDS IN SERUM ON THE MICRO-SCALE

### 3. MEASUREMENT OF SERUM BRANCHED CHAIN AMINO ACIDS

This problem may be considered in three parts: (1) separation by thin-layer chromatography (TLC); (2) tests of recovery with radioactive amino acids; (3) quantification by the use of ninhydrin.

#### 3.1. Separation by TLC

In the serum of patients with kwashiorkor the concentrations of the essential amino acids are depressed, particularly those of valine, leucine and isoleucine (Holt et al, 1958; Whitehead and Dean, 1964; Saunders et al, 1967) and it has been suggested that this is a possible early indicator of sub-clinical states of the disease (Whitehead, 1965). It will be useful to look at the changes in the concentrations of these amino acids by methods such as chromatography, which can be made simple and reproducible, are reasonably quickly carried out and which might therefore be used to study marginal protein nutrition in individuals or populations.

Attempts have been made in the past to separate amino acids in either deproteinised or non-deproteinised serum or plasma by paper chromatography (PC) or TLC. Whitehead (1965) and McEvoy-Bowe and Thevi (1966) used deproteinised serum and PC. Efron, Young, Moser and MacCready (1964) used PC with non-deproteinised serum and the procedure included desalting and autoclaving of the sample. Whitehead (1968) separated amino acids in deproteinised serum with two-dimensional PC. Culley in 1968 used non-deproteinised serum with TLC; he then introduced a variation in his method which utilised blood impregnated on paper discs.

The examples cited have the following attributes in common; they are time-consuming, with no attempt to quantify any of the amino acids and not less than 50 ul sample was used for a single analysis.

Recently Allen et al (1972) have used non-deproteinised serum with TLC to look at changes in valine, leucine and isoleucine concentrations but the results were semiquantitative only.

An attempt was made to develop a TLC method for separation which would have the following characteristics: (i) it would be efficient in separating the branch chain amino acids in 5 ul serum; (ii) it would be uni-dimensional rather than two-dimensional; (iii) it would employ the simplest possible solvent which would not interfere with subsequent quantification of the separated amino acids.

(a) Samples used for separation

Amino acid standards. Amino acid solutions in water were used, in concentrations ranging from 0.05 to 2.0 umols/ml.

Serum (deproteinised). 4 ml 90% alcohol were added to 50 ul serum in a small tube, stirred with glass rod, and left to stand for 20 minutes with occasional mixing. After centrifuging at 3000 rpm (2500 g) for 15 minutes, the supernatant was poured off into a small beaker, and dried under hot fan to evaporate the alcohol. The residue was dissolved in 50 ul of 10% isopropanol, and an aliquot was spotted on the chromatoplate.

Serum (non-deproteinised). 5-10 ul of serum were spotted without any prior treatment.

Whole serum (non-deproteinised serum) as compared to deproteinised serum.

TLC separation in each case was of about the same efficiency, but longer time and larger volumes than 5 or 10 ul serum were needed for deproteinised samples. After deproteinisation, picking up the

residue of free amino acids for spotting was rather cumbersome.

(b) Preparation of chromatoplates.

20 x 20 cm glass plates were used, coated with silica gel G with a Stahl-type TLC-coater.

Five clean dry plates were laid in a row on the aligning tray of the Stahl-type coater and held firmly in position. The coater was then levelled. The surface of the plates was swabbed with acetone to prevent the formation of air bubbles settling on the plates when the gel slurry was spread on them. A rectangular trough was placed at one end of the coater and adjusted to a slit-width of 0.25 mm. The composition of the gel slurry was 1 part of coating material (silica gel, 1 g) to 2 parts of distilled water (2 ml). The water was added to the gel in a beaker and stoppered. It was shaken vigorously for about one minute to produce a fine, smooth slurry and quickly poured into the trough, which was then passed in one smooth movement over the set of plates.

Drying of plates and storage. The plates were left in the horizontal position for 30 minutes for the gel to set. They were then inserted in a metal rack, and either baked in the oven at 110° for 30 minutes or left at room temperature for at least 24 hours before use. The dry plates were stored in a dust-free atmosphere until used. Plates treated in such a manner have been found to keep for at least six months.

With the help of a simple bridged ruler, the gel layer was divided into equal strips of 12.5 mm wide. This enabled equal layers or amounts of gel to be scraped off and eluted for the analysis of amino-N (described later).

(c) Solvent system and chromatography run.

Two sets of solvents were tried: (i) n-butanol/acetic acid/water, in the ratio 4:1:1 and (ii) propanol/formic acid/water, in the ratio of 20:1:5. In order to compare them, 10 ul lots of serum were spotted on two separate plates. Each plate was run in one solvent. Three hours was the migration time for 16 cm length of run of the solvent. There was tailing in the samples run in the propanol/formic acid/water solvent.

The ascending technique was employed. About 120 ml of the solvent were placed in a glass tank (24 x 24 x 7 cm) before the plate with sample spots was positioned in it. The plate was placed in the tank with the sample end dipping into the solvent in the bottom of the tank. The chamber was closed tightly and was not to be opened during the chromatography run.

(d) Method of application of sample.

The sample (5-10 ul) was taken up in a micropipette. It was found absolutely essential that the whole amount should be delivered in one continuous steady flow onto the gel. This method of application was preferred to a drop-by-drop application because a drop tended to dry off before the next one was applied. This was found to cause tailing of samples during the run and prevented separation. The drying of spots during spotting was found to be necessary for pure samples such as amino acid standards and separation was found to be satisfactory. 5 ul serum delivered in such a manner covered a circular spot of average diameter of 4 mm. 10 ul serum sample covered a larger area (between 4 and 6 mm) but separation was equally efficient.

The solvent front was allowed to travel a distance of about 16 cm. The plate was then removed from the tank and the solvent allowed to evaporate off the plate. The process might be speeded up by blowing hot air (from a hair drier) over it. The outermost spots of the sample were at least 0.5 mm away from the gel layer sides and the interval between spots was 15 mm.

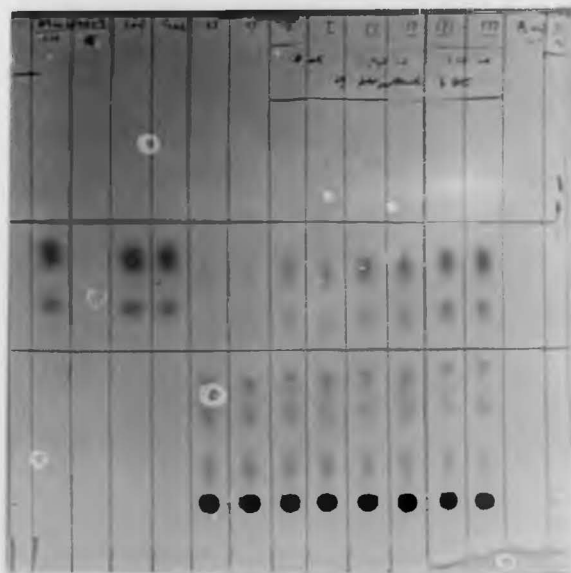
(e) Identification.

The separated branched chain amino acids may be identified by the use of (i) markers or (ii) radioactive measurements.

Markers (standards sprayed). A sample of the amino acid under investigation was spotted on the same plate as the test serum samples. A spot of serum and standard combined was also made. After the solvent run, followed by drying of the plate, these marker spots were sprayed with ninhydrin while the test serum spots were covered with a glass plate. The spots which were coloured with ninhydrin indicated the positions of the amino acids in the test serum samples. Then the lower section of the chromatogram of the serum samples was sprayed while the upper part was shielded with a glass plate. An uninterrupted separation in the lower section indicated a perfect separation in the upper area which contained the three branched chain amino acids under study. (Plate 1.)



markers



branched chain  
amino acids

uninterrupted  
separation

origin of spots

Plate 1. Serum and amino acid standards  
chromatographed  
(IDENTIFICATION)

### 3.2. Tests of recovery with radioactive amino acids

It seemed that the easiest and the quickest way of investigating completeness of separation, recovery and the factors that might influence them would be by using radioactive amino acids.

#### (a) Counting techniques.

The labelled amino acid used was  $^{14}\text{C}$ -valine.  $^{14}\text{C}$  is a 'soft' beta-emmitter, with an energy of 0.05 MeV. At the time when this work was begun a liquid scintillation counter was not available in the department. However, an old-fashioned windowless gas-flow counter was entirely adequate for the purpose, since its counting efficiency for  $^{14}\text{C}$  was about 29% (see below, i.e. counting efficiency).

In the counter which operates in the Geiger-Müller principle (Fig.11), the cathode is a metal tube and the anode is a tungsten wire, diameter 0.002 mm, which passes down the middle of the tube. A continuous flow of an inert gas, helium, with 1.3% butane added as quenching agent, is passed through the tube. If any air or moisture is present, it will not count. Counting begins when the voltage of the anode is about 1200 v, and the count rate is virtually constant over a voltage range from 1200-1600 v. (Fig.12)

Routinely the counting voltage was set at 1400 v. At this setting small fluctuations in voltage will not produce significant errors. The background count ranged between 15.1 and 20.6 counts per minute (cpm). In 32 counts the mean was 18.7 and S.D. 1.04.

Self absorption. Beta particles have only a short range, and therefore with a sample of finite thickness, when disintegrations

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It seemed that the easiest and the quickest way of investigating completeness of separation, recovery and the factors that might influence them would be by using radioactive amino acids.

#### (a) Counting techniques.

The labelled amino acid used was 14-C-valine. 14-C is a 'soft' beta-emmitter, with an energy of 0.05 MeV. At the time when this work was begun a liquid scintillation counter was not available in the department. However, an old-fashioned windowless gas-flow counter was entirely adequate for the purpose, since its counting efficiency for 14-C was about 29% (see below, i.e. counting efficiency).

In the counter which operates in the Geiger-Müller principle (Fig.11), the cathode is a metal tube and the anode is a tungsten wire, diameter 0.002 mm, which passes down the middle of the tube. A continuous flow of an inert gas, helium, with 1.3% butane added as quenching agent, is passed through the tube. If any air or moisture is present, it will not count. Counting begins when the voltage of the anode is about 1200 v, and the count rate is virtually constant over a voltage range from 1200-1600 v. (Fig.12)

Routinely the counting voltage was set at 1400 v. At this setting small fluctuations in voltage will not produce significant errors. The background count ranged between 15.1 and 20.6 counts per minute (cpm). In 32 counts the mean was 18.7 and S.D. 1.04.

Self absorption. Beta particles have only a short range, and therefore with a sample of finite thickness, when disintegrations

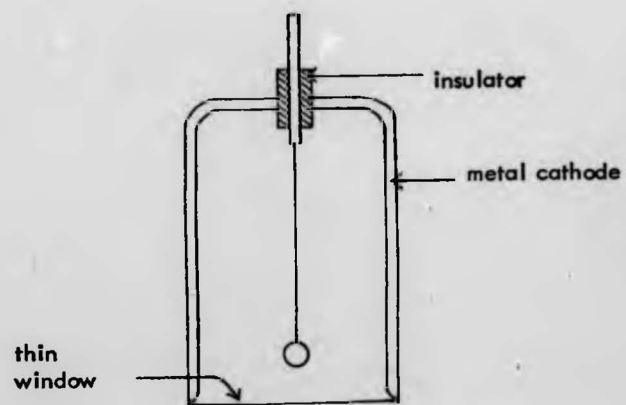
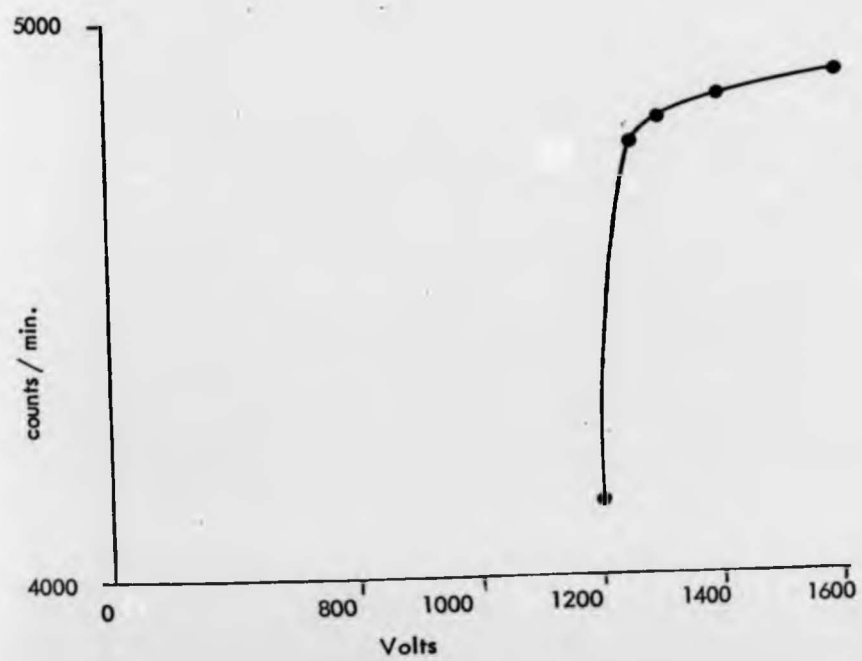
Fig 11. Geiger - Müller tube

FIG. 12 COUNTS vs. VOLTAGE



occur at the bottom of the sample, the electrons will be absorbed before they reach the anode. A correction must therefore be made for this.

Since the samples were counted on planchettes of standard size, the thickness of the sample after drying is determined by its weight. A self absorption curve was constructed by preparing a series of solutions containing the same concentration of 14-C valine but increasing concentrations of sucrose, from 2-20 g per 100 ml. Thus 200 ul of these solutions weighed from 4-40 mg, but contained the same amount of radioactivity. Duplicate samples of these solutions were counted, and the counts-rate plotted against dry sample weights. (Fig.13).

All counts-rate were related to a standard weight of 5 mg. i.e. the count rate given by each weight is expressed as a percentage of the count rate at 5 mg weight. The relationship between the percentage counts at 5 mg weight and the weight of the material on the planchette is shown in the curve referred to as the self absorption curve (Fig.14).

Preparation of sample: The samples were counted in manganese-alloy planchettes about 1 cm diameter and 1 mm deep.

Because of the problem of self absorption, it is essential that the sample should be evenly spread out. This was assured by fixing a lens paper disc (cut out with a leather punch) to the bottom of the planchette with a drop (50 ul) of 10% sucrose solution. The sample was then added, usually in a volume of 200 ul, and the planchette dried under a lamp. The planchette was weighed before use and again after drying. The self absorption curve factor was calculated from the difference in weight. Most of the samples used in the test of recovery contained about 3000 cpm and were counted for 10 minutes.

