MECHANISMS OF ANAEMIA PRODUCTION IN PROTEIN DEFICIENCY

by

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ABSTRACT

Weaned rats were used to investigate the aetiology of the anaemia associated with protein deficiency. In preliminary experiments, groups of rats were maintained on diets of four different protein contents (2, 3, 5 and 10 NDpCal%) and assessments were made of their general characteristics, of the degree and nature of anaemia and of serum proteins. It was found that the severity of anaemia correlated well with the protein content of the diet and also varied with the duration, becoming severest towards the end of the growing period (about week 8) and then gradually improving. Similar patterns were observed for the serum proteins and other characteristics. The anaemia was of mild or moderate degree and invariably of normocytic normochromic type. Hyperplasia of the bone marrow was apparent from normoblast counts, yet reticulocyte counts were in the normal range. Serum transferrin was reduced but this appeared to play little part in the aetiology of the anaemia.

Further investigations were performed on rats maintained on the 2 and 10 NDpCal% diets for 8 weeks. Erythrocytes from the protein deficient rats were observed to suffer more rapid haemolysis in control receiver rats than those from protein replete animals, while their osmotic fragility was reduced. The plasma erythropoietin

level was elevated in the protein deficient rats, in fact higher than in rats on control diet bled to the same degree of anaemia.

It was concluded that the primary cause of the anaemia was an insufficiency of protein supply at the bone marrow. The elevated erythropoietin level stimulated the bone marrow into hyperplasia but was unable to prevent the onset of anaemia. Extravascular haemolysis was a secondary cause, reflecting capture by the spleen of defective erythrocytes released from the bone marrow, but being insufficient on its own to account for the observed degree of anaemia.

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CHAPTER 1 GENERAL INTRODUCTION

GENERAL INTRODUCTION

Protein malnutrition is one of the most important problems of public health in many parts of the world today (Simmons, 1973). The widespread distribution of kwashiorkor is indicated in the map of Figure 1 (Scrimshaw and Behar, 1961), and it is certainly the most common disease in the world, affecting growth and development (Jones and Dean, 1956; Garrow et al., 1962; Graham, 1967; Chase and Martin, 1970; Frisancho et al., 1970), and finally resulting in mortality (Walt et al., 1950; Behar et al., 1958; Kahn, 1959; Kosoke, 1961; Lawless et al., 1966; Campbell et al., 1969; McLaren et al., 1969). The mortality rate for children is always found to be high and in some areas reaches 100% (Brock and Autret, 1952; Hunter, Frye and Swartzwelder, 1967).

Anaemia is an important manifestation of severe protein-energy malnutrition, which is encountered in kwashiorkor and marasmus (Trowell, 1947; Brock and Autret, 1952; Allen and Dean, 1965; Woodruff et al., 1970), but the morphological type of this anaemia appears to be variable. Normocytic normochromic anaemia has been reported most frequently (Altmann and Murray, 1948; Woodruff, 1955; Mehta, 1970), but macrocytic (Trowell, 1947; Altmann and Murray, 1948; Woodruff, 1951; Walt, 1959) and microcytic hypochromic types has also been

reported (Altmann and Murray, 1948; Stransky and Davis-Lawas, 1950). Although many investigations into the " The mechanism of anaemia induced by protein deficiency " have been performed, the precise mechanism has not yet been elucidated. Since its discovery, erythropoietin has been recognised as the haematologist's hormone, since its principal action is to regulate erythropoiesis (Kubanock, 1969; Hoffbrand and Lewis, 1972). Its role in the anaemia of protein deficiency, however, remains uncertain.

Anaemia may be classified into two types, one resulting from a loss of red cells due to haemorrhage or haemolysis and the other resulting from a decreased production of red blood cells by the bone marrow. The former is commonly accompanied by increased production of red cells and also increased concentrations of erythropoietin in blood and in urine (Jones and Klinberg, 1960; Penington, 1961; Van Dyke et al., 1961; Nakao et al., 1963). The latter may be associated with low or normal blood erythropoietin levels causing reduced erythropoiesis, or with increased levels where the increase appears to be ineffective, possibly owing to alteration or injury to the bone marrow (Penington, 1961; Hammond et al., 1968).

Some investigators have suggested that the anaemia of protein-energy malnutrition may be associated with a reduced level of erythropoietin, but, unfortunately, the

bioassay for this hormone is not yet sufficiently sensitive to allow measurement of subnormal blood levels of erythropoietin. Nevertheless there is some evidence that the erythropoietin level does not rise with this type of anaemia as it does with blood-loss anaemia. Moreover, the finding that animals with anaemia due to protein-energy malnutrition respond to administration of exogenous erythropoietin is suggestive that a lack of erythropoietin may be a causative factor in this anaemia (Morgulis, 1923; Orten and Orten, 1945; Hallgren, 1954; Gurney et al., 1957; McCarthey et al., 1959; Aschkenasy, 1963; Reissmann, 1964; Ferrari et al., 1966; Ito and Reissmann, 1966; Woodruff et al., 1970), although this finding does have alternative interpretations.

FIGURE I

Geographical distribution of Kwashiorkor

CHAPTER 2 REVIEW OF LITERATURE

REVIEW OF LITERATURE

It is estimated that between one-half and two-thirds of the world's population suffer from malnutrition (Caudau, 1963) and that approximately one-half of the present population have survived a degree of undernutrition during childhood (Graham, 1967). For these reasons, malnutrition must be regarded as one of the world's principal health problems. Infants born of malnourished mothers start life with a handicap, including impairment of growth and of physical and mental development, and their mortality rate is very high during the first year or two of life. Many infants die of malnutrition alone, while many others succumb easily to various infections owing to their malnourished state. Moreover, if the survivors remain exposed to malnutrition during growth, they will show in adulthood the ill-effects of a poor stature, a low condition of health and a comparatively low working ability (Brook and Autret, 1952; Behar et al., 1958; Caudau, 1963). Stewart and Sheppard (1970), investigating the effects of protein-energy malnutrition on rats, reported that litters produced by malnourished mothers weighed significantly less than those from a well-fed group and that the neonatal death rate was high (63%) among the congenitally malnourished animals. The survivors were also observed to grow very slowly and at

five weeks of age, were only half the weight of the well-fed controls.

Most forms of malnutrition are due to a deficiency in some essential nutrient, either because the total quantity of food taken is inadequate or because some specific nutrient is lacking in the diet, or for a combination of both these reasons. The most widespread form of malnutrition, protein-energy malnutrition, is usually a disease of childhood associated with weaning. The joint FAO/WHO expert committee on nutrition (1962) accepted the term " protein-calorie deficiency " as appropriate for this type of malnutrition, to include marasmus, marasmic kwashiorkor and kwashiorkor, but the term " protein-energy malnutrition " is now preferred. The hyphen, in either term, is indicative that the two dietary factors are associated. Protein-energy malnutrition (PEM) is now generally used to refer, in a comprehensive way, to malnutrition in the young child, and this term includes not only the severe clinical disease conditions known as marasmus and kwashiorkor but also the so-called " mild, moderate forms ", in which obvious disease is absent while retardation of growth and development, and possibly some biochemical changes, are the only evidence of the condition. As a definition " malnutrition " is preferable to "deficiency " since the former covers both 'under-' and 'over-' nutrition,

whereas the latter includes only a shortage of some essential form of nutrient. Marasmus results from total inanition, while kwashiorkor, in its acute and severe form, results from overfeeding with a diet of high carbohydrate content but low protein value, providing an energy (calorie) intake in excess of actual needs but an inadequate protein intake (McLaren and Pellett, 1973). These forms of protein-energy malnutrition occur in many parts of the world with only slight local variations in the disease characteristics (Hunter, Frye and Swartzwelder, 1967).

Anaemia has been reported to be an important clinical finding associated with severe protein-energy malnutrition (Trowell, 1947; Altmann and Murray, 1948; Brock and Autret, 1952; Adam, 1954; Trowell et al., 1954; Mehta and Gopalan, 1956; Walt, 1959; Macdougall, 1960; Shahidi et al., 1961; Woodruff, 1961; 1969; Allen and Dean, 1965; Mehta, 1970; Woodruff et al., 1970). Woodruff (1951; 1955) observed in Nigeria, a type of anaemia which did not respond to iron, folic acid or vitamin B_{12} treatment and suggested that this anaemia resulted directly from a dietary deficiency of protein. This view has subsequently been confirmed by many researchers. Latham (1960) investigated the nature of anaemia in children by dividing them into five equal groups in a random manner and administering a placebo tablet to the control group, giving antimalarial therapy to the second group, treatment for ancylostomiasis

to the third, an iron supplement to the fourth, and additional protein to the final group. The control group was observed to lose haemoglobin, whereas each of the other groups gained haemoglobin with the greatest gain being found in the protein supplemented group. From these results, he suggested that a lack of protein, especially of animal protein, might be a cause of this anaemia. Sandosi et al. (1963) also reported that the anaemia of kwashiorkor responded well to administration of a high protein diet without other haematinics, and Wharton (1967) has referred to uncomplicated anaemic kwashiorkor as an " anaemia of protein deficiency " in his studies in Kampala. Experiments in animals have provided yet more evidence that anaemia is directly associated with protein-energy malnutrition. Anaemia was a consistent finding in the investigations by Platt et al. (1964) into protein-energy malnutrition in experimental animals and the severity of the anaemia was found to be related to the protein value of the diet. Moreover, the anaemia could not be alleviated by administration of extra iron but the haemoglobin level was increased by giving a protein supplement without changing the energy intake or any other dietary constituents.

The morphology of the erythrocyte in this anaemia has been the subject of much discussion since it appears to follow no consistent pattern. The normochromic

normocytic type has been most commonly reported (Altmann and Murray, 1948; Walt et al., 1950; Brock and Autret, 1952; Adams, 1954; Trowell et al., 1954; Woodruff, 1955; 1961; Walt, 1959; Macdougall, 1960; Shahidi, 1961; Allen and Dean, 1965; Mehta, 1970), but macrocytic (Trowell and Kuwasi, 1945; Trowell, 1947; Altmann and Murray, 1948; Woodruff, 1951; 1961; Brock and Autret, 1952; Mehta and Gopalan, 1956; Walt, 1959; Adams, 1964) and microcytic hypochromic types (Altmann and Murray, 1948; Chaudi, 1950; Stransky and Davies-Lawas, 1950; Mehta and Gopalan, 1956; Trowell and Simpkiss, 1957) have also been observed.

Our knowledge of the mechanisms responsible for the anaemia of protein-energy malnutrition remains limited. It appears at present that there may be more than one mechanism involved, but the principal factor is not yet clearly established. Anaemia can result either from decreased production or from increased breakdown of erythrocytes. A review of the most important aspects of erythropoiesis under normal conditions, of the requirements for protein in red cell production, provides a useful introduction to the known effects of protein-energy malnutrition on this process, and acts as a useful aid to the elucidation of other possible changes during protein deficiency.

ERYTHROPOIETIN, ERYTHROPOIESIS AND PROTEIN DEFICIENCY

Erythropoiesis can be regarded as two stages, firstly the development of the stem cells and their reproduction to give rise to the erythron, then secondly the development of the erythron through the erythroblast stages leading to production of the red blood cells in bone marrow or spleen (Lajtha, 1966). These processes are regulated by the hormone erythropoietin.

This hormone is a glycoprotein and, in its purest form analysed until now, has been found to consist of approximately 71% protein and 29% carbohydrate (Goldwasser et al., 1962). Erythropoietin (EP) arises when a renal erythropoietic factor (REF) is released by the kidney and interacts with a plasma globulin to produce the active hormone (Kuratowska et al., 1964; Gordon et al., 1967). Release of REF occurs in response to local hypoxia in the kidney, while the effect of the erythropoietin thereby produced is to stimulate the production of red blood cells in the bone marrow. Erythropoietin appears primarily to control the rate at which the marrow stem cells give rise to erythroblasts (Alpen and Cranmore, 1959a; 1959b; Erslev, 1960; Filmanowicz and Gurney, 1961; Perretta and Tipapegui, 1968), but it also exerts influence on the rates of maturation, haemoglobin synthesis and release of the red cells from the marrow into the circulation (Gallagher and

Lange, 1960; Hodgson and Eskuche, 1962; Stohlman, 1964; 1967; 1968; Boyer, 1969). These aspects of erythropoietin production and action are discussed in detail in Part 5.

The dynamic equilibrium of the erythron, upon which the rate of red cell production effectively depends, is thus controlled by the hormone at least under normal conditions of adequate nutrition. An extremely sensitive system, based on a " feed-back " principle, is thereby created for controlling a steady level of erythrocytes in the blood. This system is capable of monitoring and responding to the normal daily destruction of erythrocytes and, additionally, to changes caused by alteration of the external or internal environment. The effect of protein deficiency on this control mechanism is not yet understood. Although it has been repeatedly demonstrated that protein-energy malnutrition can lead to anaemia, the role of erythropoietin in the aetiology of this association has remained uncertain. Some investigations have indicated that plasma erythropoietin is elevated in protein-energy malnutrition (Murthy, 1965; McKenzie et al., 1967), whereas others have implied that it is diminished (Reissmann, 1964a; 1964b; Ito and Reissmann, 1966). Until this controversy has been settled it will not be possible to assess whether the anaemia of protein deficiency is related to a defect in the erythropoietin control mechanism in this condition.

1. STEM CELL

Stem cells have not been identified morphologically, but by definition, they are cells that can maintain their own numbers while continuing to supply the needs of the body. In the bone marrow, the stem cells can give rise to red blood cells by mitosis and differentiation. A model for haematopoiesis has been presented by Stohlman (1967) with the stem cell population separated into three compartments containing pleuripotential stem cells, committed stem cells and differentiated cells. It was suggested that the differentiated cell compartment, containing proerythroblasts, is not self-sustaining but is supported by an influx of cells from a precursor compartment of committed stem cells. The latter compartment is usually self-sustaining, but under conditions of increased demand, either for physiological reasons or owing to damage, it is repopulated by pleuripotential cells. The pleuripotential compartment is normally in a resting state (G_0).

2. ERYTHRON

The red cell is composed of about 63% water, 34% haemoglobin, 1% lipid, comprised predominantly of cholesterol and lecithin, and 2 % sugar, salts, enzyme protein etc. (Wintrobe, 1967).

Structurally the red cell is known to consist of a

highly concentrated internal solution including predominantly haemoglobin and a retaining membrane, though the precise relationships between the chemical and structural components are not yet entirely clear.

(a). HAEMOGLOBIN

Haemoglobin is a conjugated protein containing four haem groups and globin. The normal globin fraction consists of four polypeptide chains made up in man of a total of 574 amino acid residues, incorporating 17 of the different amino acids. The globin chains are normally in the form of two α and two non-α chains, and the haemoglobin structure can be referred to by the formula $\alpha_2 X_2$, where "X" can be ε, γ, β or δ in man depending on the stage of development, differing between the embryo, foetus and adult. The differences between these forms are related to the number of amino acid residues and their sequence (Hoffbrand and Lewis, 1972).

From observation single erythrocyte precursors at different stages of their maturation in the bone marrow, using the techniques of microspectrophotometry and interference microscopy, Thorell (1947) deduced that haemoglobin is produced at a time when these cells are rich in cytoplasmic RNA. Haemoglobin concentration was observed to rise while RNA concentration fell, and biosynthesis of haemoglobin stopped when RNA became depleted. The highest haemoglobin concentration is attained at the normoblastic

stage of development and cause the change in cytoplasm colouring from a basophilic to an orthochromatic nature (Peace, 1956; Wolpers, 1956). Observations by electron microscopy have shown that the reticulocyte loses its ability to synthesise haemoglobin when it reaches maturity and becomes an erythrocyte. There is a decrease in the production of polyribosomes during maturation until no ribosomes are demonstrable and protein synthesis stops at maturity (Burka, 1969a; 1969b; Rifkind et al., 1964; Rowley, 1967).

Magnetic resonance and electron paramagnetic resonance measurement, as used in investigations by Bolton et al.(1968) and Shulman et al. (1969), have indicated that no significant configuration change of the haem group occurs during oxygenation of haemoglobin. But a configuration change of the polypeptide sub-units does take place, leading to a different quaternary structure of the molecule and accounting for the energy of oxygenation. Such a change appears to explain satisfactorily the sigmoidal shape of the oxygenation curve. Substitution of certain amino acid residues in the globin chains can have a profound effect on the position of the oxygenation curve and these shifts can account for the different properties of the different types of haemoglobin, such as Hb S where the curve is markedly shifted to the right and Hb Kohn where the curve is shifted to the left.

In the event of a limitation in protein intake, some competition must arise between the various demands for protein synthesis. Robscheit-Robbins et al. (1943) found that, under all circumstances, haemoglobin synthesis had a high priority over plasma protein formation when protein was supplied in various forms to dogs rendered hypoproteinaemic and anaemic by maintaining of a low protein diet and bleeding. Globin synthesis in man (at a normal rate of about 8 g per day) is of such priority that it can be produced at the expense of other body proteins, but slight abnormalities in its structure can cause fatal diseases, the haemoglobinopathies. But, when there was an increased demand for protein formation, as in pregnancy or lactation, haemoglobin production was observed to be more affected than the synthesis of other body proteins in protein deficient rats (Hallgren et al., 1954). These rats lost about 30% of their carcass protein, but the maximum reduction in total haemoglobin was much greater at 55-60%.

(b). ERYTHROCYTE MEMBRANE

The major constituents of the red cell membrane are protein and lipid, the former 40-60% and the latter 10-12% (Wintrobe, 1967).

Ghosts prepared by Weed et al. (1963) from normal human erythrocytes by the method of gradual osmotic lysis were found to retain the following properties of the intact red cells from which they were prepared: (a) glucose-6-

phosphate dehydrogenase and sodium- and potassium-dependent adenosine triphosphatase activities, independently of their haemoglobin content, (b) total cholesterol and phospholipid contents, (c) their biconcave disc shape and (d) osmotic responsiveness. It was thus proposed that haemoglobin is not an essential structural component of the human erythrocyte membrane: the membrane itself is considered to be responsible for the biconcave shape of red cells.

The erythrocyte membrane can be chemically separated into several crude fractions. Extraction at alkaline pH yields a soluble fraction called S-protein and a residue called stromin (Moskowitz and Calvin, 1952). Some lipids can be extracted by ether from the stromin, leaving a residue of lipid-carbohydrate-protein complex, referred to as elinin and now recognised as still a crude fraction. The blood group A, B and O antigens, which were originally thought to be a part of the elinin, have now been separated and identified as a specific glycoprotein fraction (Whitemore et al., 1969).

The specific roles of the membrane chemicals in the structural arrangement have not yet been completely elucidated, although there have been a number of hypothesis proposed to account for the biconcave, discoid shape of the erythrocyte. Nakoa et al. (1961) suggested that the shape is maintained partly by an ATP-dependent contractile

protein, which had been called elinin by Moskowitz and Calvin (1952). Rosenthal et al. (1970) have attributed contractile and shape-forming properties to a group of membrane proteins with Ca^{++}-dependent ATPase activity and a capability of forming fibrils, and helical filaments of protein on the inner aspect of guinea-pig red cell membrane have been detected with electron microscopy by Marchesi and Palade (1967). This filamentous protein has been called spectrin and may serve a structural role (Marchesi and Steers, 1968; Marchesi et al., 1970; Tillack et al., 1970). Another feature of the surface membrane of the erythrocyte is its net negative charge (Eyler et al., 1962), which is attributable to the carboxyl group of sialic acid residues localised in the glycoprotein of the exterior membrane surface (Winzler, 1969). This negative surface charge is probably sufficient to produce an intercellular repulsive force strong enough to prevent the cells from touching one another. The effectiveness of this electrostatic repulsive force has been demonstrated by the observation of enhanced agglutination when the surface charge is reduced or abolished (Marikovsky and Danon, 1969).

The lipid component of red cells has been found to comprise a wide variety of phospholipids, glycerides, glycolipids and cholesterol (van Deenen and de Gier, 1964). These lipids interact with the proteins in the structural

organisation of the membrane (Hanaban, 1969), and it has been suggested that cholesterol may play an important role in the shape of the erythrocyte (Murphy, 1962). The arrangement of the membrane components within the membrane has not yet been fully defined, but various models for the membrane structure have been proposed (Davson and Danielli, 1943; Whittam, 1958; Kavanau, 1966) and some of these concepts will be discussed in detail in Part 4, as will the relationship between the membrane structure and the extent of haemolysis. It should be noted that normally the erythrocyte membrane is not rigid but possesses viscoelastic properties essential to the erythrocyte's movement through the microcirculation. Although the membrane is generally considered to be responsible for the biconcave shape of the red cell (Weed et al., 1963), there has also been some evidence of shape-controlling factors in the interior of the cell (Shrivastav and Burton, 1969).

RELATION BETWEEN ERYTHROCYTE AND THE ROUTE OF DESTRUCTION

The main causes of haemolytic destruction of erythrocytes appear to be related to changes in the red cell membrane (Weed and Reed, 1966), although an intracellular abnormality may sometimes be responsible for such changes. Results to date indicate that there are four major routes leading to the destruction of any

altered erythrocytes, these being: (a) colloidal osmotic lysis, (b) primary perforation of the red cell membrane, resulting in direct loss of haemoglobin and other macromolecules, (c) fragmentation, and (d) erythrophagocytosis. The principal route for destruction and the rate of haemolysis depend on the nature of the red cell defect.

(a). Colloidal Osmotic Lysis

Increased cation permeability will lead cellular swelling, owing to rapid entry of sodium, and distension of the membrane and its " pores " allowing also a loss of macromolecules and finally leakage of haemoglobin, resulting in haemolysis. Spherocytic cells, for example, are more susceptible than normal red cells to this type of haemolysis since they have a smaller capacity for excess water.

(b). Primary Perforation of Erythrocyte Membrane

If a defect in the membrane is sufficient, it may enable haemoglobin to diffuse from the cell. Such a perforation defect has been produced by anti-A isoantibody or, in the case of erythrocytes from a patient with paroxysmal nocturnal haemoglobinuria, by exposure to acidified human serum in vitro (Scott et al., 1966; 1967).

(c). Fragmentation

A piece (or pieces) of the red cell membrane may

become separated from the cell, possibly with some direct loss of haemoglobin with the fragment. This process results in a decreased surface area/volume ratio, with the red cell becoming spheroidal and increasing in rigidity. Such changes interfere with the ability of the erythrocyte to undergo plastic deformations while traversing the narrow passages of the microcirculation, particularly in the spleen, thus promoting sequestration and resulting in a diminished life span.

(d). Erythrophagocytosis

Damaged red cells may be eliminated by phagocytosis, either intravascularly through the agency of monocytic and polymorphonuclear leucocytes or in the reticuloendothelial system through the agency of its phagocytic cells.

THE RELATION BETWEEN PROTEIN-ENERGY MALNUTRITION AND HAEMOLYSIS

Dalmonte et al. (1964) have suggested that a structural defect of erythrocytes is responsible for the increased haemolysis in protein-deficiency anaemia, though commenting that the defect might alternatively lie in the serum of protein-deficient rats providing the red cells with less protection than provided by normal serum against chemical, alkali and mechanical trauma. This effect of serum has been observed in vitro and may also contribute to the haemolysis in the protein-deficient rats in vivo. Lemakowsky et al. (1967) reported that the reduced erythro-

cyte survival in protein-energy malnutrition (marasmus and kwashiorkor) appeared to be due both to corpuscular and to extra-corpuscular factors. Since erythrocyte survival improved on realimentation and, furthermore, since this improvement occurred on a protein diet of low iron content and without haematinics or vitamin supplements, it was considered that protein depletion was mainly responsible for the shortened survival. Haemolysis does not appear to be a major factor in the causation of anaemia in protein deficiency, as Woodruff et al.(1970) found only a statistically insignificant shortening of the life-span of erythrocytes in protein-malnourished dogs.

Lankowsky (1967) observed significant increases in erythrocyte osmotic resistance and thermal resistance in some cases of protein malnutrition, and found that improvements occurred following protein feeding without haematinics. The erythrocyte membrane in Ugandan children with kwashiorkor has been found to contain larger amounts of lecithin than normal (Coward et al., 1971), and it is thought that this increase in lecithin content might account for the membrane rupturing less easily.

CHAPTER 3 NUTRITIONAL PROBLEMS IN THAILAND.

NUTRITIONAL PROBLEMS IN THAILAND

Protein-energy malnutrition is a major health problem in many countries of the world. The extent of malnutrition varies considerably from one country to another, but the nature of the problem remains similar. A convenient way to examine the medical and sociological aspects of this disease is to use one particular country as an illustrative example for detailed discussion. Thailand has been chosen for this purpose.

Thailand is largely an agricultural country with more than 85% of its population (34.7 million) engaged in farming and is self-sufficient as regards food production. The country is divided into four geographical regions, Central, North, North-east and South (Figure 1). The Central region is a low fertile plain consisting mostly of paddy fields; the Northern region is hilly and forested, and the people derive their living mostly from foresting and growing fruit and vegetable; the North-east region is a high dry plateau and is the poorest of the regions, the people taking their living from rice growing; and the Southern region is undulating and has a high rainfall, while the main sources of income here are extensive mining and rubber plantations.

As there is a surplus of some food stuffs such as rice and maize, these are exported to neighbouring countries

FIGURE 1

Thailand: Provinces and regions.
(Taken from SEADAG PAPER, 1973)

FIGURE 1

Thailand: Provinces and regions.
(Taken from SEADAG PAPER, 1973)

TABLE 1

NUTRITIONAL DISEASES IN THAILAND* TAKEN FROM THE REPORT OF THE DIVISION OF HEALTH 1967

NUTRITIONAL DISORDERS	NUMBER
Protein-calorie malnutrition	11,328
Anaemia	9,569
Bladder stones	6,110
Thiamine deficiency (Beriberi)	5,869
Simple goitre	1,865
Riboflavin deficiency	1,742
Vitamin A deficiency	524
Vitamin C deficiency (Scurvy)	395
Niacin deficiency (Pellagra)	116
Unknown nutritional diseases	150

* Data from 62 Provinces (Total of 71 Provinces)

(From Nondasuta, A. : J. Med. Assoc. Thailand. 52: 27, 1969).

FIGURE 2

Provinces where nutrition surveys have been conducted.

NUTRITION SURVEYS (Figure 2)

The symbols used on Figure 2 indicate provinces where nutrition surveys have been made. Capital letters refer to the particular deficiencies studied, or to general nutrition surveys.

N - General nutrition status :-
Chiang Mai, Chon Buri, Khon Kaen, Lop Buri, Phra Nakhon, Phrae, Songkhla, Ubon Ratchathani, Udon Thani.

B - Beriberi :-
Chiang Mai, Chiang Rai, Phrae, Ubon Ratchathani, Udon Thani.

G - Goitre :-
Chiang Mai, Chiang Rai, Phrae, Ubon Ratchathani, Udon Thani.

E - Expanded nutrition project :-
Ubon Ratchathani.

U - Urolithiasis :- Ubon Ratchathani.

Y - Yao village :- Chiang Mai.

and few items need to be imported. In spite of this surplus, the nutritional status of the people is still below the acceptable level for a number of reasons, including ignorance, food taboos, poverty and false beliefs about diet (Suvarnakich, 1950; Suvarnakich and Indrambarya, 1962; Indrambarya, 1964). The nutritional problems of Thailand are indicated in Table 1 (Mondasuta, 1969), while the areas in which nutritional surveys have now been performed are shown in the map of Figure 2.

PROTEIN-ENERGY MALNUTRITION

a. Clinical PEM (Kwashiorkor and Marasmus)

The report of Mondasuta (1969) has suggested that protein-energy malnutrition is the most important nutritional problem in Thailand (Table 1), and clinical cases of protein-energy malnutrition (kwashiorkor and marasmus) have been reported from various parts of the country (Metraseri and Metraseri, 1955; Viramuvatti et al., 1963; Valyasevi, 1964; Thanangkul et al., 1966; Cunningham et al., 1970).

Metraseri and Metraseri (1955), after 4 years of case-observations, indicated that there was clear evidence that protein malnutrition existed in Thailand, showing the typical symptoms of kwashiorkor and with more than 50% of the 54 cases diagnosed in Bangkok (Central region) being

in 1-2 year old children. These authors indicated that all their patients were from the lower socio-economic classes and that infections such as measles, ascariasis, amoebiasis and bacillary dysentery were common precipitating factors. In addition, half their patients had ocular lesions associated with vitamin A deficiency, whilst most had angular lesions. A nutritional survey, conducted by the Interdepartmental Committee on Nutition for National Defense (ICNND) in 1960, found that the protein intake among Thais over 5 years of age was generally satisfactory, although there were population subgroups such as young children with protein deficiency. A further nutritional survey by ICNND in 1962 extended these findings, by combining the results of the earlier survey and those of Netraseri and Netraseri (1955) with data obtained through conversations with paediatricians in Bangkok and concluded that protein-energy malnutrition was indeed a problem in Thai children of 1-2 years of age. Viranuvatti et al.(1963) reported that the incidence of malnutrition was 5% of the total number of patients admitted between January 1961 and June 1963 to Vajira Metropolis Hospital in Bangkok (Central region). The blood protein levels and the presence and nature of associated vitamin deficiencies were used to classify the malnutrition of the 184 children studied into three types, marasmus, kwashiorkor and nutritional oedema.

Investigations into the frequency of protein-energy

malnutrition have also been made in other regions of Thailand. For example, Stahlie (1961) made a study of children upto 4 years of age from families of low income living in 7 regions, 6 of which were rural while 1 was in Bangkok, and reported a diagnosis of marked malnutrition bordering on kwashiorkor in 17 cases from a total of 1,050 children. He also suggested that second degree malnutrition occurred in 1 of every 100 rural Thai children. For the Northern region, Thanangkul et al. (1966) reported that PEM accounted for 9% of the total paediatric admissions to Chiang Mai Hospital, with kwashiorkor diagnosed more frequently than marasmus, and that 75% of the 111 cases admitted to this hospital were in children between 1 and 4 years of age. Haemoglobin levels of less than 10 g/100 ml were found in 69% of these patients upon admission, and it was thought that this anaemia was probably due to multiple deficiencies in the diet.

b. Sub-clinical PEM

The number of clinical cases of kwashiorkor and marasmus does not really give a true indication of the extent of protein-energy malnutrition, either in Thailand or in other parts of the world, since the number of occurrences of sub-clinical protein-energy malnutrition, that is malnutrition of a mild or moderate degree, may greatly exceed the number of clinically-diagnosed cases.

Unfortunately, however, there are as yet no biochemical techniques to clearly identify sub-clinical protein-energy malnutrition, which by definition cannot be clinically recognised either, and no adequate anthropometric standards for various regions to provide a valid comparison against suspected cases and thus to enable a realistic assessment to be made of the extent of sub-clinical protein-energy malnutrition (Hegsted, 1972).

At present, it is considered that body weight provides the best indication of the current level of nutrition. A widely-used method of classification of malnutrition involves a comparison of body weight with a standard for the same age and this method is under consideration as an evaluative procedure. Height measurement has a similar usefulness as long as the patients age is known, but, since height usually increases until the later years, it provides a longer term indication of nutriture and is less easily correlated than weight with current levels of nutrition. It has not yet been proved, however, whether a useful comparison of height and weight can be made between people in developing and in developed countries, and it is thus considered necessary to collect weight-height data from children of good nutrition but exposed to similar environmental conditions as the children with suspected sub-clinical malnutrition, if a reliable comparison is to be made. Recent surveys of heights and weights of children

in the Mekong area of Thailand (North-eastern region) have provided the data listed in Table 2, which must be regarded as only a rough guide, however, since accuracy of the ages in some cases could not be relied upon (SEDAG PAPERS, 1973). The growth rate of Thai children, measured in terms of weight, was similar to that of children of the U.S.A. for the first 6 months of age, but after this age weight gain was markedly lower than that of the U.S. children, as shown in Figure 3. The qualification that the environmental conditions to which the children were subjected differed considerably between Thailand and the U.S.A. should not be forgotten, but the data of Table 2 and Figure 3 suggested growth retardation in the Thai children, and this possibly reflected an inadequate dietary intake of protein and energy.

Measurement of serum albumin is the chemical procedure most commonly employed to make a biochemical assessment of protein nutritional status, and most workers consider albumin concentrations of less than 3.5 g/100 ml to be low. Determination of total serum protein can also be informative, and Vachanands et al. (1966) reported that, in Ubon (North-eastern region), the total serum protein values of less than 6.0 g/100 ml were found in 69% of 64 pregnant women, in 8% of lactating women, and in 98% of 49 infants under 7 months of age. In addition, urinary and plasma urea levels were found to be lower in samples from inhabitants

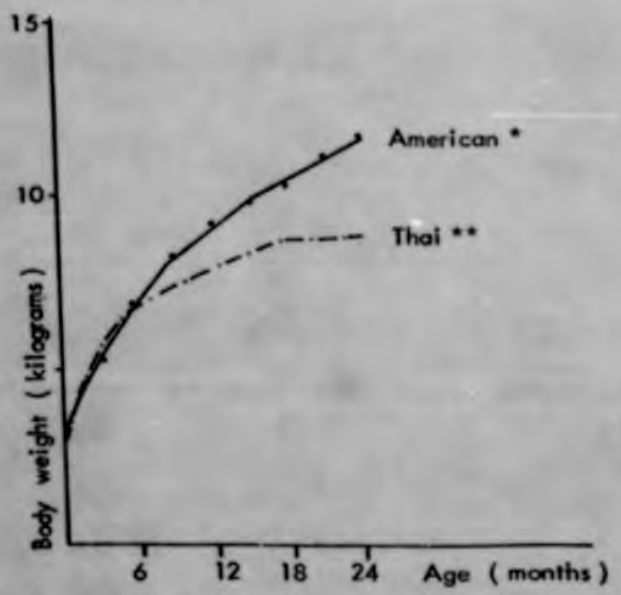
TABLE 2

RESULTS OF SURVEY OF HEIGHTS AND WEIGHTS IN THAILAND

Year	Province (Project)	Ages Tested	No. Tested	Results and Comments
1960	Udorn, Ubon and Chiang Mai	All	1,842	Average weight 5–15 kg and average height 10–20 cm lower for Thais than Americans for both sexes and all ages over one year.
1965	Chiang Mai	3–12	38	Mean weight lower than that of Bangkok children.
1968	Chiang Mai (Saraphi)	0–5	1,669	Body weight nomograms produced from the results in urban and rural samples.
1970	Chiang Mai	Birth	583	Birth weight 2995 ± 28.3 g for males; 2905 ± 27.6 g for females. Weight doubled in 5 months and tripled in 12 months.
1969	Khon Kaen	3 mos. to 7 yrs.	180	Average heights and weights lower than those of similarly aged Bangkok children.
1969	Korat (Soong Ngern)	0–2	229	After 12 months, height and weight lower than the Harvard (U.S.) standard; on the average, 70% of standard weight and 85% of standard height.
1969	Korat (Soong Ngern)	Birth	NA	Average birth weight of both males and females 3,020 g; average height 48.3 cm.
1970	Korat (Soong Ngern)	2–6	72	From ages 2–6 average height ranged from 78–100 cm; average weight from 10–15 kg. Similar for boys and girls.

FIGURE 3

Weight curves of American and Thai infants.



* From growth of the children's medical center, Boston, Mass.

** From three well-baby clinics (3490 infants) in Bankok, 1960 and 1961.

(Taken from Valyasevi, A., 1964)

of the North-eastern Provinces than in those from Bangkok and, in general, urea levels were lower in infants than in children of 2-7 years (Van Reen et al., 1970; Annual Reports, Thailand, 1965-1971). The urine hydroxyproline: creatinine (H:C) index has been determined in one group of Thai pre-school children in Khon Kaen (North-eastern Province) in 1969, when it was found that 69% of these children had values below normal, this evidence being interpreted as suggestive of marginal PEM (Annual Reports, Thailand, 1965-1971).

ANAEMIA

Anaemia is the second most important of the principal nutritional problems of Thailand (Table 1) (Nondasuta, 1969). Haemoglobin (or haematocrit) determination is widely used to diagnose anaemia and assess its severity since this method is easily performed and tends to correlate grossly with nutritional status. Recent available data for Thailand is summarised in Table 3.

Iron deficiency anaemia, in particular, is very common in Thailand (Sundharagiati, 1959; Sundharagiati et al., 1967a; 1967b; Nondasuta, 1969; Suwansri et al., 1970), and Nondasuta (1969) has reported that it is the most common type of anaemia there. One cause of this prevalent iron deficiency anaemia is hook worm infection, which has a very high incidence both in Sakon Nakhon (17%) in the

TABLE 3

HAEMOGLOBIN AND HAEMATOCRIT LEVELS INDICATED IN RECENT SURVEYS IN NORTHEASTERN AND NORTHERN THAILAND

(Taken from SEADAG PAPERS, 1973)

Survey Description	No. Tested	Hb. (g/100 ml)		Hct. (%) Mean
		Mean	% <12	
1. Ubon (1964)				
Pregnant women ≥6 mos.	70	9.3	95	29.2
Lactating women ≤7 mos.	70	9.9	84	32.3
Control women, same ages	54	10.2	91	34.5
Infants ≤7 mos.	53	9.2	98	26.2
2. Khon Kaen (1968)				
2 villages. Preschool children	128	9.1	-	38.5
3. Khon Kaen (1971). 3 villages				
ages 0-12	117	-	26	-
ages ≥13	205	-	17	-
4. School children (1971)				
Khon Kaen	390	11.7	61	38.7
(Bangkok)	(184)	(12.4)	(30)	(41.6)
5. Chiang Mai (1971)				
Saraphi Villagers				
All ages: Male	620	9.5	-	-
Female	879	8.8	-	-
Ages 20-25: Saraphi Villagers	96	9.6	-	-
Medical Students	208	13.2	-	-
6. Chiang Mai Valley (1970)				
4 villages:				
(age 1-39) Male	157	12.2	47	39.8
Female	158	11.4	65	38.2
Urban Schoolchildren:				
(ages 6-8) Male	81	11.8	46	39.1
Female	78	12.1	42	39.1

North-eastern region (Sadun and Vajrasthira, 1952) and in Chon Buri (25%) in the Central region (Sadun and Vajrasthira, 1953). Suwansri and Sundharagiati (1970) found 302 cases (21%) with blood disorders in a study of 1,457 adult patients at Vajira Metropolis Hospital in Bangkok (Central region). On further analysis of those patients with blood disorders, it was found that 69% had anaemia due to nutritional deficiencies and that 45% of the blood disorder cases were due to iron deficiency.