FIG. 13      COUNTS vs. WEIGHT

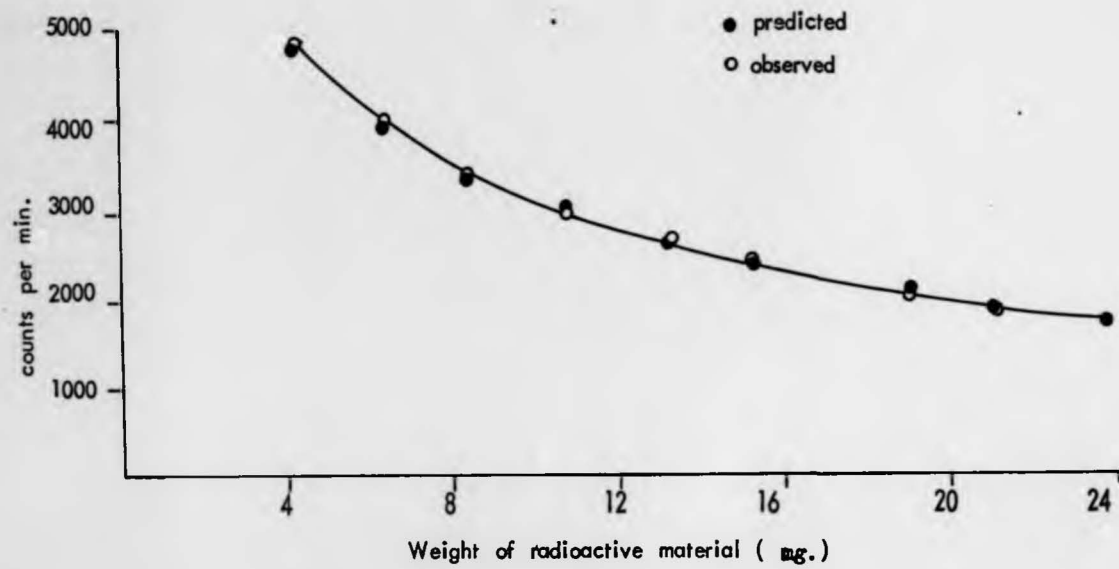


FIG. 14 SELF ABSORPTION CURVE

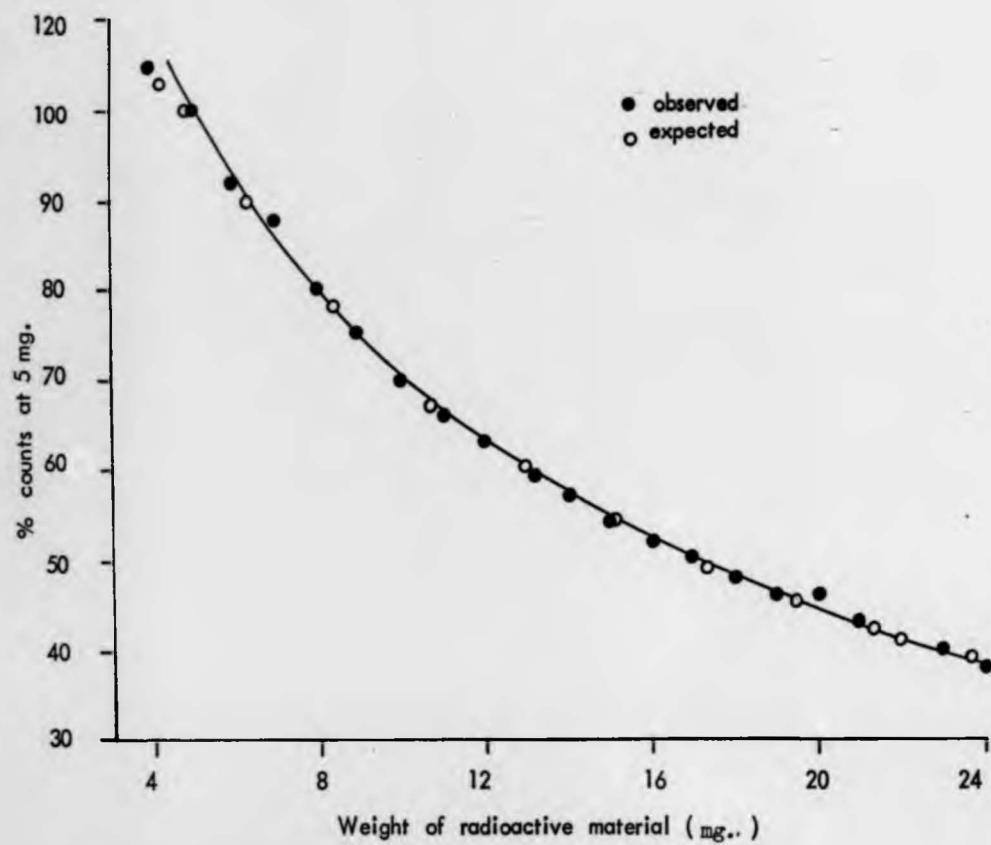




Table 3.

Weights of radioactive material  
and counts-rate

<u>wt. of radioactive material. average (mg) of duplicates</u>	<u>corrected cpm (average of duplicates)</u>
4.25	4843
6.35	3943
8.40	3400
10.25	3075
13.20	2618
15.20	2388
17.30	2136
19.45	2039
21.30	1880
23.70	1721

It is observed from Table 3 that the counts-rate decreases with increase in weight of radioactive material. The relationship between 10000/count and weight of radioactive material produced a good fit (Fig. 15.). The coefficient of correlation was 0.99. The counts and weight of the radioactive material were estimated to be related by the regression equation:

$$\frac{10000}{\text{counts}} = 0.18976 \times \text{weight} + 1.2971$$

From this equation counts could be predicted if weight was known:

$$\text{counts} = \frac{10000}{(0.1897 w + 1.2971)}$$

Counts were worked out (from the equation) for other radioactive weights ranging from 4-24 mg. All counts-rate were related to a standard weight of 5 mg. The results are shown in Table 4.

The curve obtained in this way, shown in Fig 16, is used to correct for self absorption in all subsequent measurements.

FIG. 15 WEIGHT AND COUNTS RELATIONSHIP

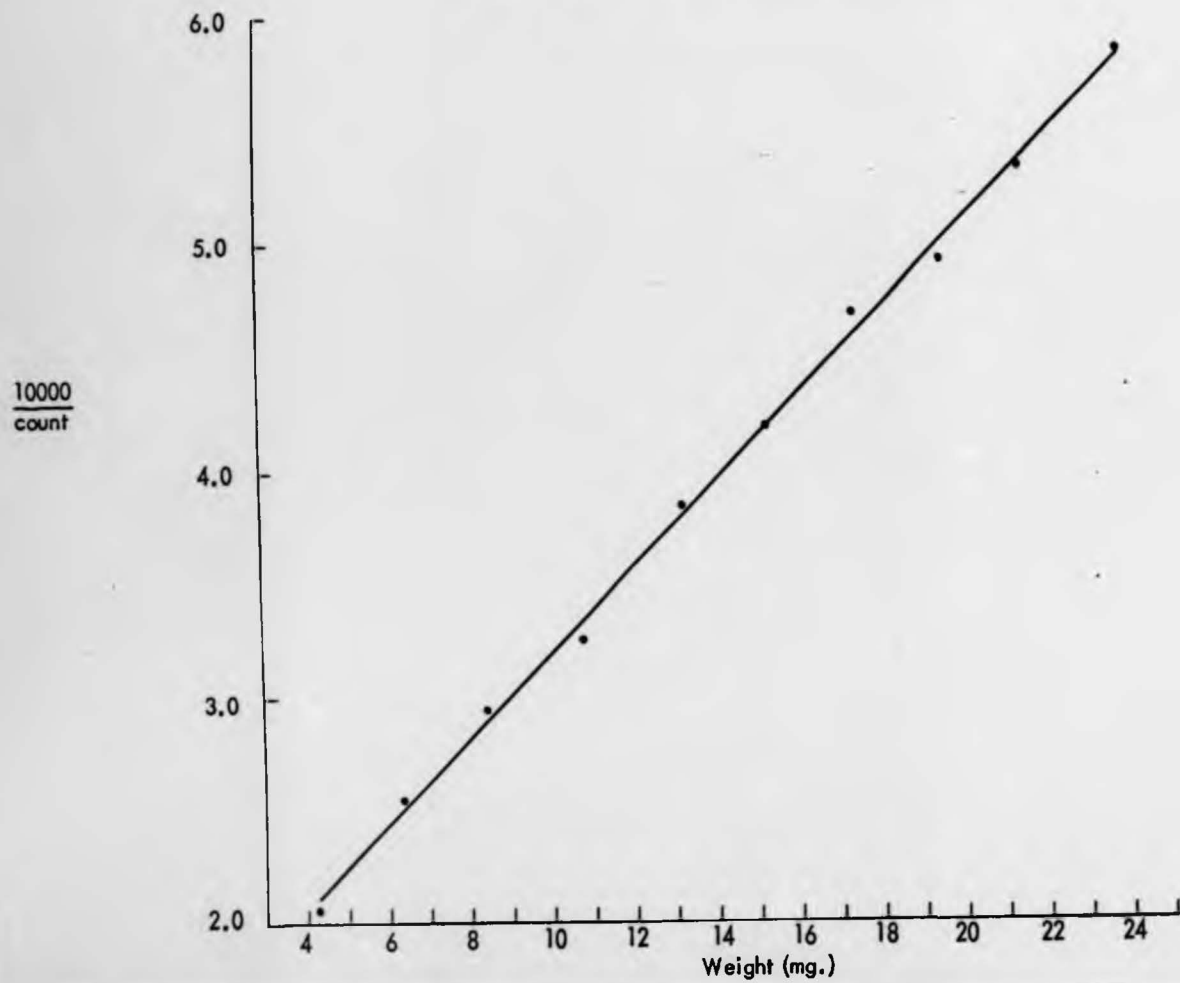
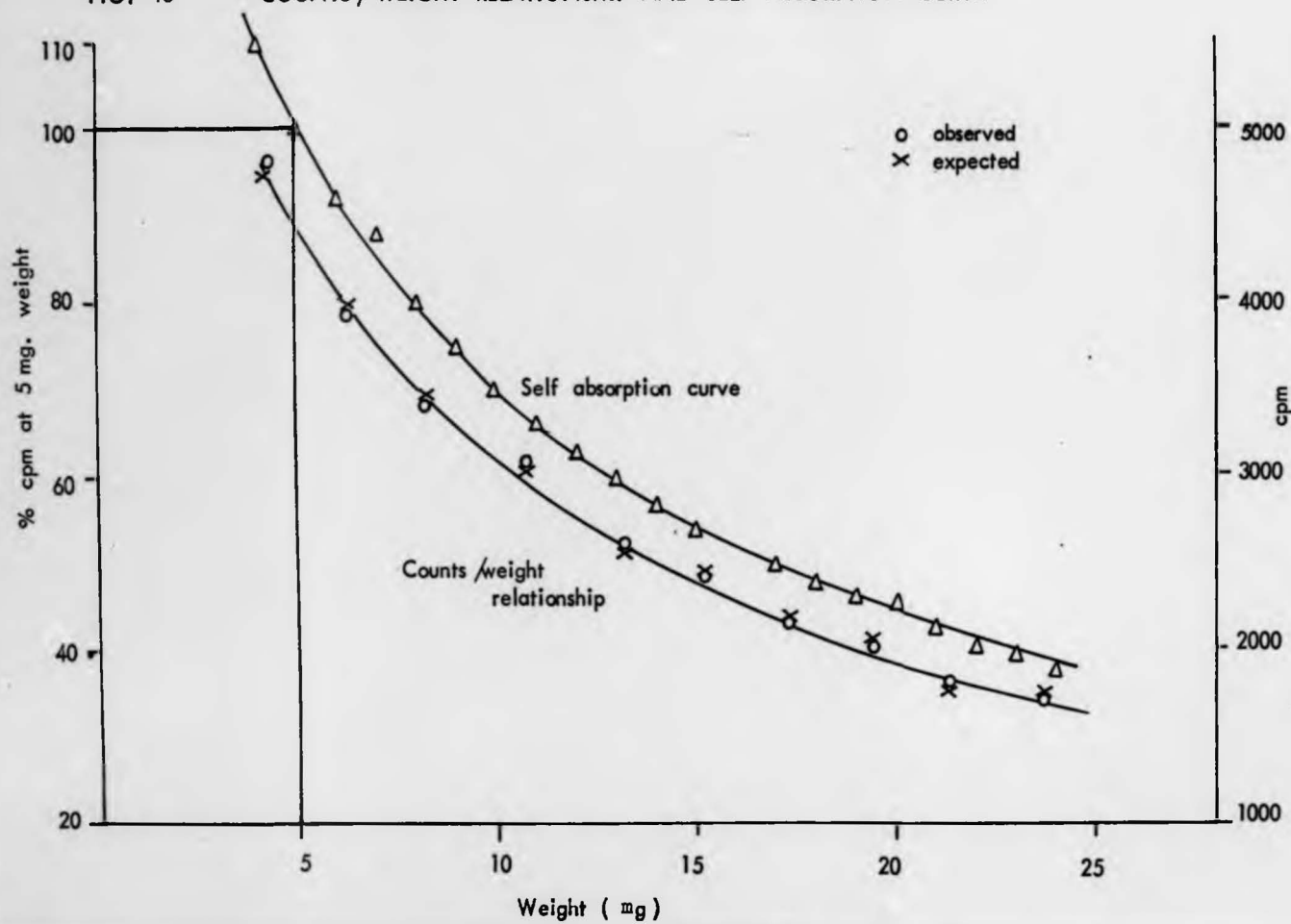


Table 4

Observed and predicted counts. Relationship  
of counts-rate to a standard weight of 5 mg.

weight of radioactive material (mg)	counts per min		% cpm at 5 mg weight
	observed (from counter)	predicted (from equation)	
4		4878	110
4.25	4843	4754	107
5		4452	100
6		4107	92
6.35	3943	3997	90
7		3809	88
8		3552	80
8.4	3400	3460	78
9		3329	75
10		3131	70
10.75	3075	2997	67
11		2955	66
12		2798	63
13		2657	60
13.2	2618	2631	59
14		2530	57
15		2413	54
15.2	2388	2392	54
16		2308	52
17		2211	50
17.3	2136	2184	49
18		2122	48
19		2040	46
19.45	2039	2005	45
20		2030	46
21		1935	43
21.3	1880	1873	42
22		1828	41
23		1767	40
23.7	1721	1726	39
24		1709	38

FIG. 16 COUNTS/WEIGHT RELATIONSHIP AND SELF ABSORPTION CURVE



Counting efficiency: From Table 5, it is seen that the counting efficiency falls off at very high counts-rate, i.e. above 170,000 cpm. All experimental samples had much lower counts-rate than this.

The absolute efficiency of the counter can be determined from these figures. For example, 1 nCi (i.e. 5 ul of 14-C-valine, 0.0053 umols/ml, i.e. 0.2 uCi/ml) gave a counts-rate of 643 cpm.

The true number of disintegrations produced by 1 uCi = 36700/sec.

$$\begin{aligned} \text{True count therefore of 1 nCi (0.001 uCi)} &= 36700 \times 10^{-3} \times 60 \text{ cpm} \\ &= 2202 \end{aligned}$$

$$\therefore \text{efficiency} = \frac{643}{2202} \times 100$$

$$= 29\% \text{ at a sample weight of 5 mg.}$$

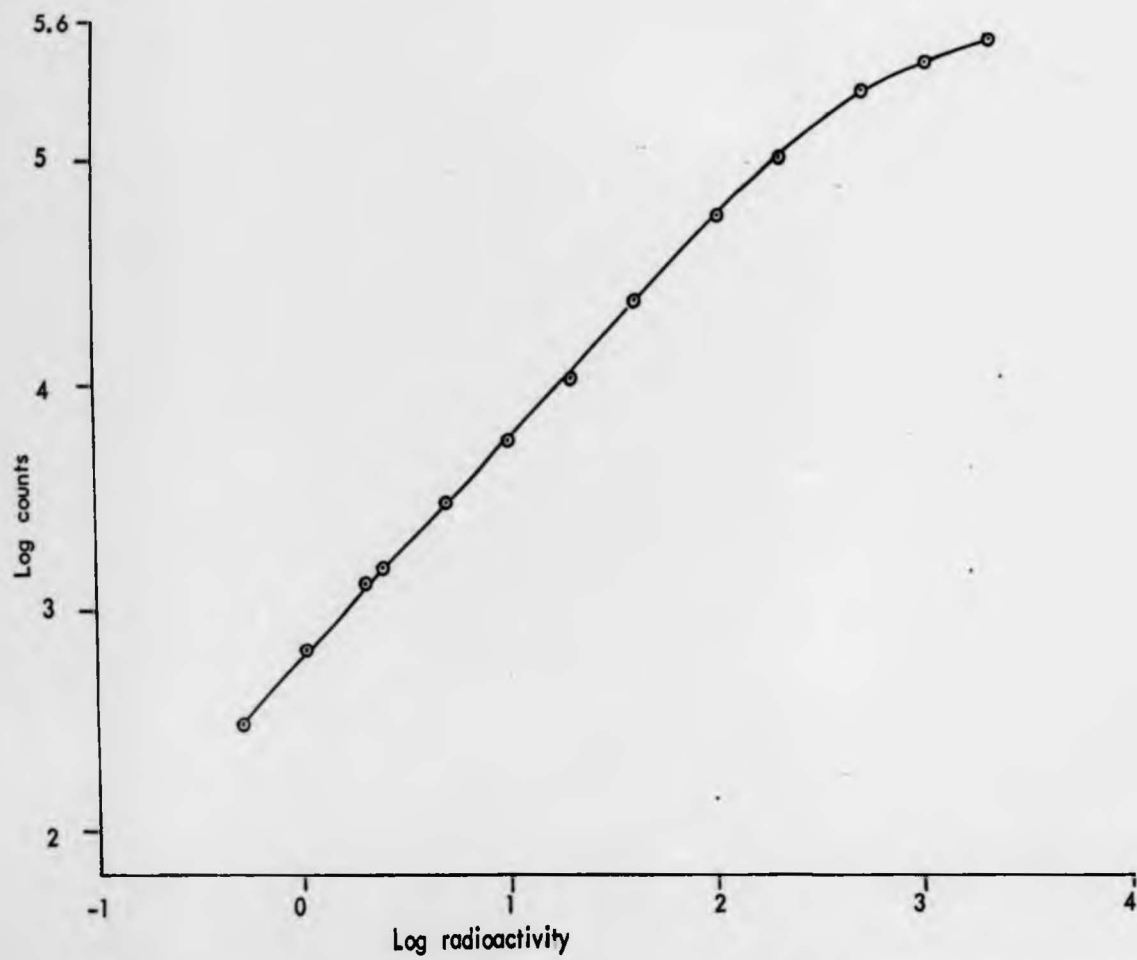
Table 5.