CAUSES OF NUTRITIONAL DISORDERS IN THAILAND

Many nutritional problems in Thailand are probably due to improper food eating habits, many of which have been practised from generation to generation and are bound up with superstitious beliefs. Information on local traditional beliefs, obtained by interviewing, has provided some knowledge of the food habits of pregnant women, lactating mothers and infants. In rural areas, breast milk is generally consumed until a child is 2 years old, unless this period is interrupted by a further pregnancy. Supplementation of the infant's diet is common but may be insufficient. Pregnant women are reported to eat less than usual and they are not allowed eggs, fat, sweets, sweet potatoes or young coconuts, since these foods tend to result in a larger foetus with consequent difficulty in delivery. The diet of lactating women, for possibly 2

months after delivery, consists solely of rice, salt and paper. They are not allowed certain green leafy vegetables, fatty fish or certain meats, as these could possibly cause dizziness and vomiting and there is a belief that such foods could be toxic both to the mother and to her child. In some areas it is believed that eggs are an unsuitable diet for children and most food is withheld if they are sick. Moreover, there appears to be a general preference for raw or half-cooked foods and this can spread parasitic diseases (SEADAG PAPERS, 1973). With a knowledge of this restriction in food intake during pregnancy and for 2-6 weeks after delivery, it is not surprising that biochemical studies at Ubon (North-eastern region) have revealed that the serum albumin concentrations of 97 pregnant women examined were all deficient (less than 2.5 g/100 ml), compared with the levels recommended by the U.S. National Research Council (Valyasevi, 1964). These dietary habits are thought to play an important role in infant mortality and in their susceptibility to infections, and are considered to be a principal cause of the deficient nutritional status and of the nutritional anaemia both in children and in mothers (SEADAG PAPERS, 1973).

NUTRITIONAL PROBLEMS IN THAILAND IN THE CONTEXT OF WORLD MALNUTRITION

Thailand is fortunate in that it is able to grow

sufficient quantities of the staple foodstuffs to feed its population, whereas many other countries are unable to do this. Although malnutrition, and protein-energy malnutrition in particular, is certainly a health problem in Thailand, the number of clinical cases and the severity of malnutrition are not as great as in a number of countries. The principal reasons for the persistence of protein-energy malnutrition in Thailand appear to be related to false beliefs, ignorance and taboos about food, especially in regard to the nutritional requirements of pregnant women and lactating mothers. Similar false beliefs and taboos may be responsible in part for malnutrition throughout the world, but complete shortages of food are likely to be the major problem in many areas, particularly when unfavourable climate conditions intervene to spoil a vital food crop. Nevertheless, many features of the protein-energy malnutrition found in Thailand are common features of this condition throughout the world. Severe protein-energy malnutrition is most frequently found in young children and it is frequently associated with anaemia both in children and in pregnant women. The extent of sub-clinical protein-energy malnutrition is difficult to assess in any country but another universal feature may be that mild and moderate forms of malnutrition are more frequent than cases of clinical protein-energy malnutrition. The frequent association of anaemia with protein-energy

malnutrition underlines the importance of understanding this association in order that a fully effective treatment for this condition can be devised.

CHAPTER 4 PURPOSE OF STUDY

PURPOSE OF STUDY

It is clear from the preceding review that the role of protein-energy malnutrition in the causation of anaemia has not been elucidated. At present, so far as is known, there are two main factors that are considered most likely to account for the anaemia associated with protein-energy malnutrition. One factor is haemolysis and the other is decreasing bone marrow activity, due either to a reduction in or a lack of substrates required for erythropoiesis in the bone marrow or to a fall in the plasma concentration of the hormone erythropoietin (Woodruff et al., 1970).

The purpose of this study is to elucidate whether increased haemolysis or decreased bone marrow activity, or both these effects, can provide possible mechanisms to account for the anaemia of protein-energy malnutrition. One major problem, that presents difficulties in the elucidation of the mechanisms responsible for this type of anaemia in protein-energy malnutrition (kwashiorkor and marasmus) in man, is that almost invariably these conditions are associated with a deficiency of other nutrients (such as minerals or vitamins) and also with many kinds of infection. The clinical and metabolic pictures are complicated by such nutrient deficiencies and infections (Woodruff, 1955; 1961; 1969; Woodruff et al., 1970; Scrimshaw, 1964; Mehta, 1970). In attempting

to study the effects of uncomplicated protein deficiency, there are thus many advantages in the use of animals as experimental models for protein-energy malnutrition.

The present investigations into the anaemia of protein deficiency had the following three aims:-

1. To provide a clear demonstration of anaemia in uncomplicated protein deficiency and to assess the severity of the anaemia and its relationship to the protein content of the diet.
2. To investigate the relationship between haemolysis and anaemia in protein malnutrition.
3. To investigate the relationships between erythropoietin, bone marrow activity and anaemia in protein malnutrition.

PART 2

MATERIALS AND METHODS

PART 2

MATERIALS AND METHODS

This part describes the experimental animals and their diets, and details the basic haematological and biochemical techniques.

1. ANIMALS

The animals used for these investigations were male, weaned, hooded rats, with black and white fur. They were 3 weeks old and weight 35-45 grams at the start of each experimental diet, all animals being maintained on the same protein-sufficient diet until this time.

2. DIETS

The experimental diets were divided into 2 classes:-

a. Low protein diets- 2, 3 and 5 NDpCal%.

b. Control diet- 10 NDpCal%.

Diets of these different protein values prepared by varying primarily the casein content, using the diet formulae shown in Table 1. Protein values were expressed in NDpCal%, as indicated by Platt et al. (1961).

3. BASIC HAEMATOLOGICAL TECHNIQUES

Collection of Blood

Each animal was anaesthetised by ether and its

TABLE 1

Percentage Composition of Diets

Ingredient	Diet in MDpCal%			
	O2*	O3*	O5*	O10*
Rolled oats (Quaker Oats Ltd.)	25	45	45	45
Dripping (beef)	25	25	25	25
Casein	0	0	4	22
Maize starch	43.8	23.8	19.8	1.8
Salt mixture (Jones & Foster, 1942)	5	5	5	5
Mixture of B vitamins ✦	1.1	1.1	1.1	1.1
Fat-soluble vitamins ‡	0.1	0.1	0.1	0.1
Protein values of the diets in MDpCal% (Platt, Miller & Payne, 1961)	2	3.1	5.2	9.8

* Code names for diets with oats as the main ingredient.

✦ Contained thiamin hydrochloride 0.3 mg, riboflavin 1 mg, pyridoxine hydrochloride 0.2 mg, calcium pantothenate 6 mg, nicotinic acid 20 mg, myo-inositol 20 mg, p-aminobenzoic acid 60 mg, biotin 0.02 mg, pteroylmonoglutamic acid 0.2 mg, choline 60 mg, cyanocobalamin 5 µg.

‡ Each rat received from weaning 800 i.u. retinol, 40 i.u. ergocalciferol, 1.25 mg mixed tocopherols and 0.08 mg menaphthone each week.

(Diets modified from Stewart, R.J.C. and Sheppard, H.G., Br. J. Nutr. 25: 175, 1971)

thoracic cavity was opened for blood collection by heart puncture. Blood samples were drawn into heparinised tubes (except where otherwise mentioned) for subsequent analysis.

a. Haemoglobin Estimation

The cyanmethaemoglobin method was used.

Materials

Haemoglobin pipette

Photoelectric colorimeter

Drabkin's solution

Cyanmethaemoglobin standard solution

Procedure

When this method was first used, a standard curve was constructed by adding 10 ml of Drabkin's reagent to 0.05 ml of each of a series of dilutions of the cyanmethaemoglobin standard solution and to a distilled water blank, mixing each gently and allowing it to stand at room temperature for 1 hour, then measuring the optical density at 540 nm with a photoelectric colorimeter, with the instrument zeroed for the blank. The standard curve of optical density against haemoglobin concentration of each standard dilution was found to be linear and to pass through the origin. Thus on subsequent occasions it was only necessary to use one cyanmethaemoglobin standard and a blank.

0.05 ml of whole blood was added to 10 ml of Drabkin's

reagent, mixed gently and allowed to stand at room temperature for 1 hour. Its optical density at 540 nm was measured and comparison was made with the standard cyanmethaemoglobin solution, both readings taken against the reagent blank.

Haemoglobin concentration, in g/100ml, of the blood was then calculated as follows :

$$\text{Hb. (g/100 ml)} = \frac{\text{Blood reading}}{\text{Standard reading}} \times \text{Hb. conc. of standard}$$

b. Determination of Packed Cell Volume

Blood was drawn into microhaematocrit tubes, leaving about 15 mm empty at one end, which was then sealed, and centrifuged for 5 minutes in a microhaematocrit centrifuge. A microhaematocrit reader was used to determine the PCV, which was expressed as a percentage.

c. Reticulocyte Count

This count was carried out immediately after drawing the blood specimen, as ripening of reticulocytes might otherwise occur during storage. The dry slide method was used. A drop of dye (C.5% New methylene blue solution, freshly prepared, in absolute alcohol) was allowed to evaporate to dryness on the slide; then a drop of blood was added, mixed with the dye on the slide, smeared and allowed to dry. Wright's stain was used to counterstain

the smear.

A suitable area of the smear was chosen where the cells were undamaged and undistorted, and counting was performed with the aid of an oil immersion microscopic objective. The percentage of reticulocytes was calculated from a count of 500-1000 red cells.

d. Red Cell Count

Materials

Red cell diluting pipette

Counting chamber (Neubauer chamber)

Diluting solution (10% (V/V) formalin in isotonic saline)

Procedure

Whole blood was diluted 1 in 200 in the pipette and mixed well for 2 minutes. Thus the white cells were lysed and the red cells were left in suspension. An improved Neubauer chamber was used for the count, the result of which was expressed as the number of red cells per mm^3.

e. Blood Film

A very small drop of non-heparinised blood was placed on the centre line of a slide, 1-2 cm from the end. A spreading slide was placed in contact with the drop of blood, at an angle of approx 45°, and was then moved smoothly along the slide away from the drop of blood.

The smear was allowed to dry in air, covered with one volume of Leishmann's stain and left for 2 minutes. Two volumes of distilled water were then added and further staining was allowed to occur for 5-7 minutes, during gentle agitation. The whole slide was finally washed in a stream of buffered water (pH 6.8) until the film had a pinkish tinge (approx 2 minutes) and allowed to dry in air. The slide was then ready for examination of morphology and determination of size of the red cells.

f. Determination of the Size of Red Cells

Red cell diameter were measured directly, while other estimates of size were calculated from the PCV, red cell count and red cell diameter, as follows :

Stage 1. Measurement of Cell Diameter

Materials

Eye-piece micrometer

Micrometer slide calibrated in 10 µm divisions

Microscope with 2 mm objective and x6 eye-piece

Procedure

1. The eye-piece micrometer was calibrated with the micrometer slide, to relate the divisions on the eye-piece micrometer to distances in micrometres along the slide.

2. Viewing a thin area of the blood film, the

diameters of 500 red cells were measured.

3. A frequency distribution of red cell diameters was constructed and recorded graphically, showing the numbers of cells of various sizes. This is known as a "Price-Jones Curve". The mean red cell diameter was calculated from the frequency distribution and expressed in micrometres (μm).

Stage 2. Determination of Red Cell Volume

The mean volume of the red cells was calculated from the red cell count and the packed cell volume, using the following formula :

$$\text{Mean cell volume (MCV)} = \frac{\text{Packed cell volume (\%)} \times 10}{\text{Red cell count (millions per mm}^3\text{)}}$$

The result was expressed in cubic micrometres (μm^3).

Stage 3. Determination of Mean Cell Thickness (MCT)

The mean thickness was estimated from the mean cell volume and the mean cell diameter by approximately the cells as short cylinders :

$$\text{Mean cell thickness} = \frac{\text{Mean cell volume (MCV)}}{\pi \left(\frac{\text{Mean cell diameter (MCD)}}{2}\right)^2}$$

The result was expressed in micrometres (μm).

Stage 4. Calculation of Mean Cell Surface Area

This was estimated from the formula (see appendix)

$$\text{Surface area } (\mu m^2) = 2\pi a^2 + \frac{2\pi b^2}{e} \sinh^{-1}\left(\frac{ea}{b}\right)$$

where a = mean cell radius

b = mean cell thickness

$$e = \left(\sqrt{a^2 - b^2}\right) \Big/ a$$

g. Estimation of Mean Cell Haemoglobin Concentration (MCHC)

Mean haemoglobin concentration in the red cells, regarding the red cells as carrying haemoglobin in solution, was calculated from the following formula :

$$\text{MCHC} = \frac{\text{Haemoglobin (g/100 ml)} \times 100}{\text{Packed cell volume (\%)}}$$

The result was expressed as a percentage (g/100 ml).

h. Examination of Bone Marrow Film

The May-Grunwald-Giemsa's Stain was adopted for this assessment of the bone marrow.

Materials

1. May-Grunwald's stain [diluted with an equal volume of buffered water (pH 6.8)] .
2. Giemsa's stain (diluted with 9 parts of buffered water, pH 6.8)
3. Distilled water

Procedure

1. Bone marrow from the femoral bone was smeared on a slide and allowed to dry in air.
2. The smear was fixed by immersion in methanol for 30 minutes.
3. The marrow was stained with May-Grunwald's stain (diluted) for approximately 5 minutes; then, after tipping off the excess stain, the slide was flooded with Giemsa's stain (diluted) for about 30 minutes.
4. Excess stain was washed off with distilled water, 3 or 4 times, and the slide was then allowed to stand undisturbed in distilled water for differentiation to take place (approximately 5 minutes). When this was complete, the slide was placed vertically to dry.
5. The slide was examined under microscope and the proportion of erythroid elements was determined to assess the extent of erythropoiesis.

4. BIOCHEMICAL TECHNIQUES

Collection of Blood

Blood was obtained by heart puncture, in the manner previously described, but was drawn into non-heparinised tubes and allowed to clot. The serum was then separated and stored frozen (-20°C) until required.

a. Total Protein and Albumin Determination

The micromethod of Sunderman and Sunderman Jnr. (1964) was used.

Materials

1. Microbiuret Reagent

   9.6 g potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) were dissolved in about 80 ml of distilled water in a 500 ml volumetric flask, 2.4 g copper sulphate were added and dissolved, then 360 ml of 2.5 M sodium hydroxide were added slowly. Finally 0.5 g potassium iodide was added and dissolved, and the solution was made up to 500 ml with distilled water.

2. 2% sodium sulphite solution (w/v)
3. 2.5 M sodium hydroxide
4. Di-ethyl ether

5. Spectrophotometer

Procedure

1. 100 µl of serum were added to 2.5 ml sodium sulphite solution in a test-tube mixed gently by inverting 10 times.
2. 1 ml of this mixture was immediately transferred to another test tube (labelled " T ").
3. 0.5 ml ether was added to the remainder of the solution in the tube, which was then also mixed by inverting 10 times. Finally, this mixture was centrifuged for 5 minutes at high speed, to separate the albumin (clear lower layer) from the globulins which form a surface cake with the ether.
4. 1 ml from the clear albumin layer was transferred, using a pipette inserted carefully through the ether and globulin cake, to a third test-tube (labelled " A ").
5. 1 ml of sodium sulphite reagent was placed in an empty test-tube (labelled " B ") to act as a blank.
6. 0.5 ml of microbiuret reagent was added to each of the labelled test-tubes. After mixing, they were allowed to stand for 1 hour for the reaction

to occur and for the colour to develop.

7. The contents of each tube were then transferred to a cuvet to fit the spectrophotometer.
8. After zeroing the galvanometer for the blank (tube " B "), optical density readings were taken for each of the remaining tubes at a spectrophotometer wavelength setting of 540 nm.
9. A standard curve was constructed by carrying out the same procedure as for the total protein measurements but using 100 µl of each of a series of dilutions of standard serum in place of the 100 µl of test serum. A linear plot of optical density against protein concentration of standard dilutions provided the standard curve for calibration of the measurement on test serum.
10. Protein concentrations obtained from this standard curve.

Note:- T - Total protein, A - Albumin and B - Blank.

b. Serum Transferrin Estimation

The immunodiffusion method used was modified from Mancini et al. (1965).

Materials and Reagents

1. Special Agar-Noble (Difco)

2. Barbiturate buffer, pH 8.6.

9 g sodium diethylbarbiturate and 0.5 g sodium azide were dissolved in distilled water, 65 ml of 0.1 M HCl was added, the pH was checked and adjusted if necessary, and the volume was made up to 1 litre.

3. Standard serum transferrin and anti-transferrin

(These were kindly supplied by Dr. A.H. Gordon of the National Institute of Medical Research, Mill Hill, London, U.K.)

4. Immunodiffusion plates

These plates (of size 2.5 cm x 7.5 cm x 1 mm deep) were obtained from Hyland, Costa Mesa, California, U.S.A.

5. 7.5% acetic acid

Methods

1. Preparation of agar

3 g agar were added to 100 ml barbiturate buffer and heated until completely dissolved. Distilled water added to replace losses due to evaporation. The stock solution of agar was stored at 4°C in well-stoppered tubes until required.

2. Antiserum-agar mixture

14 ml of solidified 3% agar-gel was melted in a boiling water bath, then allowed to cool to 60°C before mixing thoroughly with 0.16 ml of antiserum. Allowing

the agar to cool to 60°C was essential since higher temperatures tend to denature the antiserum. The mixture was then maintained at 60°C in a water bath until used.

3. Preparation of antiserum-agar plate

3.5 ml of the antiserum-agar mixture were pipetted onto an immunodiffusion plate and the mixture was allowed to spread itself evenly across the plate. It then solidified completely on cooling to room temperature. Circular wells for the reception of the antigen solutions were punched out in the gel by means of a capillary tube of 2 mm bore. The lid was replaced and the prepared plate was stored at 4°C until required.

4. Application of antigen samples

With the agar plate horizontal, each well was filled to the level of the agar surface with the appropriate specimen, by allowing this to drain from a capillary pipette whose tip was in contact with the bottom of the well. Each plate contained three wells for standards, at three different concentrations, in addition to wells for the test specimens of serum. The lid was then replaced and the plate was incubated at 37°C for 4 hours in a moist chamber to make the antigen-antibody reaction run to completion.

5. Measurement of ring size

After incubation, the lid was removed and the plate was dipped in 7.5% acetic acid for 2 minutes, then

rinsed with distilled water, to improve the distinctness of the rather hazy precipitin rings. The diameter of each precipitin ring was then measured by means of a hand magnifier with an attached measuring device.

6. Standard curve

The diameters of the precipitin rings for the three standards were plotted on semilogarithmic graph paper, using the logarithmic (vertical) scale for standard concentration and the arithmetic (horizontal) scale for ring diameter. The straight line of best fit was drawn through the points, and the concentrations of the unknown specimens were determined by reference to this standard curve.

c. Serum Iron Determination

Dr. Reinouts van Hoga (Laboratory for Clinical Microchemistry, Utrecht) kindly provided me with details of his micromethod for measurement of serum iron.

Materials

1. 4 mg of 2,4,6-tri(2'-pyridyl)-1,3,5 triazine (TPTZ) were dissolved in the minimum possible quantity of concentrated HCl, then added to 10 ml of 15% trichloroacetic acid (TCA) containing additionally 2% ascorbic acid.

2. 20% sodium acetate (trihydrate)

3. Iron standard (from Hyland, Div. Travenol Laboratories, Inc., Los Angeles, California, U.S.A.)

Procedure

1. 110 µl serum were placed in an iron-free centrifuge tube, equal volumes of iron standard at a series of dilutions in a further set of tubes and iron-free distilled water in a final tube to act as a blank.

2. 60 µl of the TPTZ/TCA reagent was added to each tube and mixed thoroughly.

3. The tubes were left to stand for 10 minutes before centrifugation at 10,000 r.p.m. for 10 minutes.

4. 100 µl of the supernatant were aspirated, taking care not to disturb the precipitate, and transfrred to a test-tube.

5. 150 µl of 20% sodium acetate solution was then added to each test-tube. The final pH was checked and the samples were discarded if not in the range pH 4.7-5.0.

6. The extinction at 593 nm was recorded for each sample, and serum iron concentration calculated from a standard curve.

4. DETERMINATION OF BODY FLUID

Procedure

1. The weight of each rat was recorded whilst alive

(= wet weight).