Radioactivity and counts

<u>nCi</u>	<u>cpm</u>
0.5	303
* <u>1.0</u>	* <u>643</u>
2.0	1297
2.5	1436
5.0	2921
10.0	5471
20.0	10693
40.0	23821
100.0	52228
200.0	94878
500.0	168260
1000.0	237490
2000.0	293827

The log of the nCi values were plotted against the log of the counts-rate. The relationship is shown in Fig. 17.

FIG. 17 RADIOACTIVITY : Log Amount ( nCi ) vrs, Log Counts ( cpm )



Standard radioactive planchettes were counted at intervals over a period of seven months. The scaler was found to give fairly consistent counts:

(Standard solution, 0.5 umols/ml  $^{14}\text{C}$ -valine)

<u>vol. of</u> <u>std. soln</u> <u>on planchette</u>	<u>Date</u>		
	<u>4/4/73</u>	<u>5/4/73</u>	<u>11/11/73</u>
5.0 ul	11040	11071	11197
2.5 ul	5628	5640	5737

It looks as if there may be a slight falling off in counting efficiency (about 2%) at the higher counts-rate.

(b) Tests of recovery of valine eluted with different solvents.

In these tests 5 ul samples of  $^{14}\text{C}$ -valine, containing 0.53 umols/ml and 0.00476 uCi (4.76 nCi) were spotted on TLC plates and chromatographed as described in section 3.1. (above). After the plates had been dried, the valine area on the plate was located with a marker alongside, in which colour was developed with ninhydrin, bearing in mind the  $R_f$  value of valine (0.5-0.52). The gel was scooped from the plate into a plastic tube, 7.5 x 1 cm, and 500 ul of solvent was added to elute the amino acid from the gel. The sample was mixed in a whirl-mixer, stood for ten minutes with occasional mixing, and centrifuged for 15 minutes at 3000 rpm (2500 g). An aliquot (300 ul) of the supernatant was removed for counting and amino-N analysis.

The solvents tested for elution were methanol, water, n-propanol and chromatography solvent BAW (butanol/acetic acid/water). The results derived from radioactivity measurements are shown below (Table 6).

Recoveries are calculated after correcting for dilution of the sample during elution.

Table 6  
Solvents for elution

<u>Solvent</u>	<u>number of samples</u>	<u>mean cpm</u>	<u>S.D.</u>	<u>percentage recovery</u>
methanol	20	1942	191	70
water	4	2396	215	87
BAW	4	2719	125	98
n-propanol	8	2761	277	100

Eight samples of 5 ul of 14-C-valine solution were counted without being chromatographed. The mean counts/min was 2763, SD 91.4. Water appeared superior to methanol. Both n-propanol and BAW gave higher recoveries than water, but from the point of view of colour development (see below) water was considered preferable.

When suspensions (irrespective of solvent used) were counted of the gel containing the radioactive material, without elution, percentage recoveries of about 100% were possible. This suggests that all valine was concentrated in the spot analysed and that valine was not spread on other parts of the plate. The latter point was again verified by testing radioactivity in the gel on the other parts of the plate. Those areas were found to contain no radioactivity.

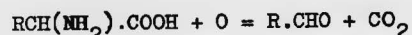
It is expected therefore that 100% colour recovery could be made also; at least 87% was found in the supernatant if water was used as solvent to elute amino acid from the gel.



### 3.3. Quantification by the use of ninhydrin

Quantification was based on the reaction between ninhydrin and amino acid to form a purple colour which is read at 570nm. Two possible ways of carrying out the reaction were; (i) colouring the amino acid on the chromatoplate before eluting the colour and (ii) eluting amino acid from gel with appropriate solvent before carrying out colour development in the supernatant.

Ninhydrin (triketohydrindene hydrate; indane-1,2,3,-trione hydrate) is a powerful oxidizing agent. It causes oxidative decarboxylation of alpha-amino acids producing  $\text{CO}_2$ ,  $\text{NH}_3$ , and an aldehyde with one less carbon atom than the parent amino acid. It is a quantitative oxidative deamination of alpha-amino acid according to the equation:



The reduced ninhydrin then reacts with the liberated  $\text{NH}_3$  forming a blue complex which maximally absorbs light at 570nm (Rodwell, 1969).

#### (a) Development of the colour with ninhydrin before elution

Standards containing 2.5 to 5.0 nmols  $1\text{-}^{14}\text{C}$ -valine were spotted on the gel. Some spots were chromatographed (TLCed) and others not (not TLCed). The spots were sprayed with 0.5% ninhydrin in butan-1-ol and eluted with methanol, and the colour was measured in microcuvettes in the spectrophotometer. The ODs were too low for accurate measurements although the expected ODs were in the range 0.15-0.30.

The results obtained with radioactive valine, described in the previous section, showed that good recoveries were possible if the spots were eluted without treatment with ninhydrin. One possible explanation for the poor results of elution after spraying with

ninhydrin was that the derivative formed might be bound to the glass plate. However, no improvement was found when the plates were first washed with a solution of 'cold' valine to saturate possible binding sites. Perspex was tried instead of glass, and again no evidence of binding was found. However, perspex cannot replace glass in the preparation of chromatoplates because the gel does not form a smooth layer on it.

Another possibility was that the reaction with ninhydrin was incomplete, since it was difficult to be sure that the amount of ninhydrin received or the heating of the plate was adequate. Evidence of incompleteness of the reaction was obtained from radioactive measurements. Since the labelled carboxyl carbon in the  $1-^{14}\text{C}$ -valine is driven off as  $\text{CO}_2$  on reaction with ninhydrin, there should be no radioactivity in the eluate after spraying with ninhydrin. In fact appreciable number of counts were found in the eluate, and the radioactivity increases when increasing amounts of cold valine were added to the spot on the chromatogram (Table 7). The radioactivity was not in the dissolved  $\text{CO}_2$ , because after precipitation of  $\text{CO}_2$  by the addition of barium chloride, there was only a 10% reduction in counts in the eluate.

The results suggested that under the conditions used the ninhydrin reaction was not complete, and that spraying with ninhydrin before elution was unlikely to be satisfactory.

(b) Elution before treating with ninhydrin

Valine samples (1.25 nm) were spotted onto the gel; some were chromatographed and others were not. The spots were eluted with water and treated with ninhydrin (Jagenburg, 1969). Background gel samples were treated in the same way.

Table 7

14-C-valine and cold valine or serum spotted together. Recovery: percentage of expected counts after ninhydrin spray.

(expected cpm from 5 ul of 14-C-valine=52000)

14-C-valine (ul)	serum (ul)	cold valine (nmols)	observed counts (cpm)	recovery (% of expected counts)
5			2842	5.0
5	5		4950	9.5
5		1.25	2870	5.6
5		8.55	12517	24.0
5		21.37	23500	45.0
5		176.00	31000	60.0
5		252.00	41000	79.0

The first difficulty encountered was that the background OD was extremely high. Before the solvent run the mean background OD was 0.069, and after the solvent run it was 0.151. It seemed possible that some colour was being contributed by residual ammonia in the acetic acid of the solvent; or that oxidation of acetic acid to aldehyde might be contributing to the colour. It was found that the background could be substantially reduced by blowing hot air continuously over the plate for at least 30 minutes before the valine spots were scrapped off. The gel background (OD 0.083) was then no higher than the standard blank (OD 0.085).

The second difficulty was that when the eluted valine spots were treated with ninhydrin, the OD was too low for accurate measurement. Since the radioactive measurements (Table 6) showed that it was possible to obtain 80-100% recovery of valine after chromatography and elution, the only possible explanation seemed to be that the conditions for the ninhydrin reaction were unsatisfactory, so that colour development was incomplete. It was found that contact with gel made the eluate acid, which reduced the colour. This was counteracted by increasing the pH of the buffer used in the reaction from 5.0 to 5.6. Also, the concentration of ninhydrin in the reaction mixture was doubled. These two measures produced a considerable increase in the optical density. The addition of pyridine to the reaction mixture, as recommended by Mortimore and Mondon (1970) had no consistent effect in increasing the colour.

Another source of error was that with these samples of very small volume, significant dilution could occur by condensation in the tubes of water from the bath during heating (Table 8).

Table 8.  
Metal caps and tin-foil: Effect  
on colour

volume of standard	net OD*	
	Tubes with metal caps	tubes with tin-foil
5	35	56
10	98	113
15	145	179
20	164	261

\*(net OD multiplied by 1000)

One problem which remained unsolved was that the eluate of  $^{14}\text{C}$ -valine spots, after treatment with ninhydrin, still contained some radioactivity. As pointed out in the previous section, with  $^{14}\text{C}$ -valine this should not happen if the ninhydrin reaction is complete. However, when efficiency of the reaction was improved, as shown by increase in OD, there was no decrease in the counts. The only explanation of this seems to be that some of the radioactivity was in carbon atoms other than the 1-carbon.

The method finally adopted is set out in appendix VI. The standard curve is shown in Fig. 23. It is apparent that the sample should contain 6-10 nmols amino-N for accurate measurements to be obtained. The amounts of the branched chain amino acids in normal rat serum are given as follows by Scharff and Wool (1966):

valine = 0.25  $\mu\text{mols per ml}$   
 leucine = 0.12       "  
 isoleucine = 0.16       "

In order to obtain enough colour for accurate measurement on 5  $\mu\text{l}$  of serum it is therefore necessary to elute all three branched

chain amino acids; valine alone is not enough. Therefore for further measurements a standard solution was made containing equal parts of the three branched chain amino acids to a total concentration of 2 umols per ml (10 nmols per 5 ul). Table 9 shows the results of six chromatograph runs with this standard at intervals of a week. The mean recovery of colour was 81% and of radioactivity 82.5%.

#### 3.4. Reproducibility of results

For 10 nmols of the standard solution applied in the method, the mean and SEM were 238 and 4.72 respectively. This SE of only 2% indicates constancy in the optical density values over the period. Constancy of values was also obtained when 10 nmols of the standard was chromatographed. The mean and SEM respectively were 193 and 3.4, a standard error of 1.8%.

Percentage colour recovery and counts recovery. Student's 't' test was used to test for a significant difference between the percentage recovery of colour on one hand and the percentage recovery of counts on the other during the 6-week period under consideration. No significant difference was found, indicating good agreement. The coefficient of correlation was 0.6 and the percentage recovery in the range of 72-88%.

Table 9.

Reproducibility and sensitivity of measurement  
of branched chain amino acids in successive weeks

(All samples contained 10 nmols mixed amino acids, the valine being labelled with 14-C. All values are OD x 1000)

	zero week	1st week	2nd week	3rd week	4th week	5th week
A. (standards not chromatographed)						
	250	248	248	210	247	250
	<u>251</u>	<u>248</u>	<u>250</u>	<u>238</u>	<u>219</u>	<u>223</u>
mean=	250	248	249	224	233	236

Total weekly mean  $\pm$  SE = 238  $\pm$  4.72

B. (standards chromatographed)

	167	233	185	178	232	203
	170	180	161	191	175	163
	190	185	225	177	175	178
	<u>192</u>	<u>216</u>	<u>220</u>	<u>212</u>	<u>212</u>	<u>220</u>
mean=	181	203	198	189	201	191

Total weekly mean  $\pm$  SE = 193.8  $\pm$  3.41

nmols=(7.24) (8.18) (7.95) (8.44) (8.97) (8.09)

Colour. Percentage recovery (B/A x 100)

72%    82%    79%    84%    86%    81%.    Weekly mean = 81%

Counts. Percentage recovery over 6 weeks (5 ul  
14-C-valine chromatographed)

	78	94	94	85	94	85
	71	104	97	83	79	86
	86	(46)	88	88	82	80
		(50)	85	91	94	86
				<u>93</u>	<u>84</u>	<u>88</u>
mean=	78%	74%	83%	88%	87%	85%.    Weekly mean = 82.5%

CHAPTER FOUR

## RESULTS



#### 4.1. Body weights and food consumptions.

Table 10 and Fig.18 are a summary of the weight gains and food consumptions over a period of 35 days. The results from all five experiments are included.

The weights of food consumed by each group were not significantly different except for group A; the smallest amount was 9 g/day/rat in the rats on 8% protein diet as opposed to 11-13 g/day/rat in the other groups. The corresponding protein consumed per rat per 35 days ranged between 25 g and 97 g. The range, 11-13 g, is in agreement with the findings of previous workers; Salem et al (1973) using young albino rats, and Horie & Ashida (1973) also using growing rats of the Wister strain. Kirsch, Brock and Saunders (1968), working on young albino rats, have reported an average daily food consumption per rat as 15 g.

There was a stepwise increase in weight gain proportional to the protein content of the diet and to the amount of protein eaten. Fig.18 shows the relation between protein consumption and percentage increase in weight. All the diets supported growth; nevertheless, the groups of rats on diets of different protein content were clearly differentiated by differences in their weight gain.

The protein efficiency ratio (g weight gain/g protein eaten) fell slightly with increasing protein content of the diet. Since the diets were iso-energetic, and the amounts of food consumed were the same, except in group A, the rats on diet of lower protein content were consuming more energy in relation to the weight they gained. Carcass analyses were not done, so it is not known whether this extra energy was deposited as fat or dissipated as heat. The measurements of muscle mass (Table 22) do not suggest any great difference in fat content of the body in the different groups. Other evidence from this department suggests that rats on low protein diets do dissipate more

Table 10

Mean food intake, initial weights and weight gain of groups of young rats fed ad lib for five weeks on the diets shown.

(figures in parenthesis are the number of rats in each dietary group)

Group	% protein in diet	food consumed (g)		protein (g) consumed per rat per 35 days	mean body weights (g)			gain per rat per day	PER*
		per rat per 35 days	per rat per day		initial	gain per rat per 35 days	% increase per rat per 35 days		
A	8(4)	314	8.97	25	79	50	63.3	1.43	2.0
B	12(20)	402	11.48	48	67.4	93.2	138	2.66	1.95
C	15(8)	446	12.74	67	79.2	119.5	151	3.00	1.78
D	18(16)	439	12.54	77	69.7	123.2	177	3.51	1.60
E	24(4)	407	11.63	97	67	159	237	4.54	1.63

(\* PER = protein efficiency ratio)

energy ( Miller and Payne, 1962; Neale and Waterlow, in press).

The mean weekly body weights of rats on the various protein diets appear in Table 11 and Fig.19. The weekly weights of rats on 18% protein (group D) were lower than those of the group on 15% protein (group C). This is explained by the fact that group D started off at a weight 10 g lower than group C.