2. Rats were then killed and placed in an oven at 60°-70°C until drying was complete after approximately 2 weeks. The dry weight was then measured.

3. Body fluid was expressed as a percentage of the original wet weight by the following formula:

$$\text{Body fluid} = \left(\frac{\text{Wet weight} - \text{Dry weight}}{\text{wet weight}} \right) \times 100$$

PART 3

SELECTION OF OPTIMAL CONDITIONS FOR SUBSEQUENT DETAILED ANALYSIS OF THE HAEMATOLOGICAL CHARACTERISTICS OF ANAEMIA IN RATS ON PROTEIN DEFICIENT DIETS

SELECTION OF OPTIMAL CONDITIONS FOR SUBSEQUENT DETAILED ANALYSIS OF THE HAEMATOLOGICAL CHARACTERISTICS OF ANAEMIA IN RATS ON PROTEIN DEFICIENT DIETS

The purpose of this initial part of the study was to assess the effects of differences in dietary protein content and of duration of diet on the general characteristics of rats maintained on a protein deficient diet, on the severity of anaemia as judged by the normal criteria and on plasma proteins, and thereby to select the optimum dietary conditions for further detailed investigations (Parts 4 and 5) into the mechanism (s) responsible for anaemia during protein deficiency in rats.

Male weaned hooded rats, three weeks old, were used since the aim was to reproduce the equivalent of protein-energy malnutrition (PEM), a disease occurring predominantly in young children soon after weaning. The rats were divided into four groups. Rats in one group were fed on a diet providing 2 NDpCal%, those in a second group on a diet of 3 NDpCal% and a third group on 5 NDpCal%. The final group was a control group fed on a diet providing 10 NDpCal%. The animals were weighed and their general characteristics were recorded each week. Also a sample of rats was taken from each group at weeks 2 and 4 and then at four-weekly intervals upto week 24 and sacrificed for biochemical and haematological examination.

The investigations of Part 3 have been divided into the following chapters :-

Chapter 1. Changes in general characteristics of the rats during protein deficiency.

Chapter 2. Characteristics of the anaemia produced by protein deficiency.

Chapter 3. Effects of protein deficient diets on serum proteins.

CHAPTER 1. Changes in general characteristics of the rats during protein deficiency.

CHANGES IN GENERAL CHARACTERISTICS OF THE RATS DURING PROTEIN DEFICIENCY

Effects of diets containing different protein values on:-

1. General appearance, behaviour, body weight and size.
2. Body fluid.
3. Fur loss.
4. Mortality.

Section 1. GENERAL APPEARANCE, BEHAVIOUR, BODY WEIGHT AND SIZE

(a). General Appearance

Protein deficiency affects the general appearance of the child or animal suffering from this condition in many different ways. Growth is retarded, the subject is emaciated, changes occur in skin and hair, oedema may be present and the subject appears apathetic and miserable (Wayburne, 1968; Gopalan, 1968). Various biochemical changes underlie these changes in appearance in this condition and many of the major biochemical aspects are discussed in later chapters.

Most manifestations of protein deficiency appear to be most severe during the growing period when the

growth rate is normally fastest and the protein demand is greatest. Although these manifestations may be alleviated at later stages, the appearance and stature still remain abnormal unless protein deficiency is mild.

RESULTS

Some effects of protein deficiency on general appearance are illustrated in Figure 1 and 2, which show a typical rat on the control diet (10 NDpCal%) and on the 2 NDpCal% low protein diet, respectively, at a diet duration of 12 weeks in each case. By this time, there had infact been a slight improvement in the general appearance and stature of the protein deficient rats which had survived the severest effects of the protein deficiency. Nevertheless, some marked differences in general appearance were immediately apparent in comparing the control and protein deficient animals. A reduction in size (see following pages) and a substantial loss of fur (see section 3) were the most obvious features of the rats fed on the 2 NDpCal% diet, while these rats were also clearly emaciated, their skin had a wrinkled appearance and they looked unhealthy even after the end of the growing period.

FIGURE 1.

Showing a rat fed on control diet (10 NDpCal%), at the 12th week of the experiment.

FIGURE 2

Showing a rat fed on low protein diet (2 NDpCal%), at 12th week of the experiment.

(v). Behaviour

It has been reported that apathy is usually found in kwashiorkor (Brock and Autret, 1952); Behar et al., 1958; Gopalan, 1967; Wharton, 1968; Rajalakshmi and Ramakrishnan, 1969; Whitehead and Alleyne, 1972; McLaren, 1973) but alertness in marasmus (Rajalakshmi and Ramakrishnan, 1969; McLaren, 1973). Trowell et al., (1954) described how apathy in kwashiorkor became a stupor that deepened into coma and sometimes resulted in death. This behaviour pattern was also observed by Gopalan and Ramalingaswami (1955).

Gopalan (1967) suggested that when a child was subjected to the stress of protein-energy malnutrition its tissues responded in such a way as to enable the child to adapt itself to the deficiency. From this point of view, growth retardation and restriction of physical activity can be considered as the clinical manifestations of this adaption. Wharton (1967) found that more children with moderate to severe kwashiorkor were fully conscious, though some of these were apathetic, on admission for treatment, but during the next few days they slept more and more and became very drowsy even when awake. These changes occurred in about a fifth of the children and in most cases it was self limited.

In two such cases, however, the drowsiness deepened into coma and resulted in death, while another child died suddenly after a few hours of drowsiness. As yet there is no definite clue to the cause of drowsiness, although Wharton suggested that the drowsiness and coma are forms of hepatic encephalopathy.

It is generally realised that the intellectual development of the kwashiorkor child is poor (Barrera-Moncada, 1963; Cravioto and Robles, 1965; Rajalakshmi and Ramakrishnan, 1969), and this is consistent with the clinical picture of severe apathy and a disturbed EEG pattern (Engel, 1956; Nelson, 1959). Similar findings have been noted in children in a marasmic condition (Stock and Smythe, 1963; Monckeberg, 1968). Platt (1961) has commented that animals and children suffering from severe protein deficiency die a " central nervous death ", as a result of a failure of their central nervous system.

RESULTS

In the present study, some of the rats fed on the 2 and 3 NDpCal% diets showed signs of decreased activity and drowsiness, and the rats showing these symptoms were often observed to die soon afterwards. Moreover, these manifestations were commonly found during the period from week 4 to week 7, which was similar to the period when the mortality rate was highest (refer to Section 4 of this chapter). These behavioural effects were not apparent in rats fed on the 5 NDpCal% diet.

(c). Body weight and size

It has usually been considered that the small size of many people is genetically determined, but such a view disregards the possibility that malnutrition in early life may have an important effect. At the present time, it is accepted that the greatest effects of protein-energy malnutrition on body weight occur during the first four years of life, when mortality from protein-energy malnutrition is also found to be very high, though tending to decrease beyond the first year(Bengoa, et al., 1959; Galvan and Calderon, 1965). Half of the world's population is estimated to have experienced a degree of undernutrition in early life (Graham, 1967), while the consequences of childhood undernutrition include increased mortality, increased

susceptibility to various infections, restricted growth and mental retardation, which is possibly permanent. It is significant that, the earlier the malnutrition, the more profound is the psychological retardation. It is also recognised that both body size and the development of the central nervous system in man (Grunewald, 1963) and in animals (Chow and Lee, 1964) are influenced by the effects of intrauterine undernutrition.

Starvation and protein-energy malnutrition result in a decreased growth rate or a reduction in body weight (McCance and Widdowson, 1962), but the effect on individual organs and cell types is not uniform. A major part of the body weight deficiency is accounted for by reductions in liver and in skeletal muscle tissues under most conditions of undernutrition, whereas brain tissues, for example, appear to be spared. Graham (1967) studied the effects of malnutrition on the growth of infants and children and concluded that the prognosis for growth could be improved by provision of an optimum diet. The severe growth deficits, particularly in head size, however, apparently could not be made up by an improved diet. Although bone age, as determined by radiological examination, generally parallels height age suggesting a lag in growth that might be made up by an extended period of growth, this was not found to be the case in a large undernourished population as the age of cessation of bone

growth was similar to that in well-nourished children. These observations suggested that a proportion of malnourished children will be permanently stunted (Graham, 1967).

The rates of growth and development during the first year of human life are so much faster than those at any later period that interference with growth during this period is much more likely to have an irreversible effect. Chase and Harold (1970), in a study of the relation between undernutrition and childhood development, found that a group of undernourished children had lower than normal values of height, weight and head circumference, while the magnitude of these deficits in development appeared to be closely related to the duration of undernutrition, as well as to its severity, in the first year of life. Moreover, Widdowson and McCance (1963) found that prolonged undernutrition in early life had important permanent effects on growth in animals and their finding was confirmed by Winick and Noble (1966), who concluded that malnutrition retards growth both in animals and in children. The earlier the animal or child becomes the victim of severe malnutrition, the greater is the likelihood of permanent stunting, while recovery of normal stature on refeeding also depends, in part, on age at onset of deprivation. Winick and Noble (1966) subjected one group of rats to malnutrition for a 21 day period starting at birth, others

at weaning and a third group at 65 days, after which they were all fed on a normal diet until adulthood. It was found that those rats with malnutrition from birth to weaning did not recover normal growth on adequate refeeding. For those rats undernourished from weaning to 42 days, refeeding resulted in recovery of normal weight only for the brain and lungs, resulting in an animal with retarded body growth but with normal-sized brain and lungs. Finally, in the case of the rats suffering malnutrition from 65 to 86 days, each organ except the thymus recovered its normal size on refeeding. To summarise, the rats did not recover from the effects of early malnutrition but were able to do so when malnutrition occurred at a later stage of growth.

It appears that permanent reductions in adult size result from undernutrition in early life, although there is little direct proof of this in man in Thailand but population statistics from Britain and America do give proof of it. Further research should provide more definite information about the effects of malnutrition on body weight and stature.

RESULTS

Mean body weights (in grams) of the rats fed on diets containing 2, 3, 5 and 10 NDpCal% are shown in Table 1 and Figure 3. The rats fed on the low protein diets (2, 3 and 5 NDpCal%) were lighter in weight than those fed on the control diet (10 NDpCal%) throughout the experimental period. Two further tables have been constructed from these data to emphasize various aspects of the differences in growth rate of rats on these different diets. Table 2 indicates the differences in mean body weight between rats fed on the low protein diets (2, 3 and 5 NDpCal%) and those on the control diet (10 NDpCal%), while Table 3 provides assessments of the rate of growth of the rats on each diet at various times. In the latter table, weight changes have been averaged over a 4 week period to smooth out some of the random variations that may affect weekly figures.

Rats fed on the diet providing 2 NDpCal%

Most of the rats (90 from a total of 98) lost weight during the first week of feeding on the diet providing the lowest protein value (2 NDpCal%) and the mean body weight continued to drop slightly up to week 3, by which time it had fallen 4 g (or about 10%) below its initial value at the commencement of the diet. From week 4 onwards

TABLE 1

Mean body weight (g) of rats fed on diet 2, 3, 5 and 10 NDpCal%

Week of diet	Diet in NDpCal%			
	2	3	5	10
0	38.4 ± 0.4 (98)	42.4 ± 0.6 (56)	41.6 ± 1.0 (25)	43.4 ± 0.7 (72)
1	35.3 ± 0.4 (98)	45.0 ± 1.0 (67)	49.4 ± 1.6 (25)	62.5 ± 1.2 (65)
2	34.5 ± 0.5 (97)	47.2 ± 1.1 (67)	61.8 ± 2.8 (25)	82.3 ± 2.1 (65)
3	34.4 ± 0.5 (96)	50.0 ± 1.1 (62)	72.7 ± 3.2 (33)	99.3 ± 2.7 (68)
4	34.7 ± 0.6 (92)	52.8 ± 1.7 (61)	86.8 ± 4.4 (33)	119.2 ± 3.6 (68)
5	35.5 ± 0.8 (76)	59.7 ± 2.5 (50)	105.9 ± 4.2 (34)	150.6 ± 4.2 (66)
6	36.4 ± 1.0 (63)	65.7 ± 3.1 (50)	125.3 ± 5.1 (34)	174.4 ± 3.7 (66)
7	38.0 ± 1.1 (53)	69.7 ± 3.7 (48)	139.3 ± 5.4 (34)	196.2 ± 4.6 (66)
8	38.8 ± 1.5 (45)	74.2 ± 4.0 (47)	154.2 ± 6.1 (34)	214.1 ± 4.0 (66)
9	43.2 ± 3.7 (9)	87.3 ± 6.2 (29)	178.7 ± 6.4 (25)	232.9 ± 4.2 (48)
10	44.2 ± 3.8 (9)	94.9 ± 6.5 (29)	195.6 ± 7.0 (25)	247.1 ± 4.8 (48)

Note. 1. ( ) = the number in parenthesis is the total number of each group.

2. The mean body weight of weanling = 41.2 g (244).

TABLE 1 (continued)

Week of diet	Diet 1 : NDpCal%			
	2	3	5	10
11	44.5 ± 4.0 (9)	98.1 ± 7.2 (29)	205.8 ± 6.2 (25)	259.7 ± 5.4 (48)
12	46.0 ± 4.1 (9)	109.8 ± 9.0 (24)	217.5 ± 7.5 (25)	273.2 ± 6.0 (45)
13	-	115.4 ± 9.4 (20)	229.6 ± 8.0 (16)	287.2 ± 5.5 (41)
14	-	125.3 ±10.2 (20)	239.3 ± 8.7 (16)	297.9 ± 5.9 (40)
15	-	124.7 ±10.3 (20)	252.3 ± 8.5 (16)	307.4 ± 5.2 (40)
16	-	125.5 ±10.1 (20)	258.1 ± 9.3 (16)	317.6 ± 7.1 (40)
17	-	136.0 ±13.1 (13)	258.4 ± 9.9 (9)	319.3 ± 9.3 (31)
18	-	138.4 ±13.5 (13)	266.0 ± 9.3 (9)	325.5 ±10.3 (31)
19	-	142.6 ±14.1 (13)	288.5 ±10.3 (9)	344.4 ±13.2 (21)
20	-	152.0 ±10.8 (10)	299.6 ±11.5 (9)	346.1 ±13.0 (21)
21	-	167.9 ± 8.5 (7)	309.7 ±16.3 (7)	355.9 ±18.8 (7)
22	-	170.9 ± 8.5 (7)	319.0 ±19.0 (7)	364.6 ±19.4 (7)
23	-	172.0 ± 9.1 (7)	333.8 ±18.4 (7)	371.2 ±18.3 (7)
24	-	173.3 ± 9.1 (7)	338.4 ±18.7 (7)	375.6 ±17.6 (7)

FIGURE 3 Body weights of rats on different protein diets

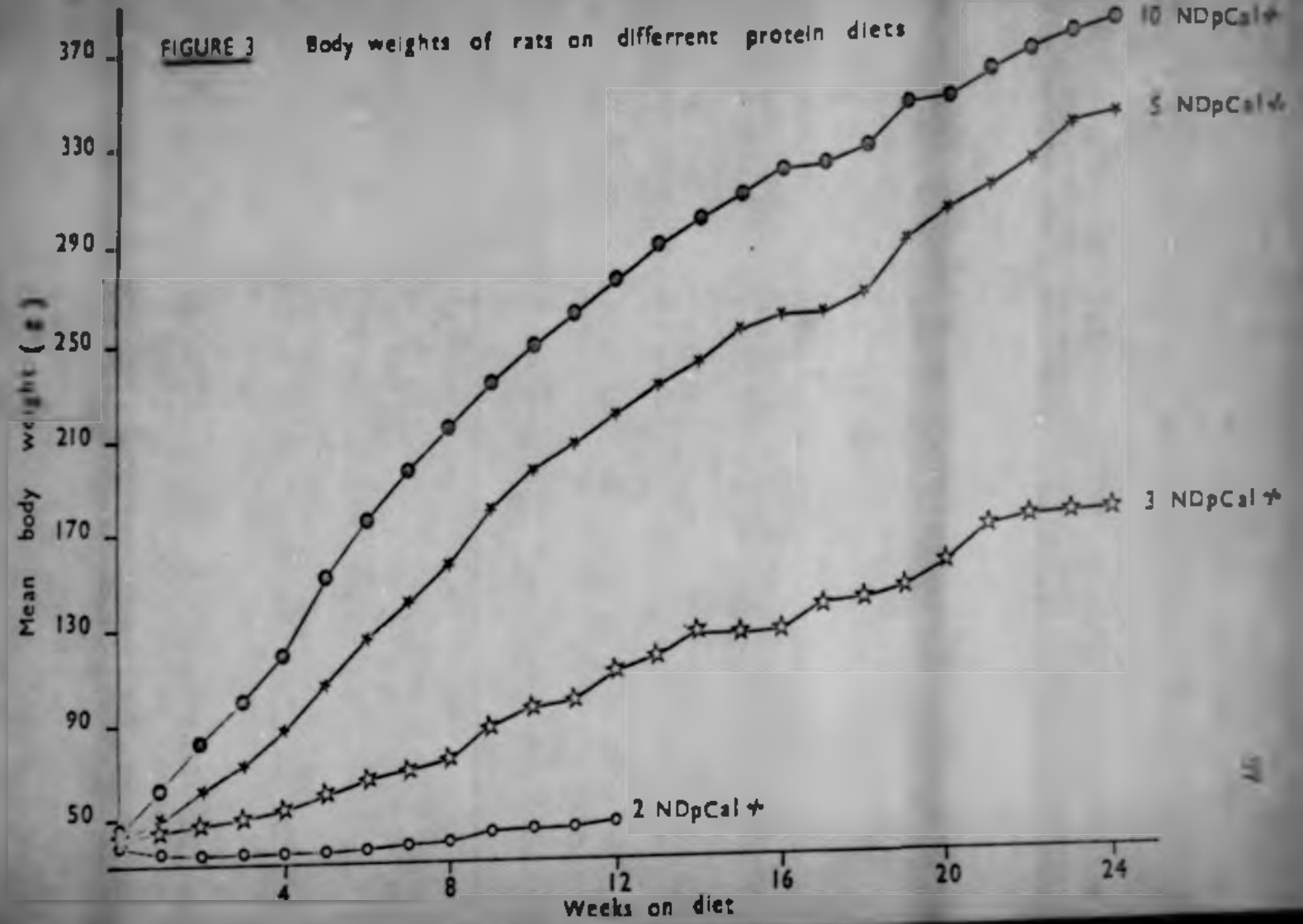


TABLE 2

Differences in mean body weight between the rats receiving diets of 2, 3, and 5 NDpCal% and those receiving 10 NDpCal%, expressed in terms of absolute weight and as a percentage of the weight of the 10 NDpCal% group at the same dietary duration

Week of diet	2 NDpCal%		3 NDpCal%		5 NDpCal%	
	gram	%	gram	%	gram	%
2	-47.8	-58.1	-35.1	-42.6	-20.5	-24.9
4	-84.5	-70.9	-66.4	-55.7	-32.4	-27.2
6	-138.0	-79.1	-108.7	-62.3	-49.1	-28.2
8	-175.3	-81.9	-139.9	-65.3	-59.9	-28.0
10	-202.9	-82.1	-152.2	-61.6	-51.5	-20.8
12	-227.2	-83.2	-163.4	-59.8	-55.7	-20.4
14	-	-	-172.6	-57.9	-58.6	-19.7
16	-	-	-192.1	-60.5	-59.5	-18.7
18	-	-	-187.1	-57.5	-59.5	-18.3
20	-	-	-194.1	-56.1	-46.5	-13.4
22	-	-	-193.7	-53.1	-45.6	-12.5
24	-	-	-202.3	-53.9	-37.2	-9.9

TABLE 3

Mean rates of growth (weight gain) of the rats fed on diets of 2, 3, 5 and 10 NDpCal%

Week of diet	Rate of gain of body weight* (gram/week)			
	2 NDpCal%	3 NDpCal%	5 NDpCal%	10 NDpCal%
2	-0.9	2.6	11.3	18.9
4	0.5	4.6	15.9	23.0
6	1.0	5.3	16.9	23.7
8	1.9	7.3	17.6	18.2
10	1.8	8.9	15.8	14.8
12	-	7.6	10.9	12.7
14	-	3.9	10.1	11.1
16	-	3.3	6.7	6.9
18	-	6.6	10.4	7.1
20	-	8.1	13.3	9.8
22	-	5.3	9.7	7.4

* Rate of gain for week n calculated as $\frac{1}{4}\left[\bar{w}_{n+2} - \bar{w}_{n-2}\right]$ where $\bar{w}_{n+2}$ is the mean weight at week (n+2).

there was a very gradual gain in weight (Table 3), but even at week 12 the mean weight was only 7.6 g (or about 20%) above its initial value. In contrast, the rats on control diet (10 NDpCal%) gained 229.8 g in this initial 12 week period. The mean body weight of this protein deficient group was less than half that of the control group after 2 weeks on the diet and the percentage weight difference progressively increased (Table 2) until the rats on the 2 NDpCal% diet weighed only about a sixth of the mean weight of control rats by week 12.