In one experiment two groups of rats, one on 12% and the other on 18% protein, were weighed once a week in the evening then fasted overnight and weighed again in the morning for 5 weeks. This was in order to find out whether an overnight fast affected the values for albumin and branched chain amino acids (Table 18 and Table 29).

The overnight fast caused a weight loss of up to 9 g (Fig.20). However, the growth rates calculated either from fed or from fasted values did not differ significantly (see Table 12).

In this experiment, after 5-week feeding period, the rats on 12% protein were changed to 18%, and those on 18% were changed to 12%. These diets were maintained for two weeks. The amount of food consumed by each group in the 14-day period was the same, the rate being 15.5 g/day/rat. The growth rates are shown in Table 12. Whereas during the first five weeks the rats on 12% protein grew at 80 per cent of the rate of those on 18% diet after the change the 'well fed' rats put on the lower protein diet grew at only 40 per cent of the rate of the poorly fed rats put on the higher protein. This suggests that on the lower protein intake the rats are programmed to grow more economically.

FIG. 18 FOOD INTAKE AND WEIGHT GAIN PER RAT PER 35 DAYS

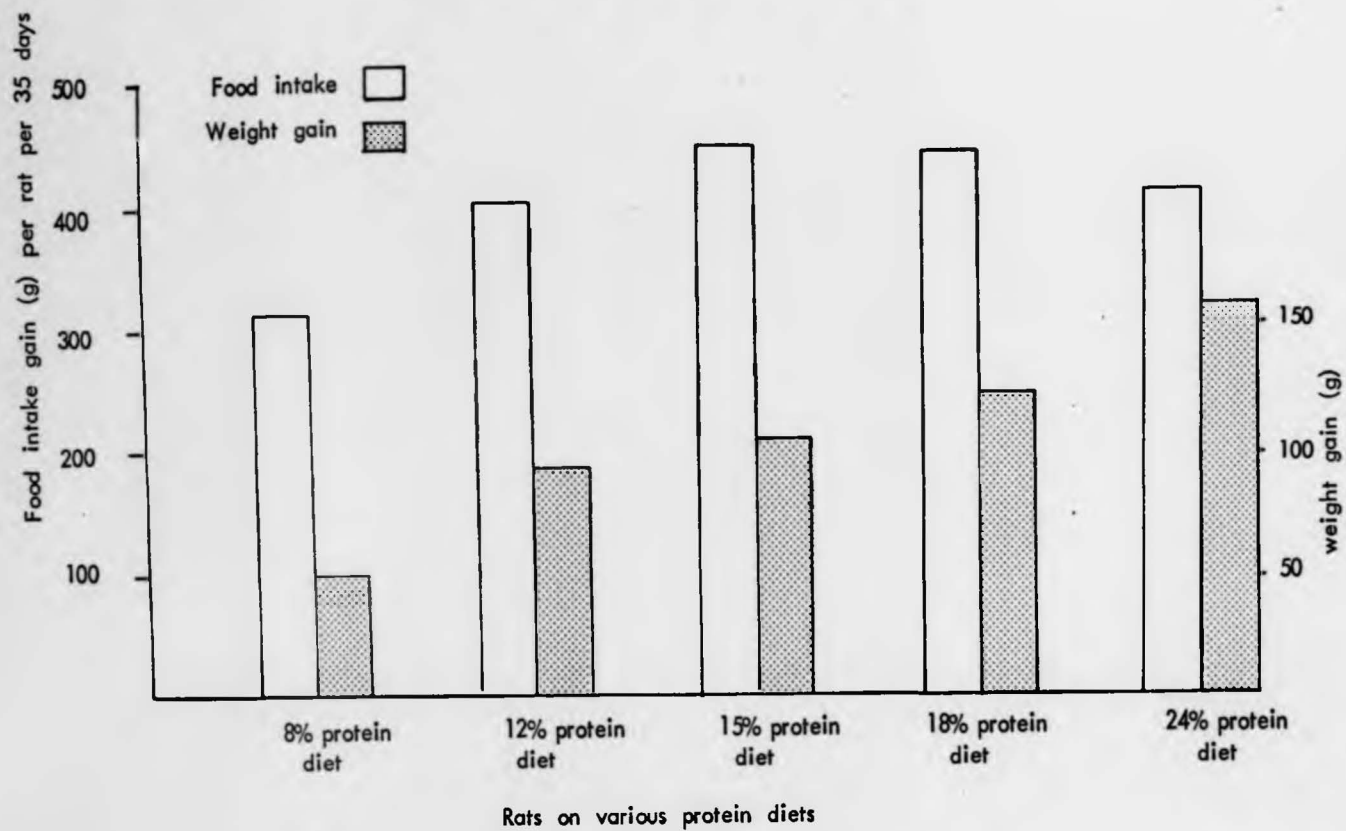


FIG. 19 RAT GROWTH CURVES

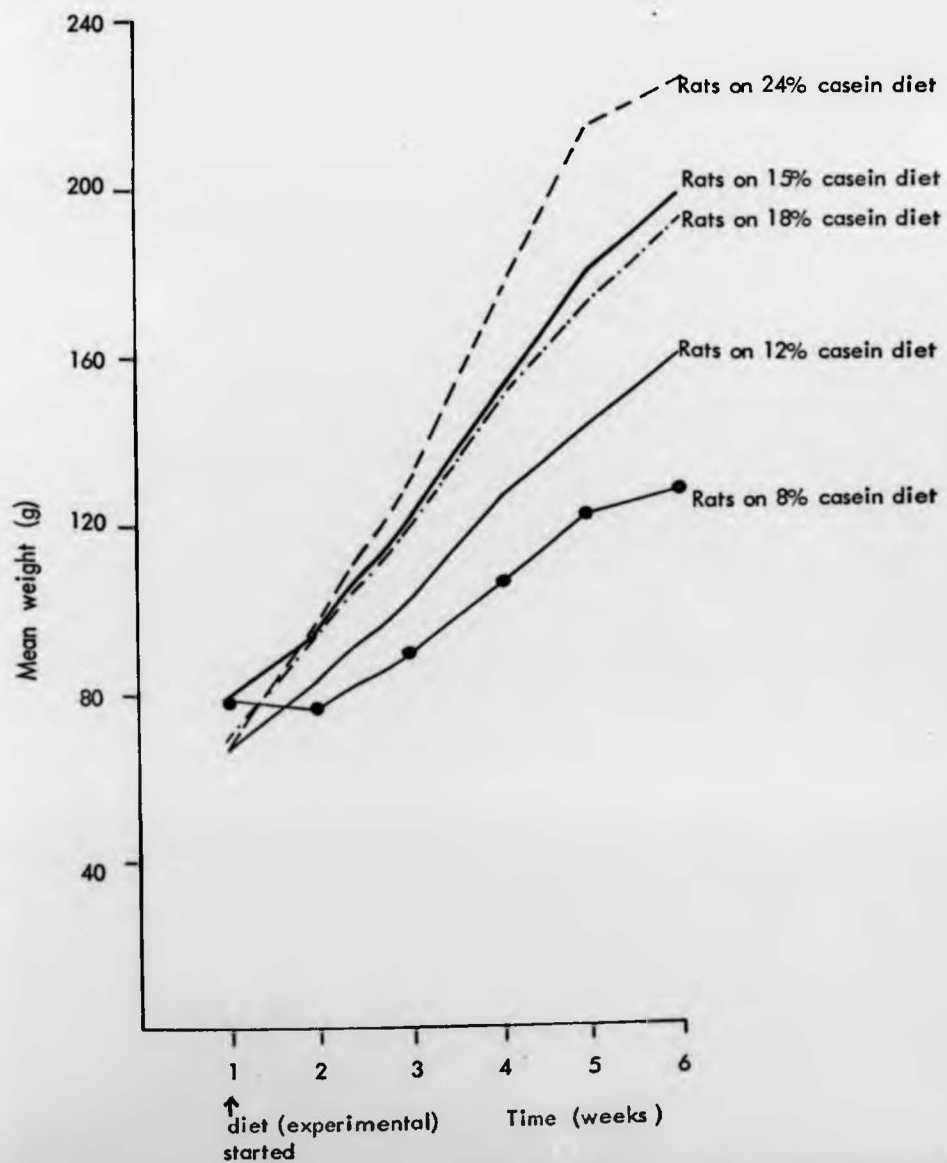


FIG. 20 GROWTH CURVES (Fasting and fed weight measurements The effect on growth rate of change in dietary protein content. Arrows indicate fasting positions.

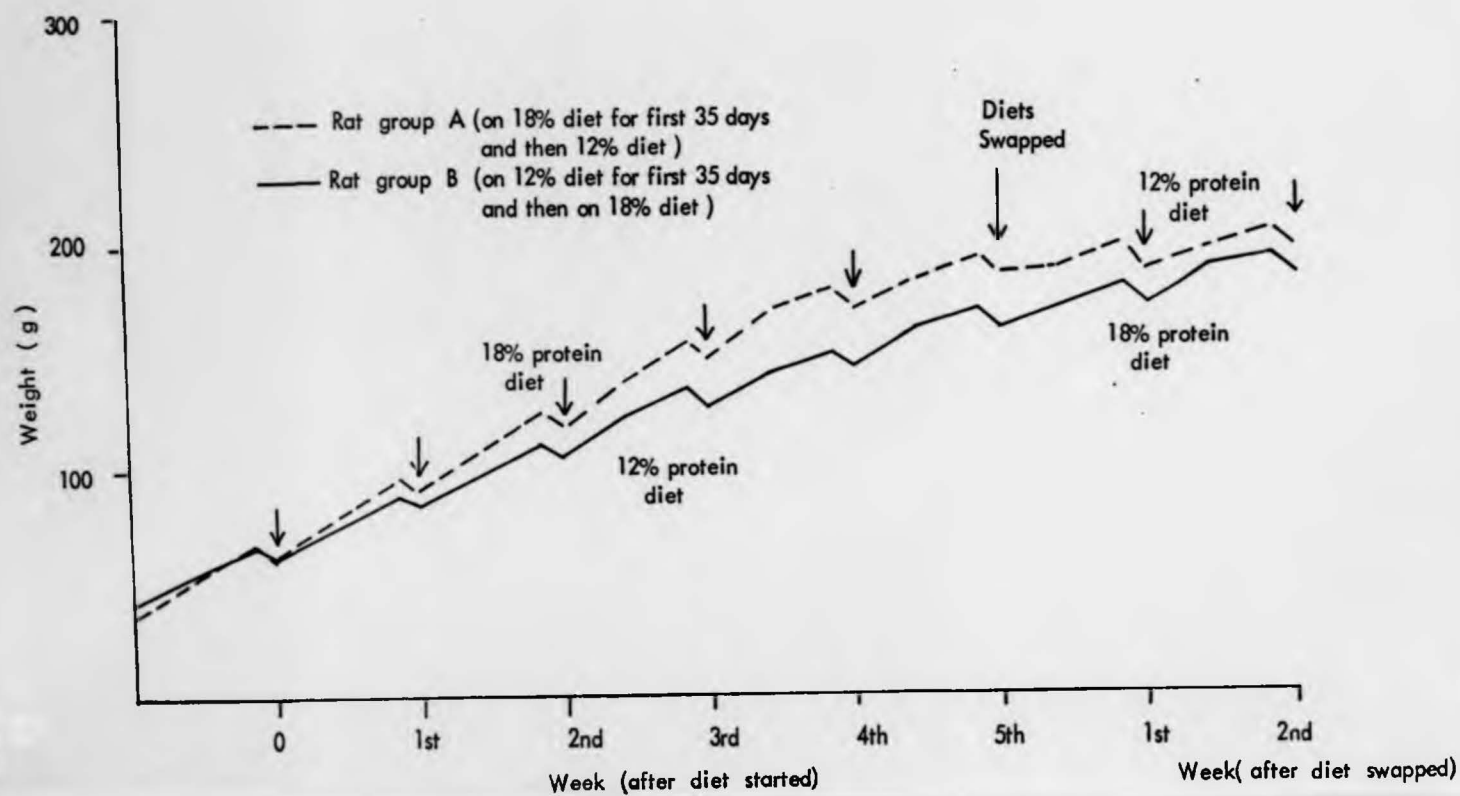


Table 11

Growth rate. Mean of weekly body weights.  
(number of rats in each dietary  
group shown in parenthesis)

	<u>% protein in diet</u>	<u>initial weight</u>	<u>1st week</u>	<u>2nd week</u>	<u>3rd week</u>	<u>4th week</u>	<u>5th week</u>
A	8(4)	79	77	90	107	123	129
B	12(20)	67.4	84	103.6	127.2	144	160.6
C	15(8)	79.2	96	123	153	180.5	199
D	18(16)	69.7	99.4	120.6	151	173	192.5
E	24(4)	67	96	132	176	215	226

Table 12

Effect of diet swapping on rats growth rate

	<u>Growth rate during first five weeks (g/rat/day)</u>		<u>Growth rate after diet change (g/rat/day)</u>	
	<u>12%</u>	<u>18%</u>	<u>18% to 12%</u>	<u>12% to 18%</u>
<u>Fasted values</u>	2.8	3.5	0.6	1.5
<u>Fed values</u>	3.0	3.6	0.6	1.4

#### 4.2. Total serum protein.

In Table 13 the serum total protein in each group from the initial week through to the end of the experimental period is shown. In addition the weekly mean and the SD for each group for the whole period are given.

Total serum protein for all the rats ranged between 5.80-7.10 g per 100 ml (mean 6.85, SD 0.47). This is in agreement with the range (6.67-7.12) given by Salem et al (1973). The value of  $6.04 \pm \text{SD } 0.25$  has been reported by Moore, Levin and Smelser (1945). A comparatively low figure, 4.78 g per 100 ml, has been given by Cabak et al (1963).

Salem et al used the biuret method for the total protein estimation whereas Cabak and co-workers employed Kjeldahl/Markham distillation. Moore et al used the method of salt fractionation for total protein estimation in 3-4 months old male rats of the Long-Evans strain. Younger rats were used by the other workers.

The levels in all groups in the initial week were much the same (5.8-6.2, mean  $6.06 \pm \text{SD } 0.17$ ). There seems to be a fairly large increase after one week on the diets.

The overall means are fairly similar in all groups. If one compares the extremes, group E is only about 5% higher than group A (7.10 against 6.73).



Table 13.

Total Protein (g per 100 ml serum)

(The number of rats in each dietary group is shown in parenthesis)

Protein content in diet	initial week	1st week	2nd week	3rd week	4th week	5th week	Mean	SD	SE
A 8% (4)	6.2	7.1	6.9	6.7	6.8	6.7	6.73	0.30	0.12
B 12% (8)	6.0	6.7	6.7	6.9	6.7	7.1	6.68	0.37	0.15
C 15% (4)	6.2	6.5	7.0	7.1	7.1	7.2	6.85	0.40	0.16
D 18% (4)	6.1	6.3	7.4	7.1	7.0	7.1	6.83	0.51	0.21
E 24% (4)	5.8	7.7	7.1	7.3	7.2	7.7	7.10	0.70	0.28

#### 4.3. Normal rat serum albumin levels.

The dye-binding method (manual) was used to measure albumin levels in the rat sera. The dye employed was bromocresol green (BCG). At the present time the use of BCG for the estimation of serum albumin in man is considered by some to be the ideal method (Webster, 1974).

The average weekly mean values of albumin (g per 100 ml) in all the five experiments are shown in Table 14. In each experiment there were two or three groups of rats designated A, B, C, D or E according to increasing dietary protein content. Each group was made up of four rats. During the week before the experiment the rats were fed a stock diet followed by the experimental diets for the next five weeks.

The mean and SD of all the albumin estimations (312 estimations) for the various groups of rats was  $4.17 \pm 0.57$  g per 100 ml with the range 3.40-5.04. This mean and range are high compared with the range 2.72-3.12 g per 100 ml given by Schultz et al (1954) and the mean of 2.39 reported by Cabak, Dickerson and Widdowson (1963). In both the examples quoted the albumin fraction in the plasma was calculated as percentage of total protein after separating the various fractions by electrophoresis. It is possible that in the rat BCG gives higher values (see discussion) so that the absolute figures reported in this work may not be correct, but its use for distinguishing rats on diets of different protein content may be justified.

The means of the albumin concentrations in each group for all the experiments are given in Table 14 with the SD for groups B, C and D. The SD is not shown for groups A and E because they were used in only one experiment, so that values are available for only four rats. The mean and SD of all these averages are  $4.15 \pm 0.54$ .

The mean initial albumin levels when the rats were on stock diet (i.e. at week 0) ranged from 3.40 to 5.04 g/100 ml, mean  $4.19 \pm \text{SD } 0.53$ .

Table 14

Serum albumin concentration (g/100 ml).  
 Combined results for all five experiments,  
 grouped according to protein content of diet.  
 In each experiment there were four rats in  
 a group. The total number of rats in each  
 group is given in parenthesis.

		Week---->	<u>0</u>	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>
<u>Group</u>	<u>Expt. No.</u>							
<u>A</u> 8% casein diet(4)	1st		3.50	3.25	3.05	3.23	3.41	3.39
<u>B</u> 12% casein diet(20)	1st		3.40	3.25	3.22	3.57	3.59	3.77
	2nd		4.37	3.81	4.15	4.05	4.22	4.60
	3rd		4.03	3.51	3.84	3.77	3.81	3.79
	4th		5.04	3.56	3.61	4.24	3.86	4.29
	5th		4.32	4.76	4.29	4.91	4.55	4.79
	mean±		(4.23±	(3.78±	(3.82±	(4.11±	(4.01±	(4.25±
	SD		0.59)	0.58)	0.43)	0.52)	0.38)	0.46)
<u>C</u> 15% casein diet(8)	2nd		4.35	4.15	4.49	4.40	4.67	4.94
	3rd		4.20	3.64	3.90	4.17	4.07	4.26
	mean±		(4.27±	(3.89±	(4.19±	(4.28±	(4.37±	(4.60±
	SD		0.1)	0.36)	0.42)	0.16)	0.42)	0.48)
<u>D</u> 18% casein diet(16)	2nd		4.30	4.14	4.72	4.56	4.71	4.85
	3rd		3.98	3.83	4.30	4.40	4.40	4.34
	4th		4.92	3.94	4.00	4.70	4.35	5.06
	5th		4.70	5.22	4.51	4.91	4.79	5.04
	mean±		(4.47±	(4.28±	(4.38±	(4.64±	(4.56±	(4.82±
	SD		0.42)	0.64)	0.31)	0.22)	0.22)	0.33)
<u>E</u> 24% casein diet(4)	1st		3.40	3.50	3.60	3.80	3.98	4.16

The values were found to differ from one experiment to the other. The differences might be explained by the fact that rats were not exactly of the same weight (range of weight, 30-60.5 g, mean with SD, 47.04+6.94) when they arrived from the supplier and consequently when the experiments started (weight range 49-92 g, mean and SD, 71.2+9.37). There was also a tendency for the albumin concentration to fall when the rats were transferred from the stock to the experimental diet. The reason for this is not clear.

Because of the difference in the initial albumin measurements between the different experiments, it is difficult to interpret the values when all the results from each group in all the experiments are averaged. In all groups the albumin concentrations tended to increase as the rats grew older. The mean increases on the different diets over the four or five weeks are shown in Table 15 (a) and (b) respectively. The increase is very small in the single group of rats on the 8% casein diet. It is larger in the other groups, but the size of the increase in albumin concentration during growth does not seem to be related to the protein content of the diet.

When one looks at the albumin concentrations in the separate experiments (Table 16) it is evident that the mean values differ in the different dietary groups. The higher the protein content in the diet the higher the albumin level. The only anomaly was in experiment 2 where, in the fifth week, the mean albumin level was lower in group D (18% casein) than in group C (15% casein).

Although, with this exception, the differences in albumin between the different diets were consistent, they are small and within each experiment are not statistically different. However, if the individual serum albumin values for the two groups B and D (12% and 18% casein) are combined for all experiments they become highly and significantly

Table 15

Mean serum albumin increase over experimental period. The mean increase from 1st to 5th week (a) and from 0 to 5th week (b)

(a) Increase from 1st to 5th week

Expt.---->	1	2	3	4	5	Mean increase
Group						
A(8%)	0.14	-	-	-	-	0.140
B(12%)	0.52	0.79	0.28	0.73	0.03	0.470
C(15%)	-	0.79	0.62	-	-	0.700
D(18%)	-	0.71	0.51	1.12	-0.18	0.540
E(24%)	0.66	-	-	-	-	0.660

(b) Increase from 0 to 5th week

Expt.---->	1	2	3	4	5	Mean increase
Group						
A(8%)	-0.11	-	-	-	-	-0.110
B(12%)	0.37	0.23	-0.24	-0.73	-0.47	0.016
C(15%)	-	0.59	0.06	-	-	0.325
D(18%)	-	0.55	0.36	0.14	0.34	0.350
E(24%)	0.76	-	-	-	-	0.760

Table 16.

Serum albumin concentration (g per 100 ml) in each experiment. The mean values from each group are shown with their standard errors. Each group (A,B,C,D or E) contained four rats. Percentage of protein in the diet is given in brackets.

Week----->	<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>mean+SE</u>
<u>Experiment 1</u>							
A(8 )	3.50	3.25	3.05	3.25	3.41	3.39	3.30±0.06
B(12)	3.40	3.25	3.22	3.57	3.59	3.77	3.47±0.09
E(24)	3.40	3.50	3.60	3.80	3.98	4.16	3.74±0.12
<u>Experiment 2</u>							
B(12)	4.37	3.81	4.15	4.05	4.22	4.60	4.20±0.11
C(15)	4.35	4.15	4.49	4.40	4.67	4.94	4.50±0.11
D(18)	4.30	4.14	4.72	4.56	4.71	4.85	4.55±0.11
<u>Experiment 3</u>							
B(12)	4.03	3.51	3.84	3.77	3.81	3.79	3.79±0.07
C(15)	4.20	3.64	3.90	4.17	4.07	4.26	4.04±0.09
D(18)	3.98	3.83	4.30	4.40	4.90	4.34	4.29±0.15
<u>Experiment 4</u>							
B(12)	5.04	3.56	3.61	4.24	3.86	4.29	4.10±0.22
D(18)	4.92	3.94	4.00	4.70	4.35	5.06	4.49±0.19
<u>Experiment 5</u>							
B(12)	4.32	4.76	4.29	4.91	4.55	4.79	4.60±0.10
D(18)	4.70	5.22	4.51	4.91	4.79	5.04	4.86±0.10

different. Analysis of variance applied to these groups indicates that the albumin concentrations in the two groups were significantly different at the 0.5% level (i.e.  $p < 0.005$ ) as far as diets, weeks and experiments were concerned. The two groups could thus be clearly distinguished on the basis of their albumin levels. The F values, after the analysis of variance, for differences between diets, weeks, and experiments were 31.9697, 5.3679, and 19.3548 respectively, all of them being highly significant at the 0.5% level. The relevant figures for the analysis of variance are shown in Table 17.

The results for serum albumin indicate that there are differences between groups but the test is not very sensitive when only a few samples are analysed. Therefore very large groups, much larger than those used in these experiments, might be needed to show the sensitivity of albumin measurements in differentiating rats on diets with only small differences in protein content.

All the albumin measurements reported above were made on serum samples from fed rats. It is shown in Table 18 that fed levels did not differ significantly from fasting levels in any particular group. Both fed and fasting values show differences between the groups, but with these small numbers the differences are not significant.

Table 17  
Analysis of variance

Source	Sum of squares (SS)	Degrees of freedom (DF)	Mean square (=SS/DF)	F (= mean square/ error mean square)
Diet	7.29	1	7.29	31.9697 (p<0.005)
Week	6.12	5	1.22	5.3679 (p<0.005)
Experiment	13.24	3	4.41	19.3548 (p<0.005)
D x W	1.45	5	0.29	1.2670 NS*
D x E	0.35	3	0.11	0.5057 NS*
W x E	13.69	15	0.91	4.0070 (p<0.005)
Error (residual)	36.26	159		

Total

191

Error mean square(EMS) = 0.2280

Standard deviation(SD) = 0.4774

\* NS = non significant



Table 18

Albumin concentration, fed and fasting levels (g per 100 ml serum). Group B was on 12% casein diet and group D on 18% casein diet. 4 rats in each group.

<u>Fed and fasting levels in each group</u>							
Week----->	0	1	2	3	4	5	mean±SD
<u>B</u>							
Fed values	4.3	4.8	4.3	4.9	4.5	4.8	4.60±0.27
Fasting values	4.5	4.8	4.3	4.8	4.4	4.7	4.58±0.21
<u>D</u>							
Fed values	4.7	5.2	4.5	4.9	4.8	5.0	4.85±0.24
Fasting values	4.9	5.2	4.5	5.0	4.7	5.2	4.92±0.29

Fed and fasting values in two groups

Fed values	4.60±0.27
B	4.85±0.24
D	
Fasting values	4.58±0.21
B	4.92±0.29
D	

#### 4.4. Transferrin.

The mean transferrin levels for each week of the experimental period (week 0-week 5) ranged between 8.0 and 14.0 (mean 10.75 and 1.84) mg per ml of serum (Table 19). Morgan and Peters (1971) have given  $4.8 \pm 0.08$  mg/ml as serum transferrin level in male Wistar rats (7-8 weeks old) and weighing between 160-240 g. Values from human sera have been given as 2.00-3.20 mg/ml (mean 2.6) by Turner and Hulme (1971) and by Cartei, Meani, Okolicany and Naccarato (1970), and as 1.34-1.90 mg per ml serum by McFarlane, Reddy, Cooke, Longe, Onabamiro and Houba (1970).

For reasons which are not clear, very high values, compared with those obtained later were found in the initial measurement in all three dietary groups. This may have been because of lack of familiarity with the test.

In all three groups there was a small increase in transferrin level between the first and fifth week.

The values in group B tended to be consistently lower compared with the other groups. From the first to the fifth week the values were the same in groups C and D except in the second week. In the last column of Table 19 is given the mean and SD for the whole experimental period from the 1st to the 5th week. The values for the initial week have not been included in the analysis because there was too great a variation between them.

It is clear from the Table that in these experiments the serum transferrin level did not act as a sensitive indicator of small differences in the state of protein nutrition. This was disappointing in view of the claim by McFarlane et al (1969) that it is the best test for distinguishing between different degrees of severity of kwashiorkor.

Table 19

Serum transferrin levels ( mg/ml ). Each  
 Each group contained four rats.  
 (values for initial week not included  
 in the statistical analysis)

<u>Group</u>	<u>Week</u> ----->	<u>initial</u>	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>	<u>mean±SD of all weeks</u>
B(12% casein)		15.0	8.3	8.0	11.0	10.0	9.0	9.26±1.24
C(15% casein)		13.0	9.0	11.0	13.0	12.0	11.0	11.20±1.48
D(18% casein)		17.0	9.0	14.0	13.0	12.0	11.0	11.80±1.92

#### 4.5 Creatine content of muscle and muscle mass.

Muscle mass and creatine content of muscle were measured in three experiments in rats on five different levels of dietary protein. The purpose was two-fold; to find out whether there was significant differences in the proportion of muscle in the body in the different dietary groups; and to get information which would help to validate the use of urinary creatinine output as a measure of muscle mass.

Creatine content of muscle. The values found for creatine content of muscle are shown in Table 20. The colorimetric method gives results which are about 10% higher than those by the enzymatic method. Presumably this is because the latter is more specific. The results suggest that about 10% of the creatine in muscle was converted to creatinine during the processing of the tissue. Baker and Miller (1939) found a conversion rate of 10% when tissue was analysed immediately after decapitation. Probably the best estimate of the true creatine content of muscle is the sum of creatinine plus enzymatically estimated creatine. The creatine content of muscle per g or per mg N shows no consistent differences in the groups on different levels of dietary protein. This finding is of some importance for the interpretation of measurements of muscle mass based on urinary output of creatinine (see discussion).

Specific activity (SA) of creatine in muscle and carcass. One of the problems encountered by Waterlow et al (1972) in their studies of creatine turnover in rats was that the specific radioactivity of 'creatine' in muscle and carcass was not identical. It was not clear whether the difference was real, or whether it resulted from the methods of measurements. It was not possible to measure creatine enzymatically in carcass extracts, because a substantial proportion was converted to creatinine during the extraction procedure.

Table 20

Creatine content of muscle.

(Mean values, umols/g muscle)

In the last column are the  
values per mg N.Number of rats in each group given in  
parenthesis.

Group	% protein in diet	colour method		enzyme method	enzyme method	umols per mg N
		creatine	creatinine	creatine	Sum of creatine and creatinine	
A(4)	8	29.9	4.6	29.4	34.0	1.04
B(8)	12	27.7	3.3	23.3	26.6	0.98
C(5)	15	29.9	2.8	24.7	27.5	1.13
D(5)	18	28.6	2.8	26.0	28.8	0.86
E(3)	24	23.4	3.8	20.1	23.9	1.07

Therefore for both muscle and carcass specific activities have been calculated as counts per umole (creatine + creatinine) estimated colorimetrically. The mean ratio of SA in muscle to that of carcass in 26 rats was 0.94, SD 0.07, not significantly different from 1 (Table 21). Because of this small difference, the muscle mass calculated from the radioactivity was on the average slightly higher than that calculated from the colorimetric measurements.

Muscle mass. The carcass weight, as per cent of body weight, was very constant (about 50%) in all the groups (Table 22). This suggests that the fat content of the body was similar in all groups, since most of the fat is either subcutaneous, and removed with the skin or associated with the abdominal viscera.

The muscle mass as per cent of body weight ranged from 35 to 47%. This is within the range found in rats by other workers (Waterlow & Stephen, 1966; Miller, 1969; Waterlow et al, 1972). Muscle mass in man, estimated from creatine output, is of the order of 40% of body weight in normal subjects (Wilmer, 1940). There were no consistent differences in the proportion of muscle between the rats on different diets. It is not clear why muscle mass is relatively so low in group D (18% casein) and so high in group E (24% casein). Except in group D there is a clear relationship between the absolute muscle mass and the dietary protein intake.

Creatine kinase activity. Table 23 illustrates the mean creatine kinase activities in (a) rat serum and in (b) rat muscle over a period of five weeks in one experiment.

The activities were initially of low values (0.23 for serum, and 0.32 for muscle) as opposed to those obtained during the five-week period when the rats were on the experimental diets (0.41-0.54 for serum and 6.2 for muscle).

The creatine kinase activity was found to decrease with storage. A loss in activity of about 27-39% was recorded after 10 days' storage. The values for the enzyme activities were not helpful in separating rats on the various dietary groups.

Table 21  
Specific activities of muscle and carcass  
in respect of creatine

The ratios of the specific activities of creatine  
in muscle and carcass are shown for 26 estimations.

0.90	1.00	1.08	0.81	1.09
0.93	0.95	1.04	0.95	0.96
0.85	1.05	1.00	0.89	0.94
0.89	0.95	0.93	0.98	0.91
1.06	0.87	0.83	0.87	0.93
0.92				

$$\text{Mean} \pm \text{SD} = 0.94 \pm 0.07$$



Table 22

Muscle mass

(Figures in parenthesis represent percentages of body weight)

Group	% protein in diet	number of rats (av. age 60 days)	body weight at death(g)	carcass as weighed(g)	g muscle of carcass		% of carcass (from counts)
					from counts	from Cr + Cn	
A	8	4	129	67 (52)	48(37)	42(32)	72
B	12	9	157	77 (49)	65(41)	60(38)	85
C	15	5	203	94(46)	80(39)	75(36)	86
D	18	5	205	101 (49)	71(35)	69(34)	70
E	24	4	226	115 (51)	107(47)	102(45)	92

Table 23  
Creatine kinase activities  
 (number of rats in parenthesis)

## (a) Rat serum (umols/min/ml)

Week----->	<u>0</u>	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>
Group and % protein in diet						
D 18(4)	0.22	0.46	0.49	0.44	0.45	0.48
C 15(4)	0.23	0.55	0.51	0.51	0.54	0.54
B 12(4)	0.24	0.41	0.48	0.48	0.51	0.42

## (b) Rat muscle (umols/g/min)

Group and % protein in diet	<u>Initial.</u>		<u>10 days after.</u>	
	<u>umols/min</u>		<u>umols/min</u>	
	<u>per ml</u>	<u>per g</u>	<u>per ml</u>	<u>per g</u>
	<u>extract</u>	<u>muscle</u>	<u>extract</u>	<u>muscle</u>
D 18(4)	0.29	5.3	0.19	3.82
C 15(4)	0.35	6.9	0.26	5.18
B 12(4)	0.32	6.5	0.19	3.96

#### 4.6. Serum cholinesterase activity in rat serum.

In one experiment serum cholinesterase activities were measured in two groups of rats (B and D). The values are shown in Table 24(a). Measurements were made a week after the experimental diets had been fed. The activity of the enzyme ranged from 0.40 to 0.074  $\mu\text{mols/ml/min}$ . The values in group D, on the higher protein diet, were lower than those of group B.