The rats fed on the 2 NDpCal% diet were in fact divided into three subgroups, which were given this low protein diet for different experiments in this study. Although an endeavour was made to select rats of very similar age and weight at the start of each experiment, there were small differences in the mean initial weight (at commencement of diet) between these three subgroups, the values for the individual subgroups being 40.9 ± 0.7, 36.2 ± 0.6 and 38.1 ± 0.6 g. It was observed that these apparently minor differences in initial weight had a significant influence on the growth of the rats in these different subgroups. The rats in the subgroup of lowest initial weight (36.2 g) continued to lose weight until week 5, with their mean weight falling to 30.8 ± 1.2 g at that time; whereas those in the subgroup of highest initial weight (40.9 g) reached their minimum weight, of

38.7 ± 1.0 g, at week 2 and had exceeded their initial weight by week 5, attaining 45.4 ± 2.2 g at that time. The difference in mean body weights between these two subgroups had increased still further to 15.8 g at week 8. The third subgroup of intermediate initial weight followed an intermediate pattern. These differences illustrated the importance of the age of the subject at the time of commencement of protein deficiency on the severity of the effects on growth, since presumably the subgroup of slightly greater initial weight consisted of slightly older rats.

Rats fed on the diet providing 3 NDpCal%

The somewhat less severe protein deficiency provided by the 3 NDpCal% diet meant that the rats in this group were able to maintain and, in general, gradually increase their body weight throughout the experimental period (Table 1). On average a 50% increase in weight was achieved by week 6, an 100% increase by week 9 and a 200% increase by week 17. Nevertheless the rate of gain of body weight was slower than that of the control rats throughout the 24 week experimental period (Table 3), although these rates differed little between the two groups beyond week 17. Thus, the weight difference between the rats fed on 3 NDpCal% diet and those on control diet increased in absolute amount until week 16 and then remained

relatively constant (Table 2), whereas the percentage weight difference reached its maximum of about 65% at week 8 and recovered gradually thereafter.

Rats fed on the diet providing 5 NDpCal%

The animals in this group all gained weight, quite markedly in comparison with the animals on the other two low protein diets (2 and 3 NDpCal%). The mean weight of the animals in this group had increased by about 50% at week 2, by 100% before week 4, 200% by week 7, 400% by week 12 and over 700% at week 24. The weight increase in animals in this group, however, was still not as rapid as that of the control animals (Table 1). The mean body weight lagged about 28% below that of the control group after 6 weeks but this percentage difference subsequently gradually decreased so that the mean body weight of the rats on 5 NDpCal% was only about 10% below that of the control group at week 24 (Table 2). It is apparent from Table 3 that the rats on this diet were unable to maintain the fast growth of the control rats during the first 8 weeks, but for the next 8 weeks a growth rate similar to the control animals was achieved and for the final weeks a rate of gain in weight in excess of that of the control rats was attained and some of the weight difference was recuperated at this stage.

Rats fed on the control diet providing 10 NDpCal%

The animals fed on the control diet showed a more rapid growth than the rats fed on any of the protein deficient diets. These control rats achieved a weight gain of almost 50% in the first week, almost 100% at week 2, 200% by week 5, 400% by week 9 and approaching 800% at week 24. The rate of increase in body weight in these animals was most rapid during the first 8 weeks (Table 3) and this period is thus commonly referred to as the growing period. Beyond this time the growth rate was observed to gradually fall off until about week 15 and then remain for the later weeks at roughly a third of the maximum rate during the growing period.

Comparison between these diets

None of the protein deficient groups of rats were able to maintain the growth rate of the rats on control diet (Figure 3). The extent of the retardation in growth in the protein deficient rats generally correlated with the severity of the protein deficiency, with the rats on the lowest protein value diet (2 NDpCal%) losing weight during the first few weeks on the diet, those on 3 NDpCal% diet gaining weight gradually and those on 5 NDpCal% diet nearly attaining the growth rate of the control rats (on 10 NDpCal% diet). If the severity of the growth restriction is assessed by the percentage

weight difference of each protein deficient group from the control group, then the growth retardation increased rapidly in severity over the first 6 weeks on the 2 NDpCal% diet (Table 2) then remained relatively constant but without any indication of recovery, while the percentage weight differences for the 3 NDpCal% and 5 NDpCal% diets were greatest at weeks 8 and 6, respectively, with slight recovery afterwards in the former case and good recovery in the latter. The greatest effects of protein deficiency on the relative weight of the animal was thus in the growing period (the first 8 weeks), at least for the rats on the moderately protein deficient diets providing 3 NDpCal% and 5 NDpCal%. The severer protein deficiency associated with feeding on 2 NDpCal% diet appeared to have a more prolonged effect on growth rate. The magnitude of the size difference at week 8 between rats fed on the control diet (10 NDpCal%) and on the 2 NDpCal% protein deficient diet is illustrated by the photograph of Figure 4. As can be seen in this figure, it was noticed that protein deficiency had a relatively lesser effect on head size than on the size of the rest of the body.

The rate of gain in body weight of the rats fed on 5 NDpCal% diet was more than half that of the control rats fed on 10 NDpCal% diet throughout the experimental period, even during the period of most rapid growth (Table 3). A further reduction in dietary protein content

FIGURE 4

Showing the difference in size of two rats of the same age (at the 8th week of diet), the smaller rat was fed on low protein diet (2 NDpCal%) and the larger one on control diet (10 NDpCal%).

from 5 NDpCal% to 3 NDpCal% or 7 NDpCal%, in contrast, produced a more than proportional reduction in growth rate, particularly during the growing period. The first of these findings might be explained by the control diet (10 NDpCal%) providing protein in excess of the demands both for growth and for energy consumption, so that the reduction in protein value to 5 NDpCal% results in a less than proportional decrease in growth rate. In the cases of the 2 NDpCal% and 3 NDpCal% diets, however, large proportions of the dietary protein are required to provide the energy necessary to maintain basal metabolism and only a small proportion remains available for growth, which as a result is severely restricted.

In summary, it was found that the growth rate of the animals depended both on the protein value of the diet and on the period at which the dietary restriction occurred.

Section 7. [illegible]

The effects of a dietary protein-energy malnutrition on the body fluid of rats.

One of the most outstanding clinical signs of some cases of protein-energy malnutrition is oedema and this manifestation is thus used as an indicator in the international standard classification of severe protein-energy malnutrition (McLaren et al., 1967).

Oedema reflects an increase in the extra-cellular fluid (ECF) volume, but the cause of this increase during protein-energy malnutrition is not clear. It was originally considered that the reductions in the serum protein concentrations were directly responsible for the oedema in this condition and hypoalbuminaemia was frequently observed by Trowell et al. (1954) in severely oedematous patients. Montgomery (1963) pointed out, however, that marked hypoalbuminaemia can occur in the absence of clinical oedema and he found that, of 60 cases where serum albumin was less than 2.0 g/100 ml, only half had severe oedema. For this reason, and because it was observed that oedema tended to be shed during the treatment of kwashiorkor long before any significant rise in serum proteins had occurred, the role of serum albumin in the production of oedema is now disputed. A possible alternative cause is that the malnourished child is given

too much fluid orally in relation to its diminished ability to clear fluid, owing to an impairment of renal function, and this imbalance results in body-water expansion (Whitehead and Alleyne, 1972). Several authors (Gopalan, 1950; Srikantia, 1958; Gopalan, 1970) have suggested that an increased secretion, or a failure of inactivation, of antidiuretic hormone (ADH) might be responsible for the changes in renal clearance of water, as it appears that water retention is due to increased tubular reabsorption rather than to reduced renal plasma flow or to diminished glomerular filtration rate (Srikantia and Gopalan, 1959). It has been suggested that the presence of ferritin in the circulation might be responsible for these antidiuretic effects by breaking down the homeostatic mechanisms controlling the release of ADH. Moreover, Srikantia (1958) has demonstrated the presence of active ferritin in the circulation of kwashiorkor patients with oedema and found that ferritin disappeared after successful treatment with a high protein diet. In addition, ferritin cannot be detected in the blood of marasmic patients where oedema is absent. More recently, Srikantia (1968) has provided more definitive evidence that ferritin may be responsible for the association of oedema with protein-energy malnutrition. On feeding with a protein deficient diet, all monkeys in this experimental group developed oedema after 3-5 months; but only one monkey from a group

of six developed oedema when the animals were maintained on the same diet but also given chlortetracycline, a drug which prevents the release of ferritin under conditions of stress. Ferritin became detectable in the circulation 2 weeks prior to the increase in ECF volume in each monkey in which oedema subsequently appeared, while no ferritin was detected in the monkeys which did not develop oedema. Also the reductions in serum proteins were similar in the two groups of monkeys and Srikantia (1968) thus concluded that ferritinaemia caused the oedema.

RESULTS

An assessment was made of the body fluid in the rats fed on the three protein deficient diets (2, 3 and 5 MDpCal%) and on the control diet (10 MDpCal%) for various durations and the results are displayed in Table 4, expressed as percentages of body weight.

When the body fluid percentages of the rats on each low protein diet were compared against the values for the control diet at each week the differences were not significant (generally $p > 0.1$), except for the difference between the 2 MDpCal% and control diets at week 8 ($p < 0.05$). Nevertheless the mean percentage for each of the protein deficient diets was greater than that for the control diet throughout the period from week 4 to

TABLE 4

Body fluid in the rats on diets of different protein content expressed as a percentage of body weight.

Diet in NDpCal% Duration (weeks)	Body fluid (% body weight) (Mean ± S.E.M.)			
	2	3	5	10
2	68.0 0.4	68.4 0.5	- -	69.0 0.5
4	68.8 0.5	68.1 0.4	67.9 0.8	67.6 0.5
8	68.5* 0.6	68.3 0.7	67.4 0.6	66.7 0.5
12	67.8 0.8	67.5 0.8	67.6 0.8	65.7 0.8
16	- -	65.7 0.8	65.0 0.6	63.9 0.9
20	- -	66.3 0.8	64.5 1.3	63.9 1.3
24	- -	66.4 0.8	66.3 0.8	66.6 0.7

N.D. Number of rats in each group was 6, except at week 24 when it was 7.

* Difference from control value significant, $p < 0.05$, All other differences from control were non-significant.

week 20, inclusive; and, when the low protein diets were grouped together and weeks 4, 8 and 12 were pooled, the overall mean of 68.0 ± 0.2 % for the protein deficient rats for this period was significantly higher ($p < 0.01$) than the mean of 66.7 ± 0.4 % for the control rats for the same period. The difference in body fluid between the groups, however, was very small. It should be mentioned that the present estimates of body fluid are subject to slight error as a result of the method employed for their measurement; it was not considered justifiable to use a seperate group of rats just for these body fluid measurements, so the body fluid measurements were made after a blood sample had been taken from each rat of the principal group (providing all the data of Part 3). The wet weight was measured after taking the blood sample and before drying the carcass, but the body fluid assessment gives a slight underestimate since blood has a higher percentage water content than the average for the whole body. The extent of underestimation due to this effect should be only about 0.5% in the biggest rats, from which upto 12 ml of blood were taken, and upto a maximum of about 1.6% in the smallest rats, from which 2-3 ml of blood were taken. For this reason, the true difference in body fluid should be about 1% greater than that estimated, but remains very small even after this correction.

It was also apparent from the present measurements that the body fluid percentage decreased with increasing age in the animals on the control diet (10 NDpCal%). The body fluid value at week 2 was significantly higher than each of the subsequent values ($p < 0.02$), the percentage at week 4 was significantly greater than those at weeks 16 and 20 ($p < 0.05$) and the value at week 8 was significantly above that at week 16 ($p < 0.05$). This pattern has also been recognised by other workers (Light et al., 1934; Hamilton and Dewar, 1938) and it has also been observed that lean rats tend to have a higher body fluid proportion than those of average weight (Scheer et al., 1947). It is thus considered that the small difference in body fluid percentage between the protein deficient and control rats in the present study may have been related to the smallness and thinness of the protein deficient animals rather than to a real increase in body fluid. Although the rats fed on 2 NDpCal% diet became older in chronological terms, they remained similar to weanling rats in size and for that reason had a body fluid percentage more appropriate to weanling rats even after about 12 weeks on the diet.

There were no clinical signs of oedema in any of the protein deficient rats, a finding which would be consistent with the view that there was no real increase in body fluid in these animals. The clinical picture of these rats was

thus of the marasmic type of protein-energy malnutrition more than of the kwashiorkor type (see Figure 6).

Section 3. FUR LOSS

Changes in hair associated with protein-energy malnutrition

Hair consists almost entirely of the protein keratin, although it also contains about 3% of solid non-protein material and some water. It has also been reported (Pillsbury et al., 1956) that germinative hair cells proliferate at a greater rate than any other tissue, with the possible exception of bone marrow, suggesting that there is a high rate of protein synthesis in the hair follicle. Sims (1969) confirmed this with the observation that the rate of protein synthesis in cells of the cortex and matrix in hair was one of the highest in the body, and Downes (1965) has demonstrated the importance of hair as an indicator of protein status, since labelled cystine was found in the follicle within a few hours of intravenous administration. If any factor causes a reduction in the rate of protein synthesis in the body as a whole, hair acts as a sensitive reflection of such a change.

Penn Chavarria et al. (1946; 1948) demonstrated that a change in hair texture was frequently associated with protein-energy malnutrition. It was observed, for example, that the hair of African children lost its sheen and changed its colour, as a result of a reduction in pigmentation, in this condition. In Central America, a child's hair might grow normally during periods of improved

nutrition but then another band of discoloured hair could result from a further period of malnutrition, leading to the so-called " flag-sign " pattern commonly found in that country, though not in Africa. The hair is often sparse and may be plucked easily and painlessly. Many other investigators have reported a variety of hair changes associated with kwashiorkor, including hypochromotrichia, loss of natural curl, brittleness and sparseness (Trowell et al., 1954; Jelliffe, 1955; Jelliffe et al., 1963). Bradfield et al. (1967; 1968; 1969) have investigated these effects in more detail and have consistently found morphological changes in the hair roots of children suffering from protein-energy malnutrition. These changes, which tended to be reversed during protein feeding, included atrophy of the hair bulb with decreasing pigmentation, absence of the external root sheath in atrophied roots and frequently absence of the internal root sheath as well. Consistent, and significant, morphological changes in hair roots were also evident after 11 days, when a group of young men were fed on a protein-free liquid diet, which was complete in all other nutrients (Bradfield, 1971). These changes included reduction in bulb diameter, atrophy, depigmentation and absence of the root sheaths and were found to occur at a time when total serum protein and serum albumin levels remained normal, although urinary nitrogen had reached a

minimum value by 11 days. Moreover, when protein was added to the diet, these hair root changes were reversed in about 14 days. These observations, made on normal individuals fed on a diet complete except for protein, have indicated that protein is essential for the development of hair and that hair can be used as an early indicator of body protein status.

RESULTS

An assessment is made of the fur changes and of fur loss in the rats fed on diets of 2, 3 and 5 NDpCal% in Tables 5, 6 and 7. All the control rats (on 10 NDpCal% diet) had very fine fur throughout the period studied. A rough quantification of the changes in appearance of the fur of the protein deficient rats was achieved by using the following gradings :-

Grade A: This indicated that the fur was no longer smooth and that there were some signs of loss of fur.

Grade B: This indicated that there was a clearly visible loss of fur.

COLOUR OF FUR

The fur of hooded rats is naturally coloured black and white. The black hair was seen to change to a brown

colour in nearly all of the rats fed on diets of 2 and 3 NDpCal% in this study, with this change occuring after approximately 4 weeks on the diet and the brown colour remaining throughout the remainder of the experimental period (i.e. to week 12 for rats fed on 2 NDpCal% diet and to week 24 for 3 NDpCal%). There was no sign of a change in fur colour, however, for the rats fed on the 5 NDpCal% diet.

FUR LOSS

Rats Fed on 2 NDpCal% Diet

Some of the rats receiving this diet started to show grade A fur changes by week 2 and grade B by week 3 (Table 5). More than half of the rats in the group showed some signs of fur loss by week 6 and all rats showed some fur loss from week 9 onwards, by which time grade B changes had become very prominent. There was no indication of an improvement in the fur condition during the remainder of the experimental period (12 weeks).

The severity of fur loss at week 8 is illustrated by the photograph in Figure 6 of a rat on the 2 NDpCal% diet for this duration, while Figure 5 shows a typical control rat (on 10 NDpCal% diet) at the same stage of its diet for comparison.

TABLE 5

Fur loss of rats fed on diet providing

2 NDpCal%

Week of diet	Total number of rats	Rats showing fur loss (grade A or grade B)		Rats with grade B fur loss	
		Number of rats	%	Number of rats	%
2	115	14	12	0	0
3	109	30	28	5	5
4	105	38	36	9	9
5	83	27	33	12	14
6	63	39	62	11	17
7	59	33	56	15	25
8	47	26	55	12	26
9	9	9	100	6	67
10	9	9	100	6	67
11	9	9	100	6	67
12	9	9	100	6	67

FIGURE 5

Showing the very fine fur of the control rat, fed on diet 10 MDpCal% at 8th week of diet.

FIGURE 6

Showing the fur loss of a rat fed on diet 2 NDpCal% at 8th week of diet.

Rats Fed on 3 MDpCalf Diet

Rats in this group showed a generally similar pattern of fur loss (Table 6) to that found in the animals receiving the 7 MDpCalf diet. Both grades of fur loss began to become apparent at week 2, and by week 3 more than half the rats showed some fur loss while more than half had developed grade B fur loss by week 6. Nearly all the rats had some evidence of fur loss throughout the period from week 7 to week 20, inclusive, but the more severe fur loss (grade B) was most prominent over a more limited period, from week 7 to week 9. This implied that the protein deficiency had its greatest effect on fur growth between weeks 7 and 9, with the number of rats that had each grade of fur loss at weeks 7, 8 and 9 not differing significantly ($X^2 = 3.32$, $p > 0.5$). Beyond this time, there appeared to be a gradual recovery of fur and the percentage of rats exhibiting grade B fur loss slowly decreased : by week 13, this recovery was becoming significant ($X^2 = 20.94$, $p < 0.01$). Moreover, at the end of the experimental period (24 weeks), only 1 rat of the 8 remaining on this diet had fur loss of grade B, although the majority of the rats still showed some signs of fur loss (Table 6).