In another experiment in which three dietary groups (B, C, and D) were used, blood was taken from the heart. The enzyme activities in the serum from heart are shown in Table 24(b). The three groups of rats could be separated by the activities of the enzyme; the higher the protein content in the diet the higher the activity of the enzyme in the serum from the heart. The activity was 5-10 times greater than the activity in the serum from the tail. Why there is a difference between the two is not clear. The analysis on the serum from the heart was done by chance at the end of the experiment and could not therefore be repeated. It would be necessary to do more analyses from heart blood to justify any pronouncement on the difference.

In the UV method adopted for the measurement of the enzyme activity, acetylthiocholine was used as the substrate for the enzyme at  $37^{\circ}$ . Employing same substrate and at the same temperature of incubation, a range of 1.8-4.4 (mean  $2.97 \pm \text{SD } 0.77$ ) has been reported by Uete et al (1972) and by Garry and Routh (1964) in samples from healthy human adults.

Table 24  
 Serum cholinesterase ( $\mu$ moles  
 per ml per min). There are  
 4 rats in each group.

Group and % protein in diet	Week---->	<u>1st</u>	<u>2nd</u>	<u>3rd</u>	<u>4th</u>	<u>5th</u>
		(a) <u>blood from tail</u>				
B(12)		0.071	0.074	0.056	0.050	0.051
D(18)		0.071	0.066	0.040	0.045	0.040
		(b) <u>blood from heart</u>				
B(12)						0.37
C(15)						0.46
D(18)						0.69

4.7. Ribonuclease:

The activity of this enzyme was estimated by UV spectrophotometric method. The values for three groups on rats (groups B, C, and D) have been compared purely on the basis of their optical densities of 10 ul of ten times diluted serum. The ODs were used without comparing them to those of standards because commercially prepared standards were not available at the time of this particular experiment. Absolute figure for the enzyme activities have therefore not been given in the table.

The values reported here were obtained from serum samples in the last week of an experimental period of five weeks in one experiment. It is clear that the values did not follow any particular pattern. Group D had the least figure followed by group C.

Table 25

Rat serum Ribonuclease (measured as change in OD) of three groups of rats on diets indicated. Each group had 4 rats. The net OD for each rat is given together with the mean for each group.

Group	B (12% casein diet)	C (15% casein diet)	D (18% protein diet)
	74	73	60
	63	135	101
	56	76	56
	<u>51</u>	<u>92</u>	<u>113</u>
mean =	61	94	82.5

(Note: These are the ODs obtained from the 20 times diluted supernatant.)  
OD is multiplied by 1000

Table 26

Markham distillation and titration  
of two aliquots of a nitrogen digest.  
(M = muscle)

<u>Sample</u>	<u>volume of digest(ml)</u>	<u>volume of N/50 HCl (ml)</u>	<u>mg N/ml (calculated)</u>	<u>mg N/ml (mean)</u>
Blank	5	0.1	.	
Standard	5	17.45	0.972	0.982
	3	17.32	0.964	
M1	5	4.76	0.261	0.258
	3	2.84	0.256	
M2	5	5.64	0.301	0.304
	3	3.55	0.307	
M3	5	6.39	0.343	0.343
	3	3.94	0.343	
M4	5	5.09	0.270	0.269
	3	3.15	0.269	
M5	5	4.65	0.246	0.247
	3	2.92	0.248	
M6	5	4.31	0.227	0.226
	3	2.67	0.225	
M7	5	4.72	0.259	0.253
	3	2.76	0.248	
MB	5	4.26	0.224	0.218
	3	2.55	0.213	
M9	5	6.39	0.343	0.343
	3	3.94	0.343	
M10	5	6.80	0.366	0.367
	3	4.22	0.369	
M11	5	5.38	0.287	0.278
	3	3.14	0.269	

#### 4.8. Comparison of methods for measuring nitrogen content of muscle

After the muscle sample had been ground up in TCA and the supernatant removed the precipitate was dissolved in 10 ml 0.2N NaOH, an aliquot of this solution was digested in  $H_2SO_4$  for measurement of non-collagen protein nitrogen (appendix III).

Since one of the objects of this work was to test the accuracy of micromethods, it was of interest to compare measurements on the microgram scale by Nessler's method with those on the milligram scale by Markham distillation.

Commercial Nessler's solution was found to be unsatisfactory, and therefore the solution was prepared in the laboratory (appendix IV). Measurements were made on samples containing 5-20  $\mu g$  N in a final volume of 3 ml Nessler's solution. Fig.21 shows that absorbance was linear up to 20  $\mu g$  N (OD 0.6). If care is taken in the preparation of the reagents the OD of the blank does not exceed 0.05.

For testing the distillation-titration method measurements were made on 2 aliquots, one of 3 ml and the other of 5 ml, of the muscle digest (Table 26). This provides a check on the completeness of the distillation. The total amount of nitrogen measured was 1.0-1.5 mg. In eleven comparisons there was no significant difference between the samples of different volumes. The mean difference between duplicates was 0.003 mg N/ml.

Measurements for nitrogen on thirty four muscle digests were made by the two methods. The results are given in table 27. Comparison of the two methods by the paired 't' test showed that Nessler gave highly comparable results with Markham in samples with mean N content of  $0.28 \pm SD 0.05$  mg N/ml.

FIG. 21 NITROGEN STANDARD CURVE:  
OPTICAL DENSITY / CONCENTRATION RELATIONSHIP.

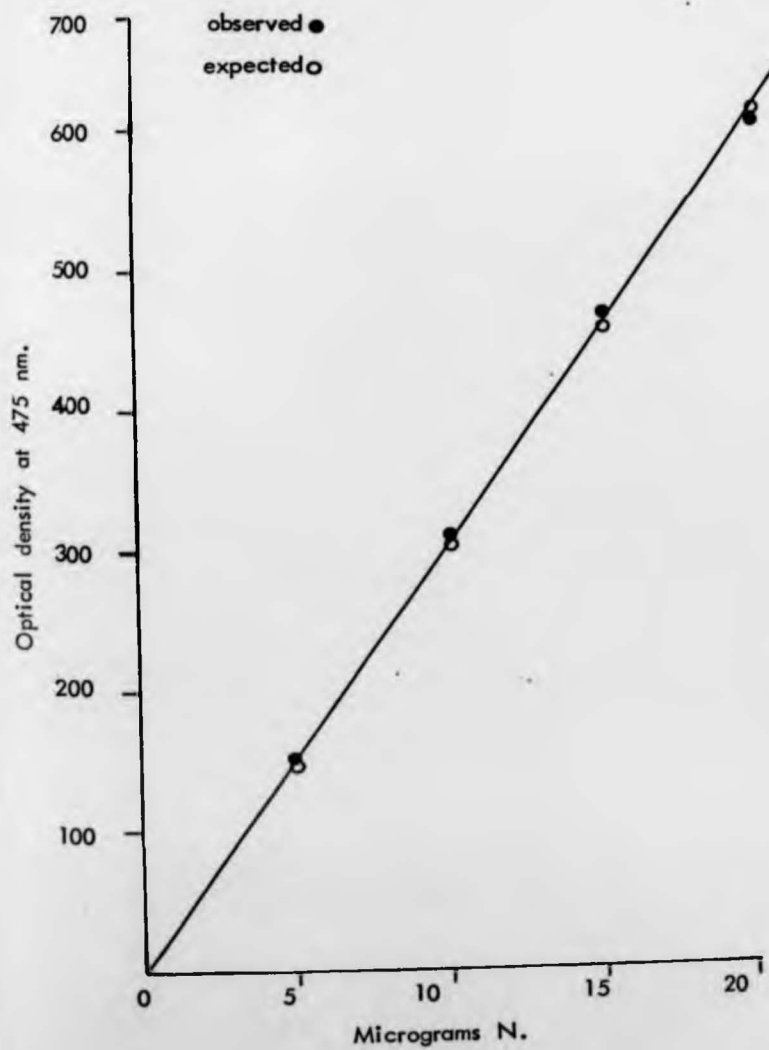




Table 27

Determination of nitrogen content of muscle by two methods (Markham and Nessler). Values given as mgN/ml of muscle digest. The values in mg N/g muscle are also given.

<u>% protein in diet</u>	<u>Nessler method</u>	<u>Markham method</u>	<u>mg N/ g muscle</u>
8	0.318	0.319	31.8
	0.214	0.240	30.6
	0.262	0.268	33.6
12	0.402	0.401	32.0
	0.250	0.245	34.4
	0.344	0.364	32.8
	0.384	0.387	32.9
	0.223	0.217	31.8
	0.279	0.276	32.3
	0.289	0.293	28.9
	0.307	0.310	29.2
	0.286	0.269	31.6
	0.240	0.218	33.4
	0.282	0.278	28.6
15	0.278	0.275	29.6
	0.444	0.449	28.6
	0.354	0.364	29.0
	0.352	0.354	28.9
	0.342	0.343	32.0
	0.252	0.226	32.0
	0.395	0.367	31.0
	0.281	0.247	32.5
18	0.323	0.316	30.2
	0.325	0.321	29.7
	0.287	0.290	30.8
	0.348	0.354	28.2
	0.253	0.258	31.4
	0.245	0.253	26.0
	0.357	0.343	26.3
	0.324	0.304	28.9
24	0.354	0.347	32.3
	0.406	0.401	33.5
	0.490	0.406	41.7
	0.308	0.381	26.8

#### 4.9. Level of serum branched chain amino acids (combined)

Serum valine, leucine and isoleucine were separated by thin layer chromatography (TLC), eluted from the gel with water and the amino-N analysed with ninhydrin as described in the sections on methods.

Two groups of rats, B (on 12% casein diet) and D (on 18% casein diet), each with four rats were investigated in one experiment. The rats were kept on the diets for five weeks. At weekly intervals they were fasted overnight. One blood sample was taken before the fast and the other the next morning after the fast in order to assess the effect of short-term variations in food intake on the concentrations of the branched chain amino acids in the serum.

The optical density (OD) of the eluted amino acids (ninhydrin-treated) was obtained by subtracting the OD of the gel background run at the same time as the test sera. When the gel background colour was impossibly high it was removed from the calculation together with the corresponding lowest blanks. The net ODs (mean of duplicates) obtained in this way are given in Table 28B for each rat in the fed and in the fasted state throughout the experimental period. The corresponding values of the ODs in  $\mu\text{mols/L}$  are given in Table 28A. These ( $\mu\text{mols/L}$ ) values were obtained by comparing the ODs with the net OD (always around 0.194) of a standard of 10 nmols amino acids run with every sample. The weekly means ( $\pm\text{SE}$ ) are also given in the table. The overall mean is 567  $\mu\text{mols/L}$ .

The values for week 0 have been ignored as the rats have come from a commercial source and were of unknown states. There is a significant difference between most of the values in the group on 12% casein diet and those on 18% casein diet in the fasted animals (Fig 21<sup>A</sup>). Some of the values in the group on 12% casein diet were so low as to be unmeasurable by the system used. This might give an exaggerated

impression of its degree of effect of the 12% casein diet. The rise of the serum branched chain amino acids at the end of the experiment is as found at **Dunn Nutritional Laboratory** (Whitehead and Lunn, personal communication).

The values lie in the accepted range in comparison with published data. Scharff and Wool (1966) found that the total concentration of the branched chain amino acids in rat serum is 530 umols per litre and Young, Vilaire, Newberne and Wilson (1973) have reported a figure of 567 umols per litre.

At the end of the last week of the experiment, and after an overnight fast, blood was taken from the tail and then from the heart, the serum separated and the concentration of the branched chain amino acids measured. The results are given in Table 29. The amino acids level in the heart blood tended to be higher than that in the tail blood. Why this is so is not clear.

Table 28A

Levels of serum branched chain amino acids (combined) in two groups, B and D, of rats on diets containing different amounts of casein. There were four rats in each group. Concentrations ( $\mu\text{mols/L}$ ) are given as mean for each rat over the experimental period.

X = levels from fed rats; 12%X and 18%X are the weekly mean( $\pm\text{SE}$ ) of the values from fed rats in each group

Y = levels from fasted rats; 12%Y and 18%Y are the weekly means( $\pm\text{SE}$ ) of the values from fasted rats in each group

Week----->	0	1st	2nd	3rd	4th	5th
Rat number						
			(Group B, 12% casein)			
↓						
1X	319	649	108	361	206	485
1Y	329	360	82	0	0	691
2X	371	969	412	515	155	619
2Y	155	330	0	0	546	608
3X	144	0	155	0	330	485
3Y	268	0	0	155	742	701
4X	845	577	907	433	0	773
4Y	845	0	732	330	454	742
	12%X= 420 $\pm$ 150	549 $\pm$ 202	396 $\pm$ 183	327 $\pm$ 114	173 $\pm$ 68	591 $\pm$ 69
	12%Y= 399 $\pm$ 153	173 $\pm$ 100	204 $\pm$ 177	121 $\pm$ 79	436 $\pm$ 157	686 $\pm$ 28
			(Group D, 18% casein)			
5X	1649	619	1412	506	464	948
5Y	1051	619	639	381	948	639
6X	1031	1340	928	515	1041	567
6Y	1536	1051	773	515	876	619
7X	742	856	278	526	1165	825
7Y	866	629	742	351	1021	506
8X	763	825	1062	598	1237	515
8Y	1165	957	1206	866	1371	608
	18%X=1046 $\pm$ 211	910 $\pm$ 153	727 $\pm$ 174	536 $\pm$ 21	977 $\pm$ 176	714 $\pm$ 103
	18%Y=1155 $\pm$ 141	814 $\pm$ 111	840 $\pm$ 125	528 $\pm$ 118	1054 $\pm$ 110	593 $\pm$ 30

Table 28B

Serum branched chain amino acids (combined). The optical densities (multiplied by 1000) of the ninhydrin-treated amino acids----corresponding to concentrations of  $\mu\text{mols/L}$  given in Table 28A

Week----->	0	1st	2nd	3rd	4th	5th
Rat number	(Group B, 12% casein)					
↓						
1X	31	63	10	35	20	47
1Y	32	35	8	0	0	67
2X	36	94	40	50	15	60
2Y	15	32	0	0	53	59
3X	14	0	15	0	32	47
3Y	26	0	0	15	72	68
4X	82	56	88	42	0	75
4Y	82	0	71	32	44	72
	(Group D, 18% casein)					
5X	160	60	137	49	45	92
5Y	102	60	62	37	92	62
6X	100	130	90	50	101	55
6Y	149	102	75	50	85	60
7X	72	83	27	51	113	80
7Y	84	61	72	34	99	49
8X	74	80	103	58	120	50
8Y	113	93	119	84	133	59