TABLE 6

Fur loss of rats fed on diet providing

3 MDpCal%

Week of diet	Total number of rats	Rats showing fur loss (grade A or grade B)		Rats with grade B fur loss	
		Number of rats	%	Number of rats	%
2	110	30	27	11	10
3	105	58	55	20	19
4	104	76	73	30	29
5	89	71	80	33	37
6	88	77	87	48	55
7	86	80	93	61	71
8	84	78	93	59	70
9	61	60	98	42	69
10	61	60	98	37	61
11	60	57	95	36	60
12	60	57	95	32	53
13	40	39	97	17	43
14	40	39	97	13	33
15	39	38	97	14	36
16	36	33	92	13	36
17	19	18	95	9	47
18	19	18	95	9	47
19	19	18	95	9	47
20	19	18	95	9	47
21	8	7	87	2	25
22	8	6	75	1	13
23	8	6	75	1	13
24	8	6	75	1	13

Rats Fed on 5 NDpCal% Diet

Animals in this group (Table 7) began to show grade A characteristics in week 2 and a few rats in the group exhibited fur loss of grade B in week 3. The severer form (grade B) of fur loss became more apparent at week 5, after which it was found in a similar percentage of the animals until week 9 and then grade B characteristics rapidly disappeared thereafter. The period during which most animals showed some form of fur loss (of either grade) was from week 7 to week 9, and the χ^2 test in fact indicated the fur loss patterns were similar throughout the period from week 5 to week 9 ($\chi^2 = 10.69$, $p > 0.2$), implying that the 5 NdpCal% diet had its maximum effect on fur growth at some stage during this period but that the actual week when the effect was severest could not be more closely defined. Beyond week 9 there were signs that new fur growth was replacing the fur losses and, in fact, significant recovery of fur was found at week 10 ($\chi^2 = 7.23$, $p > 0.05$). Moreover, all grade B characteristics had vanished by week 11 and all animals had completely recovered from the fur changes at week 20 (Table 7). The 5 NDpCal% diet thus seemed to contain sufficient protein to maintain normal fur growth in adult rats.

TABLE 7

Fur loss of rats fed on diet providing 5 NDpCal%

Week of diet	Total number of rats	Rats showing fur loss (grade A or grade B)		Rats with grade B fur loss	
		Number of rats	%	Number of rats	%
2	50	12	24	0	0
3	45	23	51	3	7
4	45	29	64	3	7
5	45	27	60	11	24
6	45	33	73	10	22
7	45	36	80	10	22
8	45	36	80	8	18
9	34	28	82	6	18
10	34	26	76	1	3
11	34	23	68	0	0
12	34	16	47	0	0
13	25	13	52	0	0
14	25	12	48	0	0
15	25	11	44	0	0
16	22	8	36	0	0
17	20	2	10	0	0
18	20	1	5	0	0
19	20	1	5	0	0
20	20	0	0	0	0
21	12	0	0	0	0
22	12	0	0	0	0
23	12	0	0	0	0
24	12	0	0	0	0

DISCUSSION AND COMPARISON BETWEEN THESE DIETS

It was clear from these observations that all three low protein diets (2, 3 and 5 NDpCal%) affected the appearance of the fur to some degree, particularly during the first 12 weeks on the diet. The very low protein diets (2 and 3 NDpCal%) were associated with changes in the colour of the fur throughout the entire experimental period, while the slightly deficient diet providing 5 NDpCal% did not affect hair colour. A loss of fur was apparent in some of the rats on each diet from the 2nd week to the 19th week and the severer form (grade B) of fur loss was found in some animals of each group from week 3 until week 10. On the basis of the observations for all three low protein diets, fur loss appeared to be most severe near week 8, after which a gradual recovery of fur could occur as a result of new fur growth, at least in the rats fed on the 3 and 5 NDpCal% diets.

Comparisons between the 2 NDpCal% diet and the diets providing 3 and 5 NDpCal% are considered to be unrealistic, since it was not possible to make simultaneous assessments of fur loss on all three low protein diets. A first experiment involved a comparison between the 3, 5 and 10 NDpCal% diets, while a separate experiment was performed to compare the 2 NDpCal% diet with the control (10 NDpCal%) diet. The qualitative nature of the gradings of fur loss

made it impossible to maintain consistent standards between these two separate experiments, but comparisons between the different weeks on any particular diet and those between the 3 and 5 NDpCal% diets should be meaningful. The fur loss of the rats fed on 3 NDpCal% diet was more severe than that of the rats fed on 5 NDpCal% diet, with the difference between the diets being significant at each week from week 4 onwards ($X^2 = 9.04$, $p < 0.02$ for week 4; $X^2 = 6.17$, $p < 0.05$ for week 5; $X^2 > 10$, $p < 0.01$ for each subsequent week).

The magnitude of the effects for growth thus appeared to correlate generally with the severity of the protein deficiency. The extent of the fur changes also varied with the age of the animal at the time of protein deficiency with the changes most severe in the growing period. The 5 NDpCal% diet provided inadequate protein for normal fur growth for the rats in their growing period but provided sufficient when the animals reached adulthood. The protein supply from the 2 and 3 NDpCal% diets, however, remained inadequate at all stages.

By the time these gross changes in fur had become apparent, there had also been a large drop in serum albumin and total serum protein (see Chapter 3) in all rats on the 2 and 3 NDpCal% diets (these proteins not measured at week 2 on 5 NDpCal% diet). It thus appeared that gross changes in fur did not precede the changes in serum protein levels

in the present study, but alterations of the hair roots should have preceded the changes in the hairs themselves and could have occurred before the serum protein levels fell, as indicated by Bradfield (1971).

Section 4. MORTALITY

In many parts of the world, a significant proportion of children born to poor families die as a direct or indirect result of malnutrition before reaching the age of 5. It is now realised that most deaths from protein-energy malnutrition occur after weaning and that the rate of mortality of children between the ages of 1 and 4 years provides the best index of the degree of protein-energy malnutrition in a given area or country (Bengoa et al., 1959). In the United States of America and in most parts of Western Europe the child mortality (1 to 4 years of age) is approximately 1 per 1,000 while in most technically underdeveloped countries the figure is much higher, varying from 10–45 per 1,000 as indicated by the following data (Scrimshaw and Behar, 1961).

SPECIFIC MORTALITY RATE, PER 1,000 POPULATION, OF CHILDREN AGED 1-4 YEARS IN SELECTED COUNTRIES (1951-1956)

Countries where kwashiorkor is rare or unknown	Mortality rate	Countries where kwashiorkor is common	Mortality rate
Argentina	3.8	Columbia	20.3
Australia	1.3	Ecuador	28.8
Belgium	1.6	Egypt	60.7
Canada	1.5	El Salvador	22.7
France	1.6	Guatemala	42.7
Japan	3.8	Guinea	55.4
Netherlands	1.2	Mexico	24.0
Sweden	1.0	Thailand	14.5
United States	1.1	Venezuela	12.5

The mortality rate after admission of infants to hospital for treatment of serious malnutrition is very high and has remained so in spite of detailed investigation and documentation over a period of 25 years. Reported mortality rates for these cases have varied from 11% to 50% (Walt et al., 1950; Gomez et al., 1956; Behar et al., 1958; Kahn, 1959; Fusoke, 1961; Lawless et al., 1966; McLaren et al., 1969). For both physiological and cultural reasons the mortality rate in marasmus is higher in children less than one year of age, while kwashiorkor is more prevalent in children during the second and third years and is a more frequent cause of mortality in the latter period (Scrimshaw and Behar, 1961).

CAUSES OF DEATH FROM MALNUTRITION

Gomez et al. (1956) analysed 733 children who were admitted to hospital with second and third degree malnutrition, and found that the degree of malnutrition, the presence of water and mineral imbalance, diarrhoea and acute broncho-pneumopathy each had a significant influence on mortality. The existence of evident oedema or of skin lesions had no apparent influence on mortality. Meanwhile, Kahn (1959) reported that death due to malnutrition was more likely when one or more of the following factors were present:- (a) advanced emaciation with a body weight below 50% of average, (b) acute

nutritional dermatosis, (c) clinically detectable dehydration associated with markedly lowered serum sodium and potassium levels, (d) marked enlargement of the liver, and, (e) hypothermic. A detailed analysis of possible factors responsible for death was made by Galvan and Calderon (1965) in a study of children with advanced malnutrition (marasmus and kwashiorkor). When these children were grouped according to their age and to the presence or absence of oedema, no significant differences in death rate were found between those with and those without oedema from within the same age group. There was a 31% overall death rate for children without oedema, and a 30% rate for those with oedema. Infants with electrolyte imbalance as well as oedema had a death rate of 44% compared with 37% for those without the oedema. Those with infection due to enteropathogenic microorganisms had a death rate of 38% if oedema was also present and of 35% if absent. Age clearly influenced the death rate in all groups, the mortality varying from 39% in the first year of life to 28% during the fourth year and 13% thereafter. Signs of terminal infection, such as bronchopneumonia and enteritis, are almost invariably found in children dying from malnutrition but these must be regarded as contributory causes only (Waterlow et al., 1960). Biochemical failure at the cellular level is probably the real cause of death. Wharton et al. (1968) have suggested that hepatic

encephalopathy is the cause of death in kwashiorkor following the development of apathy, deepening drowsiness and then coma. Apathy and disturbed EEG patterns have been observed both in kwashiorkor (Engel, 1956; Nelson, 1959) and in marasmus (Monckeberg, 1968; Stoch and Smythe, 1963), and it has been asserted that the child or animal suffering from serious protein deficiency dies a " central nervous death " (Platt, 1961).

Widdowson et al. (1960) found that all undernourished animals had an excess of extracellular fluid in their skeletal muscle, as indicated by the high concentrations of sodium and chloride and the low concentration of potassium in this tissue. In pigs that died of infection, sodium and chloride ions were also found to have entered the tissue cells and potassium to have left them, leaving a very low concentration of potassium in the tissue of the brain. Subsequently, Widdowson (1968) reported that pigs dying during the first 6 months of protein-energy malnutrition often had a large amount of extracellular fluid in the abdominal cavity and suggested that heart failure was might sometimes have been the cause of death. She also found that some malnourished pigs died more quickly than was usual and discovered that two of these animals had large gastric haemorrhages, while large rectangular ulcers were frequently observed on the lesser curvature of the stomach near the oesophagus in the protein deficient animals. A refusal

to eat was also observed in some animals given a low protein diet and their resulted rapidly in weakness and in death within a few days of starting the diet. It was considered that sick animals showing this pattern could correspond to children suffering from marasmus or kwashiorkor (Widdowson, 1968).

Many different factors have been put forward as the cause of death in protein-energy malnutrition, but there is still no agreement about which factor represents the principal cause. There is little argument, however, about the high mortality in cases of severe malnutrition in children and young animals. In very general terms, the reason for death in protein malnutrition may be best summarized by the words of Waterlow et al. (1960): " It seems probable that the real cause of death is biochemical failure at the cellular level. "

RESULTS

Some rats died from the groups fed on the low protein diets providing 2 and 3 NDpCal%, whereas all animals survived from the groups fed on the 5 NDpCal% and control diets. Nevertheless the rats fed on all three low protein diets appeared unhealthy at the commencement of the diet from weaning to puberty, and the survivors from the 2 and 3 NDpCal% diets also appeared less healthy than the control animals for the remainder of the experimental period. Table 8 shows the number of deaths at each week of the diet from amongst the rats fed on the 2 and 3 NDpCal% diet, and also the mortality rates calculated from the number of deaths in a week by expressing this number as a percentage of the number of rats surviving at the start of that week. No rats died after the 9th week on any diet. The mortality rates for these two severely protein deficient diets are also displayed in Figure 7.

RATS FED ON 2 NDpCal% DIET

In the entire experimental period, 51 rats (58%) died from the group, originally comprising 88 animals, fed on the diet providing the least amount of protein (2 NDpCal%). All these deaths occurred in the growing period (taken to be from the first week to week 8, inclusive) and the majority of deaths were in the latter half (weeks

TABLE 8

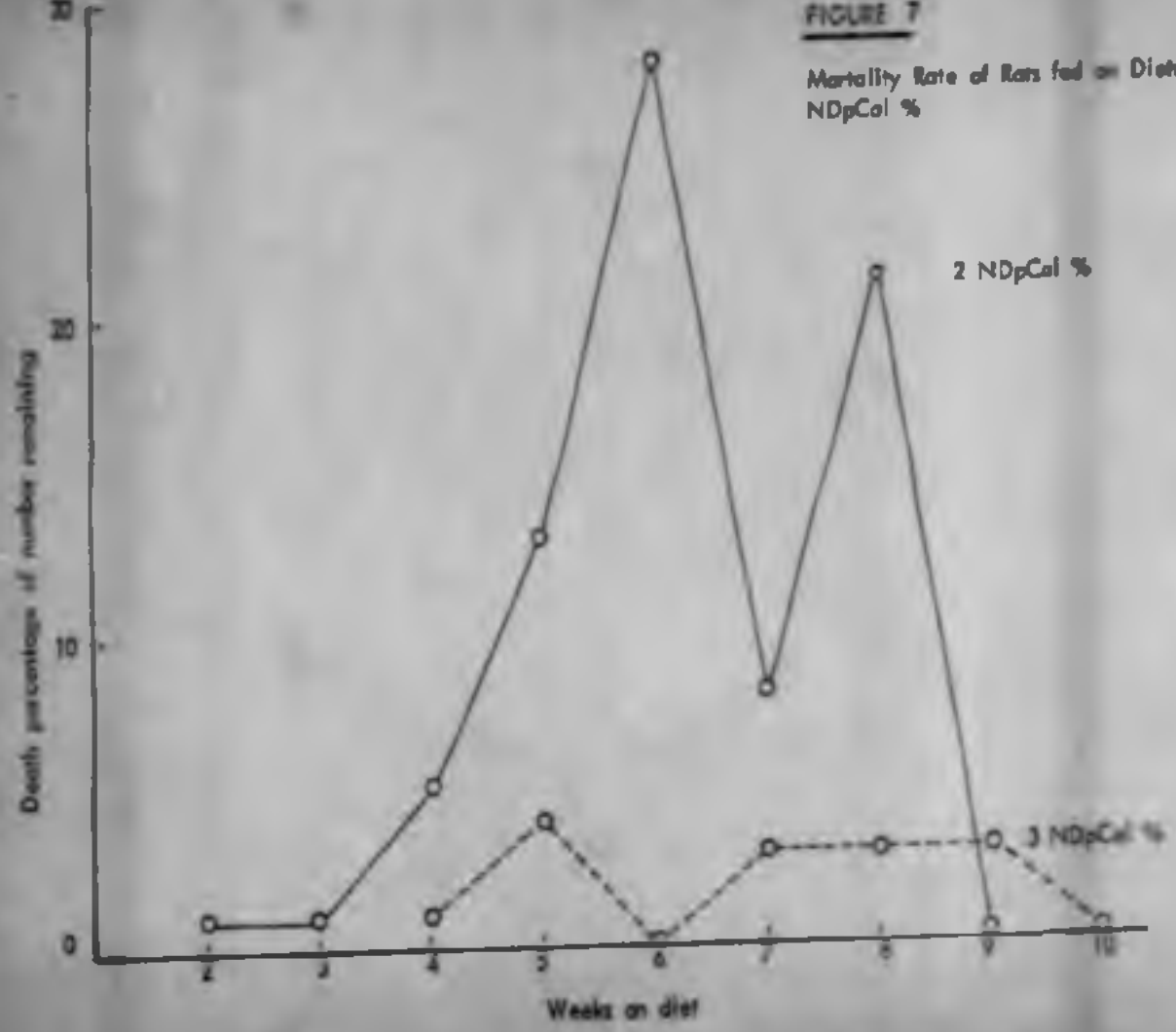
Showing the mortality rate of rats fed on diets 2 and 3 NDpCal %

Diets in NDpCal%	Mortality	Weeks on diet								Total
		2	3	4	5	6	7	8	9	
2	number of death (88)*	1	1	4	11	20	4	10	-	51
	death percentage of number remaining	1 ± 1	1 ± 1	5 ± 2	13 ± 4	28 ± 6	8 ± 4	21 ± 7	-	
3	number of death (68)*	-	-	1	3	-	2	2	2	10
	death percentage of number remaining	-	-	1 ± 1	4 ± 3	-	3 ± 2	3 ± 2	3 ± 2	

* = The number in parenthesis is the original number of each group

FIGURE 7

Mortality Rate of Rats fed on Diets 2 and 3
NDpCal %



5-8, inclusive) of this period. The results suggested two peaks of mortality rate at week 6 and 8 (see Figure 7), but statistical analysis indicated that the apparently lowered mortality at week 7 was only of marginal significance ($X^2 = 7.71$, $0.05 > p > 0.02$). On average the mortality rate over the peak period from week 6 to week 8 was 19% per week.

RATS FED ON 3 NDpCal% DIET

Altogether 10 rats (15%) died from the group, originally comprising 68 animals, fed on the diet of protein value 3 NDpCal% and the last of these deaths occurred at the 9th week of the diet. With this relatively small number of deaths, it was not possible to define a real peak in the mortality rate. It appeared instead that the deaths occurred at an approximately uniform rate of about 2% per week throughout the period from week 4 to week 9 ($X^2 = 3.39$, $p > 0.5$).

RATS FED ON 5 NDpCal% DIET

None of the rats died from the group fed on the 5 NDpCal% diet.

DISCUSSION AND COMPARISON [illegible] THESE DIETS

The overall mortality rate, in the whole experimental period, of the rats maintained on the 2 NDpCal% diet was almost four times greater than that of the rats fed on the 3 NDpCal% diet, a difference which was highly significant (X^2 = 30.41, $p < 0.001$). In addition the mortality of the rats on the 3 NDpCal% diet was significant in comparison with the lack of deaths among the group of rats maintained on the 5 NDpCal% diet (X^2 = 7.91, $p < 0.01$). Thus, the mortality rate of rats suffering protein deficiency appeared to be closely related to the protein content of the diet, with a high mortality occurring when protein deficiency was very severe.

There was no evidence of infection in the animals that died and no further investigation was carried out to determine the actual cause of death. These rats tended to lose weight, weaken and become less active before death supervened.

All deaths occurred during the period from week 2 to week 9, with the peak in mortality rate being between week 6 and week 8, inclusive, in the only group of rats in which a peak could be defined (i.e. in rats fed on 2 NDpCal% diet). The lower mortality rate in the earlier part of this growing period may have occurred because the animals had some protein reserves at the start of this

period as a result of receiving a protein replete diet until weaning. By about week 6 any such reserves were probably completely used up in the animals fed on the 2 NDpCal% diet and the rats were then liable to die as this diet provided insufficient protein and energy to maintain the normal basal metabolic rate. Any rat surviving the growing period, however, was able to survive subsequenly with less difficulty as the protein and energy demands were then lower.

The rat is normally weaned at 3 weeks, reaches puberty at 6-7 weeks and has a total life span of about 3 years. On a relative time scale, the period of high mortality found in the present study thus corresponded approximately to the high mortality period associated with protein-energy malnutrition in man.

CHAPTER 2 CHARACTERISTICS OF THE ANAEMIA PRODUCED BY PROTEIN DEFICIENCY

CHARACTERISTICS OF THE [illegible] INDUCED BY PROTEIN DEFICIENCY

INTRODUCTION

The anaemia resulting from protein deficiency can be of many types, the most common of which is the normochromic normocytic type, although macrocytic, and microcytic and/or hypochromic types have also been observed.

The aim of this chapter of the study was to establish some basic information about the anaemia induced in rats by protein deficiency, in order to be able to obtain an understanding of some of its aetiological mechanism(s). In an attempt to achieve this aim, the following aspects of the anaemia were investigated in the experimental work:-

Section 1. The effect of various values of low protein diet, at various stages during the test period, on the severity of the induced anaemia; also the time at which the anaemia reached its most extreme.

Section 2. The type of anaemia induced by the low protein diets.

Experimental Procedure

Rats were fed from weaning with various low protein diets, of 2, 3 and 5 NDpCal%, and with 10 NDpCal% control diet. Sample groups from each diet were killed by anaesthesia (ether) at weeks 2, 4, 8, 12, 16, 20 and 24. Blood was taken from their hearts and prepared for haematological examination, as described in Part 2.

SECTION 1. The effect of various values of low protein diet, at various stages during the test period, on the severity of the induced anaemia; also the time at which the anaemia reached its most extreme.

RESULTS

The haematological findings (data on Hb. and PCV) at each week are included in Tables 1 to 7. The differences in haemoglobin levels between the four different diets and the changes with duration of diet are shown in Figure 1. while analogous data on packed cell volumes are given in Figure 2.

The haemoglobin concentration and the packed red cell volume were lower in rats fed on each of the protein deficient diets than in those fed on control diet. The greatest reduction was noted to occur generally at week 8.

RATS FED ON 2 NDpCal% DIET

Their haemoglobin and packed cell volume were considerably below those of the control group throughout the experiment (12 weeks) and these differences were highly significant ($p < 0.001$ at each week). The values (Hb. and PCV) were also significantly below ($p < 0.0?$ in each case) those of the rats on the 5 NDpCal% diet. There were no significant differences ($p > 0.05$ in each case) from those of rats fed on the 3 NDpCal% diet, but the 2 NDpCal% diet values were consistently below those for the

3 NDpCal% diet. The reduction reached its greatest extent at week 8, and a significant recovery ($p < 0.001$) was seen to have occurred by the time of the next observations at week 12. The mortality of the rats on the 2 NDpCal% diet, which was very high at week 8, should also be considered in this respect, however, in that it could in itself lead to a sudden apparent recovery between weeks 8 and 12 if the individual rats with the lowest values of haemoglobin and packed cell volume were to be the ones that died.

RATS FED ON 3 NDpCal% DIET

In this group also, the haemoglobin and packed cell volume were significantly lower ($p < 0.001$ at each week) than those of rats fed on the control diet, and in addition significantly lower ($p < 0.02$) than of those fed on 5 NDpCal%, throughout the experimental period (24 weeks). The greatest reduction was again noted at week 8. Recovery was significant ($p < 0.001$) by week 12, but was not complete even at week 24.

RATS FED ON 5 NDpCal% DIET

The haemoglobin and packed cell volume were significantly lower ($p < 0.05$ in each case) than those of the control group only for a diet of duration up to 16 weeks. The extent of the reductions were found to be similar at weeks 4 and 8. There was then a gradual improvement until the

level rose to near that of the control diet by weeks 20 and 24, this recovery becoming significant ($p < 0.05$) by week 20.

DISCUSSION AND COMPARISON WITH OTHER DIETS

It was clear from the present study that protein deficiency could induce anaemia. The degree of anaemia that resulted was dependent both on the protein concentration in the diet and on the duration of the diet. The lowest value of protein content (2 NDpCal% diet) resulted in the most severe anaemia, and the effect was greatest at the 8th week after weaning, when feeding on the 2 NDpCal% diet caused not only severe anaemia but also a high mortality rate.

Neither the 2 NDpCal% diet nor the 3 NDpCal% diet were adequate to maintain erythropoiesis, and haemoglobin and packed cell volume remained below their control values until the end of the experiment, even after the growing period. During the growing period, the 5 NDpCal% diet also not quite adequate to maintain erythropoiesis, although differences from the control diet were relatively small. This diet appeared to become just adequate, however, after the growing period and the haemoglobin level and packed cell volume reached nearly the same values as on the control diet at the end of the experiment (24 weeks).

TABLE 1

Haematological data of rats fed on diets of various protein values at week 2

Diet (NDpCal%)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics. (%)
2	12.1 ± 0.4	33.3 ± 1.1	36.4 ± 0.5	0.1 ± 0.0
3	12.3 ± 0.2	33.0 ± 0.3	37.3 ± 0.3	0.1 ± 0.0
5	-	-	-	-
10	14.5 ± 0.5	39.6 ± 0.9	36.7 ± 0.6	0.6 ± 0.2

(Mean ± S.E.M. for 6,5, and 9 rats on diets 2,3 and 10 NDpCal %, respectively)

TABLE 2

Haematological data of rats fed on diets of various protein values at week 4

Diet (NDpCal%)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics (%)
2	10.4 ± 0.6	27.3 ± 1.5	38.1 ± 0.4	0.2 ± 0.1
3	11.5 ± 0.2	30.6 ± 0.7	37.6 ± 0.4	0.2 ± 0.1
5	12.9 ± 0.6	36.4 ± 2.1	35.7 ± 0.7	0.6 ± 0.4
10	14.6 ± 0.2	40.6 ± 0.4	36.1 ± 0.3	0.5 ± 0.1

(Mean ± S.E.M. for 7,9,7 and 10 rats on diets of 2,3,5 and 10 NDpCal %, respectively

TABLE 3

Haematological data of rats fed on diets of various protein values at week 8

Diet (NDpCal %)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics. (%)
2	10.1 ± 0.4	25.8 ± 1.1	38.7 ± 0.3	0.2 ± 0.1
3	11.1 ± 0.4	28.4 ± 1.1	39.0 ± 0.2	0.2 ± 0.1
5	14.3 ± 0.6	39.0 ± 1.1	36.7 ± 0.4	0.3 ± 0.2
10	16.4 ± 0.1	43.0 ± 0.4	38.1 ± 0.2	0.6 ± 0.2

(Mean ± S.E.M. for 8,10,7 and 13 rats on diets of 2,3,5 and 10 NDpCal %, respectively)

TABLE 4

Haematological data of rats fed on diets of various protein values at week 12

Diet (NDpCal%)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics. (%)
2	12.9 ± 0.5	34.2 ± 1.1	37.7 ± 0.4	0.2 ± 0.0
3	14.0 ± 0.4	36.8 ± 1.1	37.8 ± 0.5	0.5 ± 0.3
5	15.2 ± 0.4	41.9 ± 1.2	36.2 ± 0.4	1.6 ± 0.5
10	16.5 ± 0.1	43.9 ± 0.4	37.7 ± 0.3	0.5 ± 0.1

(Mean ± S.E.M. for 9,9,8 and 13 rats on diets of 2,3,5 and 10 NDpCal %, respectively)

TABLE 5

Haematological data of rats fed on diets of various protein values at week 16

Diet (NDpCal %)	Hb. (g/100ml)	PCV (%)	MCHV (%)	Retics. (%)
2	-	-	-	-
3	13.2 ± 0.4	35.3 ± 0.9	37.2 ± 0.4	0.2 ± 0.1
5	15.3 ± 0.4	42.6 ± 1.0	35.8 ± 0.3	1.1 ± 0.6
10	16.4 ± 0.2	44.9 ± 0.6	36.5 ± 0.4	1.9 ± 0.4

(Mean ± S.E.M. for 10,7 and 12 rats on diets of 3,5 and 10 NDpCal %, respectively)

TABLE 6

Haematological data of rats fed on diets of various protein values at week 20

Diet (NDpCal%)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics. (%)
2	-	-	-	-
3	13.8 ± 0.3	38.1 ± 0.8	36.2 ± 0.3	0.5 ± 0.2
5	16.0 ± 0.2	43.9 ± 0.6	36.5 ± 0.4	1.1 ± 0.2
10	16.2 ± 0.2	44.5 ± 0.5	36.4 ± 0.3	1.2 ± 0.4

(Mean ± S.E.M. for 12,8 and 12 rats on diets of 3,5 and 10 NDpCal %, respectively)

TABLE 7

Haematological data of rats fed on diets of various protein values at week 24

Diet (NDpCal%)	Hb. (g/100ml)	PCV (%)	MCHC (%)	Retics. (%)
2	-	-	-	-
3	14.3 ± 0.3	38.1 ± 0.7	37.5 ± 0.3	0.3 ± 0.1
5	16.5 ± 0.2	45.3 ± 0.4	36.1 ± 0.1	0.8 ± 0.3
10	16.6 ± 0.3	45.5 ± 0.6	36.4 ± 0.4	0.9 ± 0.3

(Mean ± S.E.M. for 7,7 and 10 rats on diets of 3,5 and 10 NDpCal%, respectively)

FIGURE I

Changes in Hb values of rats fed on diets containing different amounts of protein.

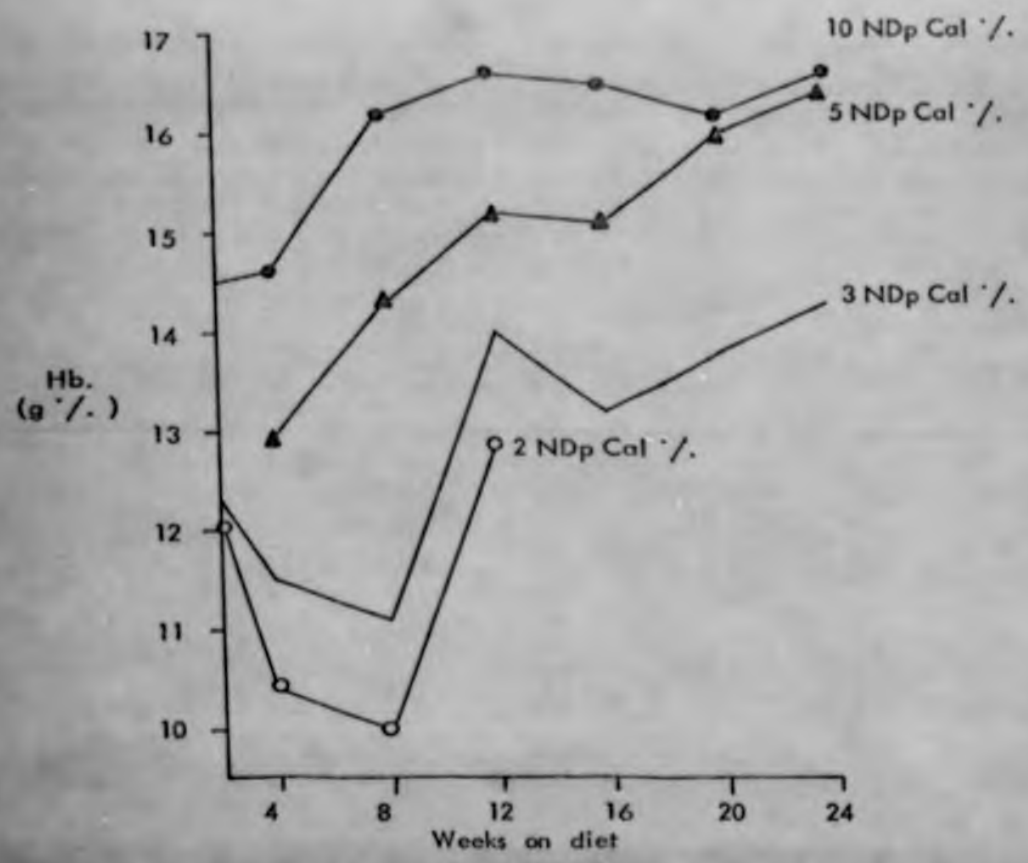
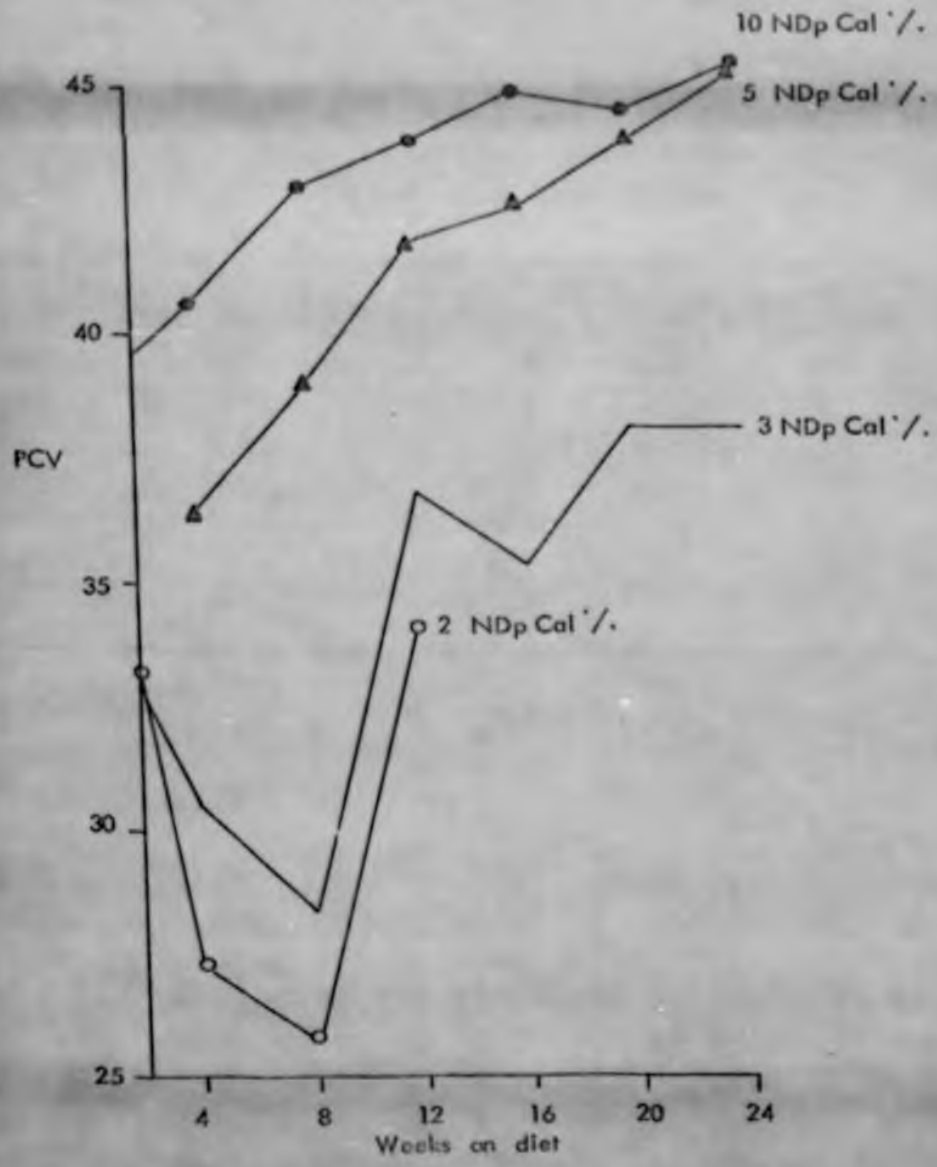


FIGURE 2

PCV values of rats on diets containing different amounts of protein.



SECTION 2. The type of anaemia induced by low protein diets

To identify the type of anaemia a more detailed haematological examination was required, including such aspects as morphology and size of the red blood cell, reticulocyte count, mean corpuscular haemoglobin concentration and bone marrow condition, in addition to the previous examination of SECTION 1.

RESULTS

1. MORPHOLOGY

Peripheral blood smears from rats fed both on low protein diets (2, 3 and 5 NDpCal%) and on control diet (10 NDpCal%) showed normocytic and normochromic red blood cells, and typical examples are illustrated in Figure 3. No erythrocytes of other types were found in any of the smears.

2. SIZE OF RED BLOOD CELLS

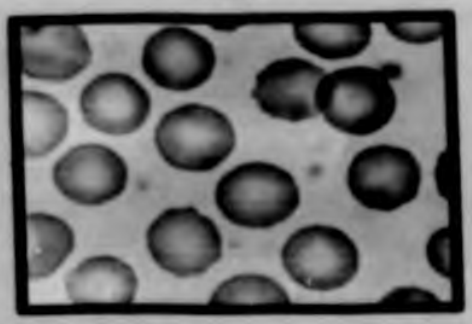
For the blood samples taken from rats fed on the 2 and 10 NDpCal% diets for 8 weeks, measurements were made of erythrocyte diameter, red cell count and packed cell volume. Estimates were then made of erythrocyte volume, thickness and surface area. The results are shown in Tables 8-12 and Figure 4 (Price-Jones curve).

The mean erythrocyte diameter did not differ significantly

FIGURE 3

Erythrocytes of rat fed on 10 NDpCal% diet (A) and
2 NDpCal% diet (B) at week 8.
(peripheral blood)

(A)



(B)

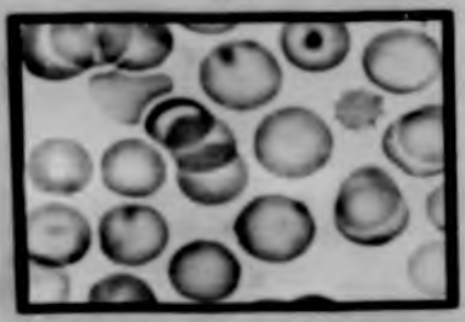


TABLE 8

Assessment of mean red cell diameter (μm) of rats fed on low protein diet (2 NDpCal %)

Number of rats	Number(%) of cells of various diameters (μm)					Mean diameter ± S.E.M (μm)
	5.1 μm	6.0 μm	6.8 μm	7.7 μm	8.5 μm	
1	1	4	59	32	4	7.13
2	1	4	61	32	2	7.07
3	-	5	68	26	1	7.01
4	15	38	46	1	-	6.25
5	10	30	58	1	1	6.42
6	19	20	47	10	4	6.47
7	18	17	56	7	2	6.45
8	12	29	51	6	2	6.45
Mean number of cells of each diameter (%)	10	18	56	14	2	6.65 ± 0.12

TABLE 9

Assessment of mean red cell diameter (μm) of rats fed on control diet (10 NDpCal %)

Number of rat	Number (%) of cells of various diameters (μm)					Mean diameter ± S.E.M. (μm)
	5.1 μm	6.0 μm	6.8 μm	7.7 μm	8.5 μm	
1	-	6	65	28	1	7.02
2	-	10	62	28	-	6.97
3	-	5	74	20	1	6.96
4	3	38	58	1	-	6.45
5	7	25	63	4	-	6.45
6	15	39	41	5	-	6.28
7	11	24	55	8	2	6.53
8	18	23	53	5	1	6.37
Mean number of cells of each diameter (%)	7	21	59	12	1	6.63 ± 0.11

TABLE 10

Mean cell volume (μm^3) of rats fed on low protein diet (2 NDpCal%)

Number of rat	Erythrocytes million/mm^3	PCV (%)	MCV (μm^3)
1	5.26	28	53
2	4.67	26	56
3	6.11	32	52
4	3.46	22	64
5	4.80	24	50
6	4.20	25	60
7	3.96	24	61
8	4.42	25	57
Mean ± S.E.M.	4.61 ± 0.29	26 ± 1	57 ± 2

TABLE 11

Mean cell volume (μm^3) of rats fed on control diet (10 MDpCal%)

Number of rat	Erythrocytes million/mm^3	PCV (%)	MCV (μm^3)
1	7.20	42	58
2	6.65	41	62
3	8.32	43	52
4	9.02	45	50
5	7.14	44	62
6	7.78	44	57
7	7.14	43	60
8	6.92	42	61
Mean ± S.E.M.	7.52 ± 0.28	43 ± 1	58 ± 2

TABLE 12

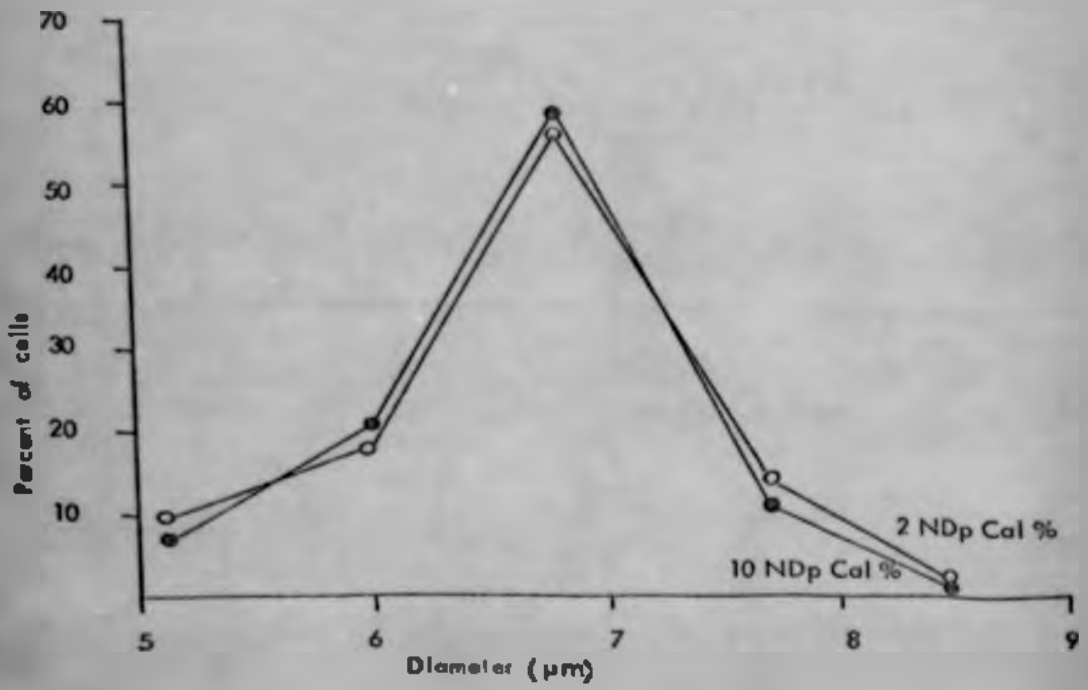
Mean size of red blood cells (volume, diameter, thickness and surface area) of rats fed on low protein diet (2 NDpCal%) and on control diet (10 NDpCal %)

Diet in NDpCal%	Number of rats	MCV (μm^3)	MCD (μm)	MCT (calculated) (μm)	Mean surface area (calculated) (μm^2)
2	8	57 ± 2	6.65 ± 0.12	1.63 ± 0.05	84 ± 2
10	8	58 ± 2	6.63 ± 0.11	1.67 ± 0.05	83 ± 2
p values		p > 0.5	p > 0.8	p > 0.5	p > 0.5

(Mean ± S.E.M.)