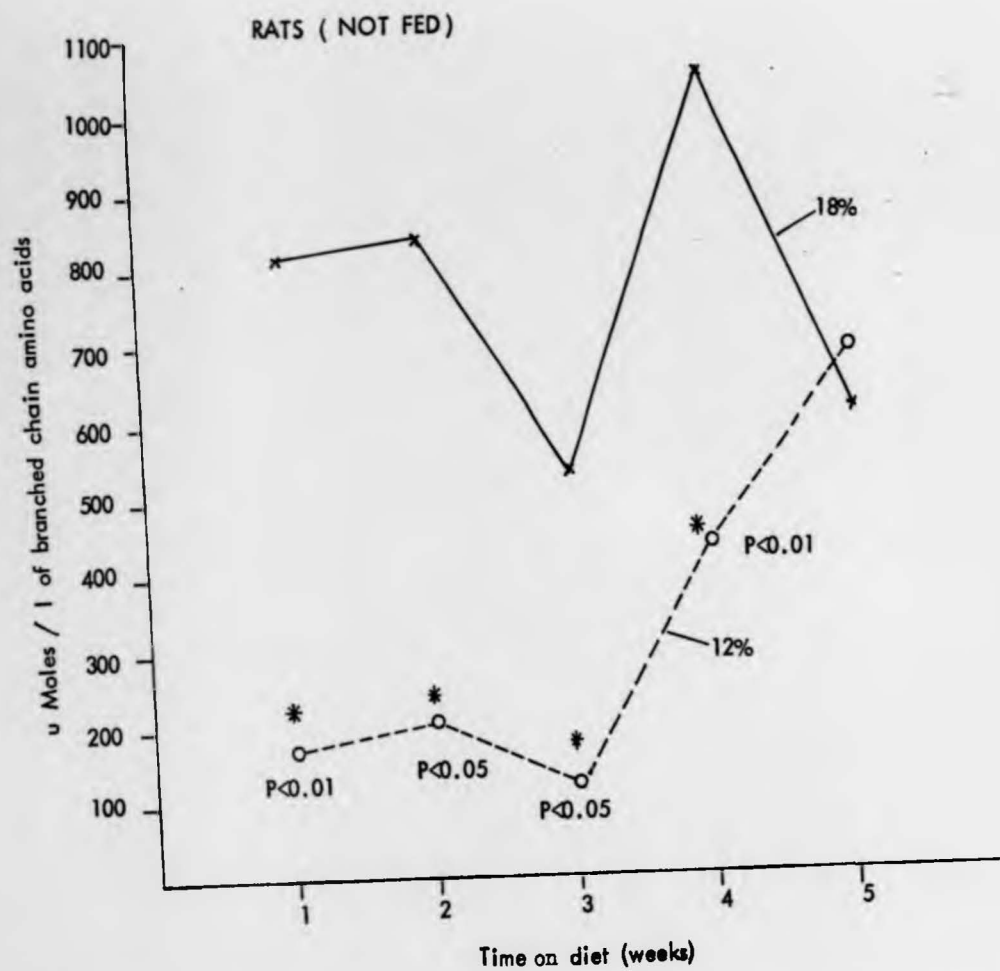
Table 29

Branched chain amino acids level (total) in serum from fasted rats in two groups (B and D) on diets with casein concentrations shown. Values are averages for 4 rats in  $\mu\text{mols/L}$ .

Rat group	serum from heart	serum from tail
B(12% casein)	691	686
D(18% casein)	910	593

FIG 21A

Effect of 12% and 18% casein diets on serum branched chain amino acids



CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

## 5. DISCUSSION AND CONCLUSIONS

Most children in developing countries suffer from PEM at some stage in their lives between the ages of 0 and 5 years. PEM causes countless deaths and untold human misery. Even more deaths, caused primarily by infections, are accelerated or precipitated by malnutrition. Malnutrition has a long-lived effect on those who survive death; there is increasing evidence that a childhood experience of severe malnutrition prevents a full realization of the individual's potential in adult life. Amman, Belshaw and Stanfield (1972), in their introduction to 'Nutrition and Food In African Economy', stressed the danger of malnutrition in slowing down economic growth.

The existence of PEM in developing countries and the realization of the enormity of the problem by both developing and to a larger extent by developed countries has acted as a powerful stimulus to research. Effective nutritional therapy can only be devised on the basis of a sound understanding of the different abnormal processes involved. There has been a great output of biochemical investigation and experimental work but in the main rather unfortunately, it has been concentrated on the acute and severe cases. We have, therefore, a fair understanding of the metabolic changes in severe cases of PEM, but this is not a matter of great satisfaction when the disease could be prevented from becoming severe.

We need more knowledge about how to diagnose and assess early stages of PEM. In spite of the pioneer work of Whitehead and his colleagues, much remains to be done in the development of new methods and the application of old ones. Methods to be used in field studies must fulfil certain requirements: they should not be sophisticated in terms of operation and equipment, but simple, precise and sensitive to



slight changes in nutritional states.

The development of methods in the present work has been carried out by using serum as the main biological specimen for analysis. The choice of serum, rather than urine, was made in view of the fact that the collection of urine under field conditions, and especially a 24-hr collection, poses many practical problems.

All the work was done in London and consequently it was concentrated on rats rather than humans, but it is realised that the rat is not an ideal model for the child. We may not in some cases be too confident in applying the results of animal experiments to man. In the rat a deficient diet imposed after weaning seems to have no permanent effect and 'catch-up' growth may be complete once an adequate intake is restored. On the other hand if there is a deficient intake of food during the suckling period, or even during pregnancy, growth may be permanently impaired (Widdowson and McCance, 1960; Chow and Lee, 1964; Venkatachalan and Ramanathan, 1966). In the rat it is difficult to produce fatty liver of any severity or oedema. The baboon may be a better experimental model. Coward et al (1972) produced a kwashiorkor-like state in baboons with changes in albumin and globulin concentrations bearing a striking resemblance to those found in children developing kwashiorkor.

#### Evaluation of tests used.

Body Weights. The rats of all groups were healthy, and gained weight over the experimental period. Throughout the experiments the weight gains did differentiate rats in the various dietary groups. All the diets were isocaloric. The greater the amount of protein consumed by the rats the higher the percentage increase in weight over the experimental period of 35 days. The increases in weight for the groups were 63%, 138%, 151%, 177%, and 237% of the initial weights in rats consuming on the average 25, 48, 67, 77 and 97 grams of protein per 35 days respectively (Table 10).

Total Protein. Rats on the various diets could not be separated by their total serum protein levels. In children with severe PEM (with or without infection) total serum protein has been found not to be helpful as a tool for diagnosis or prognosis. In severe PEM with infection, although there is a decrease in alpha- and beta-globulin concentration, the level of the serum total protein hardly changes because the concentration of gamma-globulin increases. In fact in the early stages of malnutrition the albumin concentration has been observed to fall, while the total globulins rose. The reciprocity between albumin and total globulins has also been observed in experimentally malnourished baboons and monkeys in the absence of apparent infection (Coward et al, 1972). The present finding is therefore not really surprising, in view of the observations by earlier workers cited above. Total serum protein level is probably not a good indicator for the early diagnosis of protein malnutrition.

Albumin. The study presents evidence that in apparently healthy rats on diets differing only in protein content, serum albumin levels could be used to differentiate the dietary groups: the higher the protein content of the diet, the higher the serum albumin level in the rat. However, the differences are not great, and the larger the number of samples analysed the more sensitive the albumin level becomes in distinguishing rats on the various diets.

Values for serum albumin concentration depend very much on the type of analytical system employed. Many and varied opinions have been expressed as to the specificity of BCG dye in binding to albumin. Some authors have found BCG dye binding to albumin to be specific, and have obtained comparable albumin levels by BCG and electrophoresis or salt fractionation. The contention has also been made that BCG

dye is superior to HABA dye in its specificity of binding to albumin (Reilly et al, 1970; Doumas et al, 1971; McPherson and Everard, 1972; Westgard and Lahmeyer, 1972; Miyada et al, 1972). Other workers have expressed dissenting views (Webster et al, 1974; Webster, 1974; Ferreria and Price, 1974). They have shown that BCG overestimates albumin levels in abnormal human sera and that there are discrepancies between the values obtained with BCG and those obtained by electrophoresis or salt fractionation. What seems not to be in dispute is that these discrepancies are relatively unimportant when BCG is used for measuring near normal or normal serum albumin levels.

In the estimation of albumin in this work with BCG, comparable values were obtained from diluted and undiluted sera indicating that the binding of BCG dye to albumin was not affected by dilution. This is in agreement with the observation by Webster et al, (1974). From a total of 312 estimations with BCG a mean value ( $\pm$ SD) of  $4.17 \pm 0.57$  g/100 ml was obtained. This is high compared with the values found by other workers (Caback et al, 1963; Schultz et al, 1954). It is possible, therefore, that the absolute figures are not correct but they are valid for comparative purposes.

The point which emerges from this study is that although the rats on marginally low protein intakes were growing at sub-optimal rates, they were still apparently healthy. Nevertheless, as groups they had different albumin levels. Thus it seems that serum albumin concentration could detect protein deficiency in situations where the rate of growth and the extent of growth retardation are not known.

If these results can be applied to man, it follows that, in agreement with earlier workers (Whitehead, Flood and Poskitt, 1971; Kudlicka and Kudlickova, 1973), albumin concentration could be very useful as a screening tool, for detecting protein deficiency and

differences in protein consumption in cross-sectional studies. It would be particularly valuable in children whose ages are not known, so that deficiencies in weight and height for age cannot be assessed.

Even a small reduction in serum albumin concentration may be significant not only as a sign of protein deficiency, but also because of its physiological consequences. The part played by albumin in mammalian protein metabolism is very important. In addition to its oncotic function it acts as a carrier of a large number of biologically active substances such as steroid hormones, vitamins, etc. Thirdly, it has a protein storage function, for it includes all the essential amino acids in its molecule, and can act as a mobile protein reserve (Kudlicka and Kudlickova, 1973).

Transferrin. In 1968 Antia, McFarlane and Soothill demonstrated that serum transferrin in PEM patients fell to 1/5 the levels found in controls and that the decrease was much greater than that of total protein or albumin. Later these workers claimed that transferrin levels gave a more accurate indication of diagnosis and prognosis of the early and severe states of PEM (McFarlane et al, 1969). Maletnlema (1973), working on humans, observed that the level of the serum transferrin in the mother at the 24th week of pregnancy bore a significant relation to the birth weight of the child.

There are, however, contradictory observations. Kumar et al (1972) found that the serum transferrin level was not significantly decreased in monkeys until after 12 weeks on protein-free diet, so that it was not diagnostic of early protein deficiency. Ismadi et al (1972) observed that the serum transferrin was not a useful diagnostic or prognostic tool for the various stages of the disease in a number of children. The few existing reports on the usefulness of transferrin

as an indicator of early PEM are therefore not in agreement.

In the present experiments transferrin was estimated by the immunodiffusion method. The diameter of the precipitin ring formed by the reaction between the transferrin (antigen) and antiserum to transferrin incorporated in the gel is compared to a standard diameter. In most cases ring has a diameter of 4-7 mm, and the accuracy of measurement with a simple hand measuring-device which would be convenient in field studies is limited. Moreover, in my hands the method was not very sensitive. In view of the fact that antiserum specific for human transferrin is expensive when commercially prepared and incorporated in gel plates and also difficult to prepare in an ordinary laboratory; and further, the fact that there is still a question mark on its usefulness as a marker of early protein deficiency, it could not be recommended for field studies yet.

Enzymes: Waterlow and Alleyne (1971), reviewing the results of enzyme measurements as a tool for the detection and assessment of early protein-energy malnutrition, remarked that the work done in the last ten years contributed little to the solution of the problem. The findings from the present study, in which measurements were made of serum cholinesterase, serum ribonuclease and serum and muscle creatine kinase were also discouraging.

(i) Cholinesterase. The observation was made by Schendel et al (1962) that serum cholinesterase and albumin levels correlated well with the various states of PEM in groups of children. This view was not supported by the findings of the present studies as far as detecting and differentiating between apparently healthy rats was concerned.

(ii) Ribonuclease. From a recent paper (Albanese et al, 1971) it appeared that serum ribonuclease (RNase) might offer a more dynamic

criterion of protein metabolism than nitrogen balance. A pilot study was carried out during one week in one of the experiments. The RNase activities did not differentiate the groups of rats on different diets. This finding was disappointing because Prasad and Oberleas (1973) have observed that RNase activity was increased in zinc-deficient tissues (bone, testis, thymus and kidney) and zinc deficiency presupposes growth retardation (Prasad, 1966). It seems unlikely from the present studies that RNase activity could be a sensitive method for detecting differences in individuals on diets differing in protein content. However, plasma RNase might be a useful parameter for discriminating between severe and moderate malnutrition (Sigulem, Brasel, Velasco, Rosso and Winick, 1973). It is interesting to note the observations of earlier workers: Métais and Mandel (1955) were not able to show any difference between the level of serum RNase in normal and cancer patients but contradictory observations have been made by Zytko and Cantero (1966) that the serum RNase level in 60% of patients with malignant disease was significantly higher than that found in normal subjects.

(iii) Creatine kinase. Creatine kinase was studied in view of the previous observation that the enzyme was useful as a prognostic tool in children with kwashiorkor undergoing treatment (Balmer and Rutishauser, 1968; Reindorp and Whitehead, 1971). From the observations in the present work creatine kinase activity in either serum or muscle gave no help in distinguishing rats on the various diets. It has been widely observed that serum creatine kinase levels in several subjects vary considerably on a day-to-day basis in both individuals and throughout the population (Griffiths, 1966; Thomson, 1968). Working on factors affecting serum creatine kinase activity in normal adult females, Paterson and Lawrence (1972) found that it was affected by prolonged muscular activity but not by slight day-to-day physical

movement. The present findings are in agreement with the observations by Paterson and Lawrence in that the rats studied were normal, did gain weight over the experimental period, were accustomed to a fairly moderate exercise and there were no significant changes in CK levels in all groups.

Muscle Mass. Thought-provoking observations have been made by earlier workers on the state of muscle mass in malnutrition: reduction in muscle mass is probably the best available indicator of the extent of protein depletion in the body as a whole (Waterlow, 1969). Reindorp (1970) found that in rats on diets of different protein content muscle mass correlated well with creatine output and creatine kinase activity. Muscle mass was therefore measured in the present studies with a view to studying the changes in body composition in apparently healthy rats growing at different rates.

Muscle mass was calculated from the creatine content per g muscle compared with that of the whole carcass. The comparison was made in two ways: by measurement of radioactivity after injection of  $1\text{-}^{14}\text{C}$ -creatine, and by enzymatic determination of creatine in muscle, combined with colorimetric measurement of creatinine derived from creatine in carcass. Measurements of muscle mass by the two methods compared fairly well (Table 22). The greatest difference in weights obtained by the two methods for any rat group was 6 g, and this was in group A. All other differences in weight were less than this. The percentage muscle in carcass was in agreement with that found by previous workers (Waterlow and Stephen, 1966; Miller, 1969; Waterlow et al, 1972).

In 1927 Charutin, quoted by Baker and Miller (1939), obtained an average value for creatine in rat muscle of 449 mg/100 g (about 30 umols/g muscle) as opposed to Baker and Miller's figure of 540 mg/100 g gastrocnemius (about 36 umols/g). Baker and Miller suggested that the



difference in the values which were obtained by colorimetry might be due to differences in diets or in the strain of rats employed. In the present studies, the mean and SD were  $28.16 \pm 3.73$   $\mu\text{mols/g}$  muscle with a range of 23.9-34.0. These results were obtained by enzymatic measurements of creatine in muscle. However, since there is always the possibility of some conversion of creatine to creatinine during preparation of the extracts, creatinine was also measured. The creatinine component amounted to about 15% of the total (2.8-4.6  $\mu\text{mols/g}$ ). It seems likely that our slightly low figures, compared with those obtained by earlier workers result from the fact that the enzymatic method for creatine is much more specific than the colorimetric method previously used. The ratio of creatinine plus creatine per mg Nitrogen in muscle was the same in all groups. The values ranged between 1.07 and 0.98 (mean and SD,  $1.02 \pm 0.1$ ).

It has been found in the present work that the higher the protein content in the diet the greater the muscle mass in the rats at this early stage (within 35 days of weaning) of their growth. It seems to me that rats fed with the higher protein diet will better be able to stand the ill-effect of any subsequent imposition of infection or inadequate diet. Any physiological implications in this respect, however, must await further studies.

The prospect of making measurements on muscle in field studies is remote indeed mainly because of the difficulty of obtaining samples.

Valine, Leucine and isoleucine. The levels of the three branched chain amino acids in the serum of the malnourished child are much reduced (Arroyave et al, 1962; Grimble and Whitehead, 1971). Neale (1972) has suggested that in the rat the catabolism of these three amino



acids, especially valine, is increased in malnutrition because of the increased activity of the branched chain-amino acid aminotransferases necessary for their degradation (Mimura et al, 1968). Other workers hold a contrary view (McFarlane and von Holt, 1969; Wohlhueter and Harper, 1970; Reeds, 1974; Sketcher, Fern and James, 1974). They have demonstrated that in liver and in muscle the keto-acid dehydrogenases for these amino acids are reduced so that their decreased level in the serum could not be due to increased catabolism. Since these dehydrogenases catalyse the first irreversible step in oxidation of the amino acids, the reduction in their activity represents a favourable adaptation.

What is certain is that the catabolism of the amino acids and indeed the degradation of muscle protein is regulated by plasma insulin level. Caloric restriction decreases basal plasma insulin in the rat (Grey, Goldring and Kipnis, 1970), and in normal man (Cahill, Jr., Herrera, Morgan, Soeldner, Steinke, Levy, Reichard and Kipnis, 1966) and insulin levels are low in children suffering from kwashiorkor (Waterlow and Alleyne, 1971; Iann et al, 1973). It is likely that small changes in dietary protein may have an effect on the serum level of insulin. The results of this study lend support to the observation by Perreira et al (1968) that a change in the quality and quantity of the protein in the diet of children is followed by a change in the pattern of the free amino acids. In the present experiments the level of the three branched chain amino acids in the serum of fasted rats on the 16% casein diet was significantly higher than that in the rats on 12% casein. The two groups of rat could be clearly distinguished.

Application to human studies. Duplicate analyses on a microscale could be done on 25 ul serum for total protein, albumin and transferrin.

Analysis for serum cholinesterase and ribonuclease could be carried out in duplicate on 10 ul serum. With 10 ul of serum the combined level of the three branched chain amino acids may be estimated by thin layer chromatography. A total of about 50 ul serum would therefore be sufficient for the analyses of all these biochemical parameters. With half the amount of serum, albumin and the three branched chain amino acids (the most sensitive of all the parameters) could be analysed.

In field studies the removal of such a minute amount of serum (i.e. about 100 ul blood) from the finger-tip is unlikely to meet with any opposition from the children or from the adults. A major equipment needed for all these analyses is Unicam spectrophotometer (SP 500). Albumin would be measured with the spectrophotometer which when fitted with microcuvettes could be employed for the estimation of amino-N from the branched chain amino acids. This kind of equipment will function well under tropical conditions so that for a survey in Ghana, or for that matter, in any developing country, a capital expenditure of about one thousand pounds will be sufficient. The same equipment could be adapted, without any major extra cost, to measure enzyme activities.

Time involved. With thin layer chromatography, about twenty samples could be analysed in two days. There are many operations involved but are simple and easy to teach. A worker can go through these stages quickly after a short time of training and practice (2-4 weeks). As many as fifty albumin determinations could be carried out in one day. It is possible that fifty samples could be analysed for all these biochemical parameters in less than a week by one trained worker. The methods so far mentioned have been made easy and as simple as possible to carry out and may conveniently be used in field studies in developing countries.

APPENDIX

APPENDIX 1Composition of diets(g)

	<u>8%</u>	<u>12%</u>	<u>15%</u>	<u>18%</u>	<u>24%</u>
Casein	85	127	159	191	254
Maize starch	457	415	383	351	288
Potato starch	103				
Dextrose	154				
Salt mixture*	51				
Vitamin mixture*	10				
Arachis oil	150				

Note: The amounts of potato starch, dextrose, salt mixture, vitamin mixture and arachis oil as given for 8% casein diet are the same in the other diets.

(decimal points in the figures have been rounded off)

\*(Preparation according to Payne and Stewart, 1972)

## APPENDIX II

Preparation of muscle digest for Nitrogen determination

The digest of the muscle was prepared by adding 10 ml of 0.2N NaOH to about 300 mg of wet muscle. (Wet muscle contains about 3% Nitrogen, therefore the solution was about 1 mg N/ml). The solution was centrifuged briefly after about 24 hours to get down the collagen. (supernatant contained non-collagen N). 1 ml of the supernatant was measured into a 10-ml test tube (duplicate) and 1 ml of conc.  $H_2SO_4$  added, followed by about 0.3 g grounded selenium as catalyst. The tube was kept in an oven at  $100^\circ$  for about 8 hours to evaporate the water present (volume reduced to about 0.2 ml at the end). The tube was put on a digest still until the solution became clear (6-8 hours). The solution was allowed to cool to room temperature (all ammonia kept out of the vicinity). The volume was made up to 10 ml with deionised water, the tube stoppered and shaken immediately for about half a minute. The tube was kept tightly stoppered until analysed.

## APPENDIX III

Creatinine determination on muscle extract

This was based on direct colorimetric assay by modification of the Jaffe reaction according to the method of Taussky (1956).

Reagents.

0.9% picric acid

2N NaOH

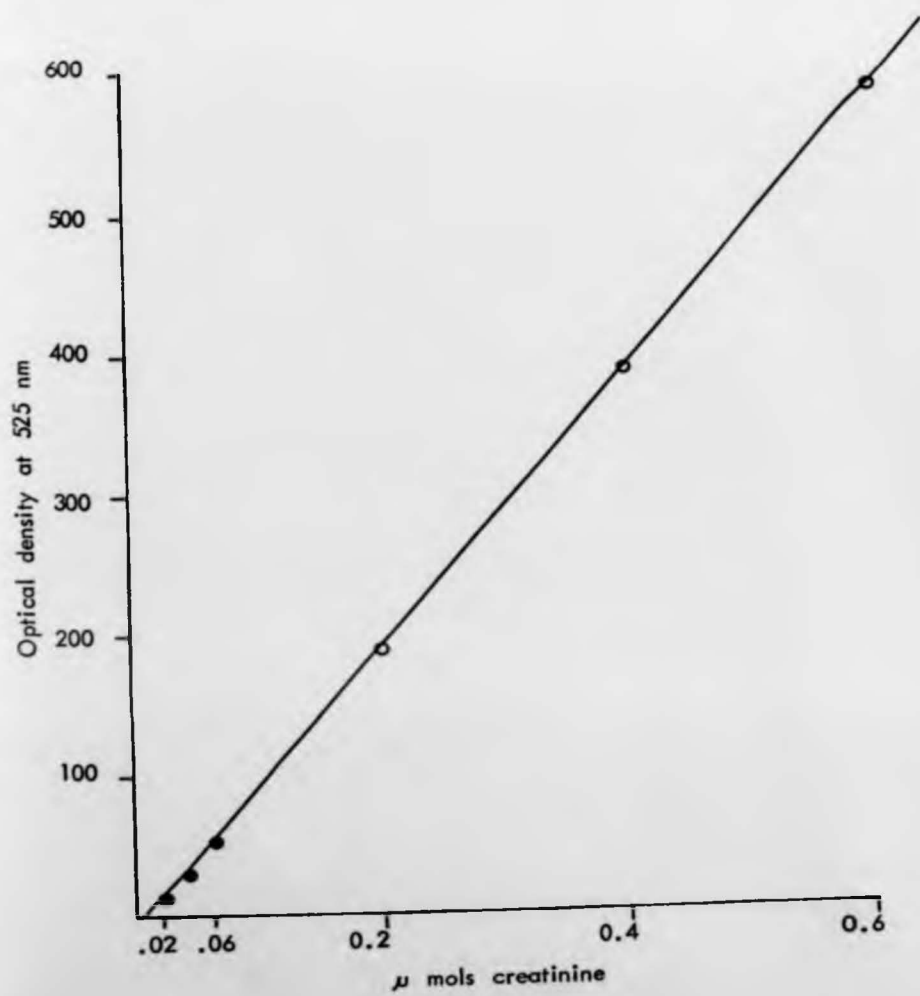
Reagent A: To 1 volume of picric acid was added 1 volume of NaOH followed by 3 volumes of water. (This reagent was prepared just before use).

Method.

To 100 ul of sample was added 3 ml of reagent A. The optical density was measured at 525nm after 20 minutes.

Standard Curve. 100 ul samples of creatinine solution containing 0.2-0.6 umols were analysed by the above procedure and the net absorbances were plotted against concentrations of creatinine to give a straight line through the origin. (Fig.22).

FIG. 22 CREATININE:  
NET ABSORBANCE / CONCENTRATION RELATIONSHIP



## APPENDIX IV

Preparation of Nessler's Reagent

- A. 8 g KI and 8 g  $\text{HgCl}_2$  dissolved in 100 ml distilled water.
- B. 2% gum ghatti solution. To about 2 g of clear crystals was added 100 ml of distilled water and boiled till the crystals were almost dissolved. The suspension was filtered through glass wool. The residue was washed with distilled water to make the filtrate up to 100 ml (the filtrate will have an OD of 0.01 at 475nm). If darker crystals were used, there had to be about two consecutive washings of the residue with 100-ml lots of warm water until one got an OD of 0.01 at 475nm).
- C. 10 ml of A plus 50 ml of B. This was made up 200 ml with distilled water (the solution keeps indefinitely).
- D. 0.8N NaOH
- E. Nessler reagent (NR). One part of C was mixed with two parts of D just before use.



## APPENDIX V

Measurement of serum ribonuclease (RNase)

The method used here is a modification of the one adopted by Zytko and Cantero (1962) for estimating serum ribonuclease. The activity of the enzyme in blood was determined by the spectrophotometric method based on the measurement of the optical density at 260nm of the acid soluble products formed by the action of RNase on ribonucleic acid (RNA) substrate.

Reagents.

Phosphate buffer, pH 7.4

1% sodium salt of RNA

0.25M sucrose solution

10% solution of sucrose in 0.25M sucrose

12% perchloric acid.

Method.

The reaction mixture was made up as follows: To 3 ml of buffer was added 1 ml of sodium salt of RNA, followed by 25-100 ul of 10% diluted serum. The mixture was incubated in water bath at 37° for 30 minutes after which time the reaction was stopped by the addition of 1 ml of 12% perchloric acid. The solution was centrifuged briefly to bring down the precipitate. The supernatant was diluted 20 times and the optical density read against water at 260nm within 90 minutes.

## APPENDIX VI

Measurement of serum branched chain amino acids.Preparation of chromatoplates

Coat plate with silica gel G (gel width 0.25 mm). Dry plate at 100° for 30 minutes or leave at room temperature for 24 hours before use. Divide gel layer into lanes of 12.5 mm.

Solvent. (n-butanol/glacial acetic acid/water) in the ratio 4:1:1 Prepare a volume of 120 ml before use.

Sample spotting and solvent run. With micropipette, spot 5  $\mu$ l sample on plate (sample must be delivered in a continuous steady flow. Must avoid drop-by-drop method and extensive drying between drops). Include spots for markers and standards. Stand plate in the tank (24 x 24 x 7 cm) containing the solvent; the level of the solvent must not cover the sample spots. Cover jar and allow run for about 3 hours (jar must not be opened before run).

Drying of plate. Remove plate from tank and blow hot air over it for 5 minutes. Spray markers (whilst shielding test sample spots) with ninhydrin. With the help of the markers, rule lines to enclose the gel areas containing the amino acid under study. Blow hot air continuously over this area for about 30 minutes (it is essential that the drying at this stage be complete otherwise residual  $\text{NH}_3$  and water molecules trapped in the gel would affect colour formation in the subsequent reaction between ninhydrin and amino-N).

Scrapping of gel and sample elution. Carefully remove gel with the help of scalpel into a plastic test tube. Add 500  $\mu$ l of distilled water. Stand for 15 minutes with occasional mixing on a whirl-mixer. Centrifuge at 3000 rpm (2500 g) for 15 minutes.

### Colour development

#### Reagents.

Citrate buffer, pH 5.6, 0.2M.

Potassium cyanide, 0.01M.

Reagent A2: (Potassium cyanide/methyl cellosolve solution), 5 ml  
0.01M KCN in 250 ml methyl cellosolve.

Reagent B2: (ninhydrin/methyl cellosolve solution), 5 g ninhydrin in  
100 ml methyl cellosolve.

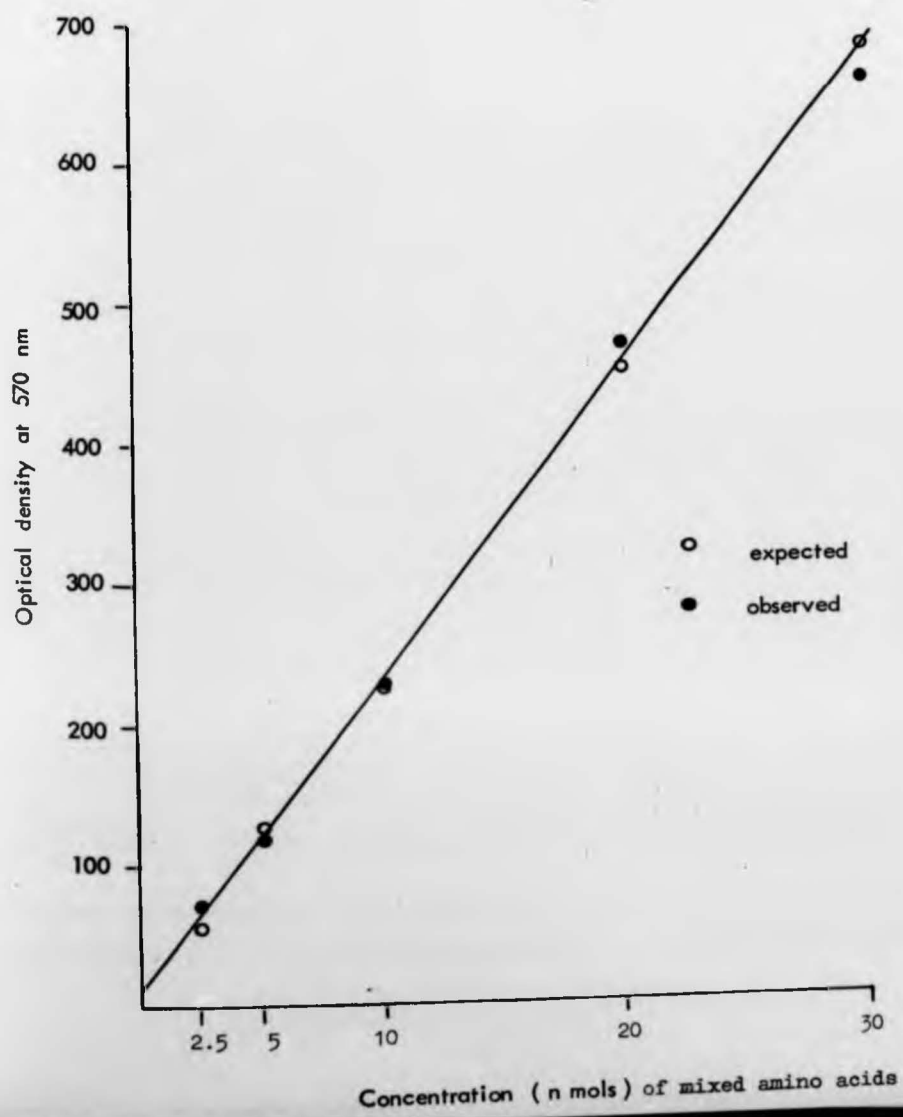
Reagent C2: (ninhydrin/KCN solution) To 25 ml of A2 add 10 ml of B2.

#### Method.

To 300-400 ul of supernatant, add 100 ul of buffer followed by 100 ul of C2. Cover tubes with tin-foil. Boil for 15 minutes. Cool tubes in running tap water. Centrifuge briefly to bring down any gel particles and also to bring into solution water drops along neck of tube. Read colour in spectrophotometer at 570nm in microcuvettes.

Standard Curve. The optical density/concentration relationship is shown in Fig.23.

FIG. 23 AMINO-NITROGEN DETERMINATION



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