FIGURE 4

Mean diameter of red cells in the blood of rats fed on control diet (10 NDp Cal %) and low protein diet (2 NDp Cal %)



($p > 0.8$) between the two groups of rats. Although PCV and erythrocyte count differed appreciably between the protein deficient (2 NDpCal% diet) and control rats (10 NDpCal% diet), these two changes were proportional and did not reflect any significant difference ($p > 0.5$) in red cell volume. As the estimated values of mean cell thickness and surface area were also both very similar in the two groups ($p > 0.5$), it was found that protein deficiency had no observable effect on any aspect of red blood cell size.

3. MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

The MCHC was similar in the rats on all the different diets throughout the experimental period, as shown in Table 1 to 7.

4. RETICULOCYTE COUNT

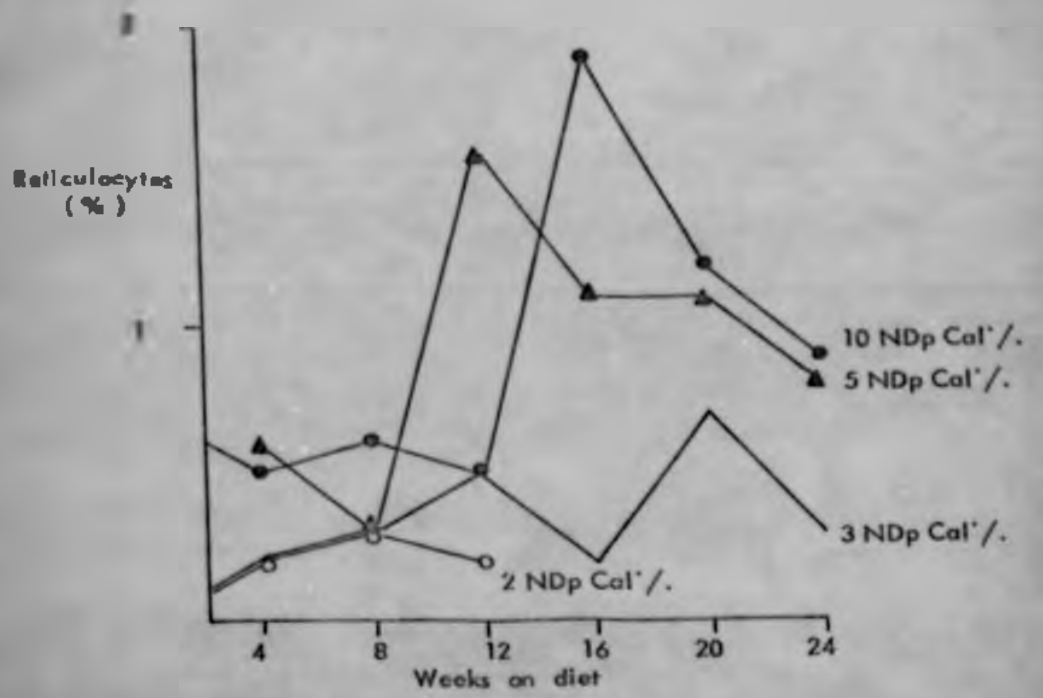
The reticulocyte count of the rats fed on diets of various low values of protein content and on control diet are shown in Table 1 to 7 and Figure 5. The reticulocyte counts for the rats on the low protein diets were generally similar to or below those for the control group throughout the experimental period. There was some indication that the reticulocyte counts of the rats on the 2 NDpCal% and 3 NDpCal% diets might be lower than those of the control group, but the differences were not significant ($p > 0.1$).

5. BONE MARROW

The myeloid : erythroid ratios and normoblast counts

FIGURE 5

Reticulocyte counts of rats fed on diets containing different amounts of protein.



for the bone marrow of rats fed on the various diets, for various durations, are shown in Table 13 and Figure 6.

At a dietary duration of 2 weeks, the normoblast count of the rats on 2 NDpCal% diet was significantly below ($p < 0.05$) that of the control group (on 10 NDpCal%) and the myeloid : erythroid ratio was significantly above ($p < 0.002$) its control value. But at week 4, and throughout the later stages of protein deficiency, this pattern was reversed. The normoblast count of the rats on 2 NDpCal% diet was significantly above ($p < 0.001$) the control level at each of weeks 4, 8 and 12. The difference from the control level was greatest at week 8 and there was a tendency for the normoblast count to recover towards the control by week 12, although this recovery was not statistically significant ($p > 0.05$). The myeloid : erythroid ratio of the rats on 2 NDpCal% diet was lower than that of the control group in the later stages but the difference was only significant at week 8 ($p < 0.002$).

Both normoblast counts and myeloid : erythroid ratios in the other protein deficient groups (3 and 5 NDpCal% diets) exhibited patterns similar to those observed for the 2 NDpCal% diet, although the differences from the control group were smaller and generally of lower significance statistically. The greatest differences in normoblast count from control level were also found to be at week 8 both on the 3 NDpCal% diet (difference

TABLE 13

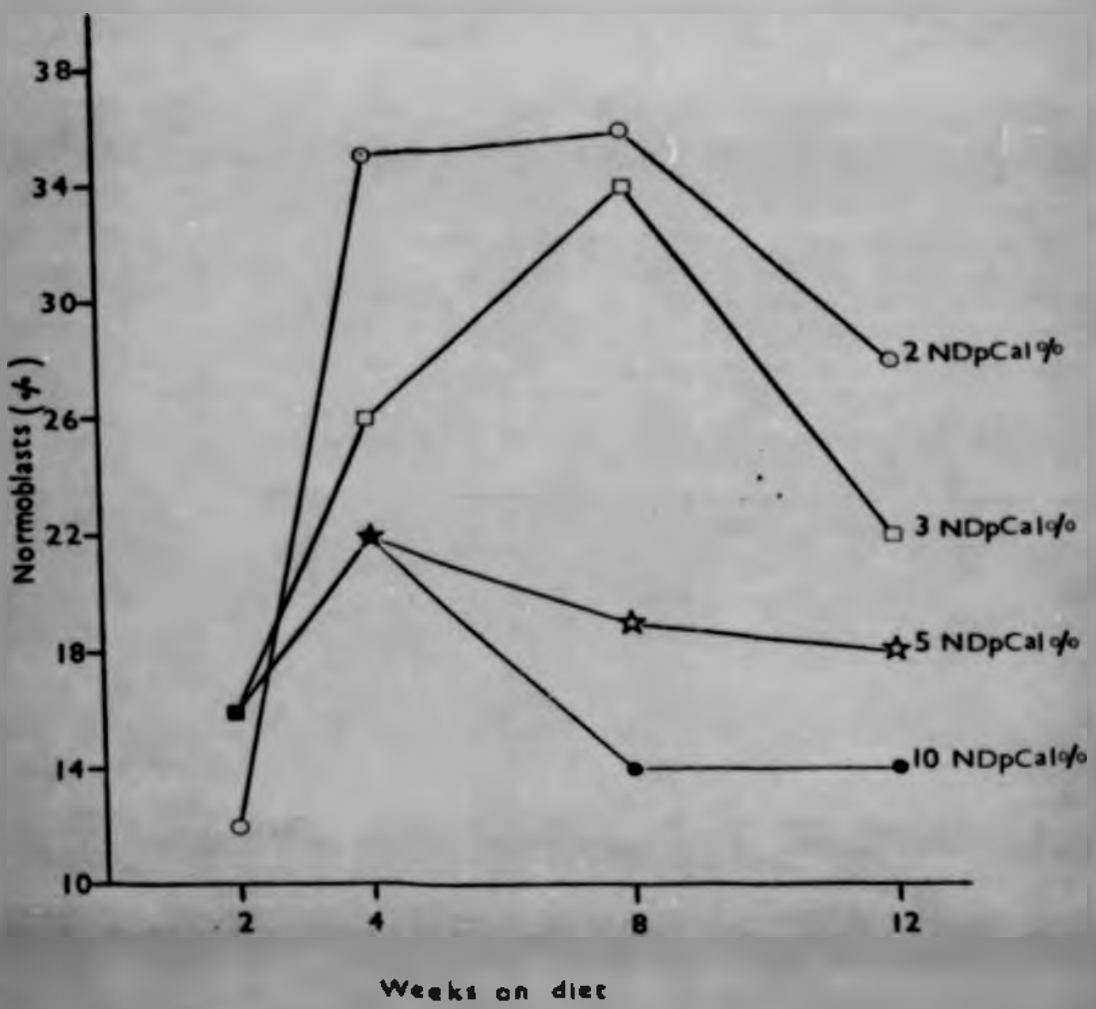
The myeloid : erythroid ratios and normoblast counts of the rats fed on various protein diets for various durations

Duration (weeks)	Diets in NDpCal %	M : E ratio (Mean ± S.E.M.)	Normoblast count (Mean ± S.E.M.)
2	2 (6)	4.0 ± 0.4	12 ± 1
	3 (5)	3.6 ± 0.7	16 ± 3
	10 (8)	2.3 ± 0.2	16 ± 1
4	2 (7)	1.3 ± 0.2	35 ± 4
	3 (5)	1.9 ± 0.2	26 ± 3
	5 (7)	1.9 ± 0.3	22 ± 2
	10 (11)	1.5 ± 0.1	22 ± 2
8	2 (7)	1.1 ± 0.2	36 ± 3
	3 (11)	1.3 ± 0.1	34 ± 3
	5 (7)	2.6 ± 0.5	19 ± 3
	10 (13)	3.5 ± 0.4	14 ± 1
12	2 (9)	1.9 ± 0.2	28 ± 3
	3 (8)	2.1 ± 0.2	22 ± 2
	5 (8)	3.0 ± 0.5	18 ± 3
	10 (12)	3.1 ± 0.5	14 ± 1

( ) = Number of rats

FIGURE 6

Normoblast counts of rats fed on diet containing different amounts of protein



significant, $p < 0.001$) and on the 5 NDpCal% diet. Significant recovery ($p < 0.01$) of the normoblast count was observed by week 12 on the 3 NDpCal% diet, but this recovery was not complete.

The increase in normoblast count, compared with the control level, was greatest for the 2 NDpCal% diet and least for the 5 NDpCal% diet at each of weeks 4, 8 and 12, and there was evidence that the increment in normoblast count on each of the low protein diets correlated well with the deficit in protein content of the diet ($r = -0.38$, $p < 0.05$; $r = -0.76$, $p < 0.001$; $r = -0.60$, $p < 0.001$ at weeks 4, 8 and 12, respectively).

In summary, the initial effect of protein deficiency, after two weeks on the diet, appeared to be hypoplasia of the bone marrow as indicated by a normoblast count below the control level. During the later stages, however, protein deficiency resulted in hyperplasia, to a degree dependent on the inadequacy of protein supply in the diet. The extent of this hyperplasia of the bone marrow was greatest at week 8. Figures 7 and 8 are photomicrographs of bone marrow smears from rats on control and 2 NDpCal% diets, respectively, showing the increased number of erythroid elements in protein deficient conditions at this stage.

FIGURE 7

Photomicrograph of bone marrow smear of rat fed on diet 10 HDpCa1% at week 8.
(x 1,750)

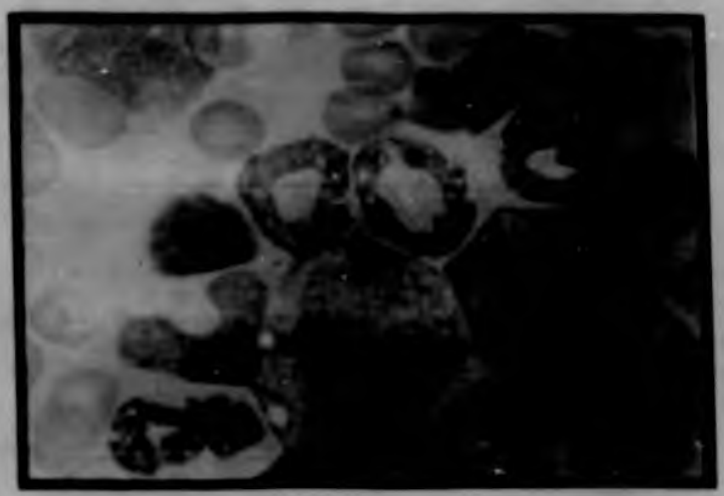


FIGURE 7

Photomicrograph of bone marrow smear of rat fed on diet 10 NDpCal% at week 8.
(x 1,750)

FIGURE 8

Photomicrograph of bone marrow smear of rat fed on 2 NDpCal% diet at week 8.
(x1,750)

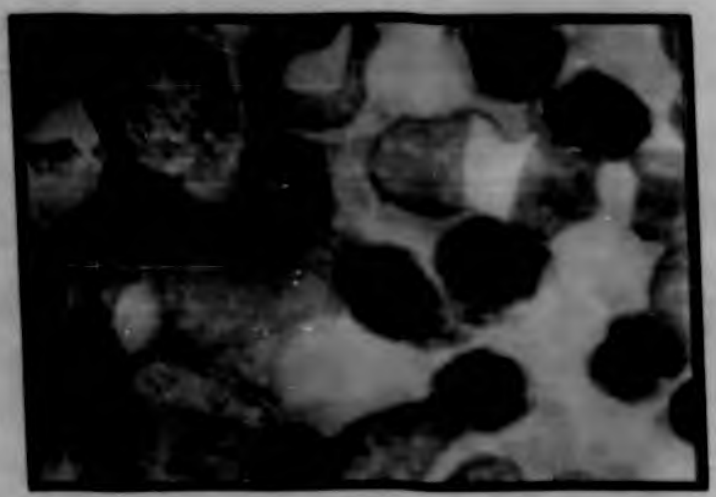
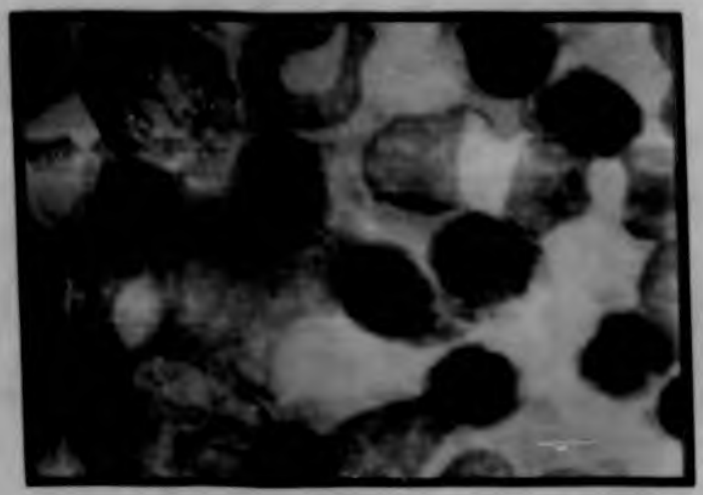


FIGURE 8

Photomicrograph of bone marrow smear of rat fed on 2 NDpCa% diet at week 8.
(x1,750)



DISCUSSION

Many authors have used animals as experimental models to investigate the cause of the anaemia produced by protein deficiency, and there have been many reports suggesting that the bone marrow tended to be functionally impaired. Ghitis et al. (1963b), from experiments on monkeys, found that normoblast and reticulocyte counts decreased when the animals were fed on a protein-free diet, but that on refeeding there was an initial erythroid hyperplasia followed by a return to normal normoblast counts. From this, they concluded that the basic mechanism producing the anaemia of protein deficiency was an atrophy of the erythropoietic tissue. Ito et al. (1964) found that the effect of protein deprivation on bone marrow of rats, after 10 days, was to make this nearly void of erythroid elements, although the few remaining cells were of normal appearance, and also observed that the reticulocyte count was low. These results indicated that the bone marrow tended to be of hypoplastic type. Experiments with dogs have similarly suggested that the main effect of protein malnutrition in relation to the anaemia was an impairment of marrow activity (Woodruff et al., 1970). The latter investigators commented that this impairment might result directly from decreased protein metabolism or might be due to a deficiency or abnormality of enzymes, all of which

are proteins. Alternatively, it is possible that erythropoietin is depressed in protein malnutrition resulting in a retardation of protein synthesis in erythroid precursors. At present, however, there is only indirect evidence of the behaviour of erythropoietin in protein deficiency. Reissmann (1964a; 1964b) found that there was a decrease in the red cell mass of rats subjected to protein deprivation for 28 days, but that this effect could be prevented by daily injection with 1.3 units of erythropoietin. Thus he concluded that either diminished erythropoietin formation or a retardation of protein synthesis in erythroid precursors, due to lowered substrate concentration, could be considered as possible causes of erythropoietic depression.

The findings of the present experiments differed considerably in regard to bone marrow condition from those of Ghitis et al. (1963b) and Ito et al. (1964). Hypoplasia of the bone marrow was observed only in the early stages of protein deficiency, in this study, and this result was therefore consistent with that of Ito et al. (1964) and with those of the other workers. This early hypoplasia could reflect directly a reduction in protein substrate in the bone marrow, or alternatively could result from a reduced level of erythropoietin, as hypothesised by Reissmann (1964b). The hyperplasia of the bone marrow, however, found in the later stages of protein deficiency and appearing to be most severe at week 8, has not been

observed by these other authors. Possible reasons for this discrepancy include differences in the duration of low protein diet, in dietary protein content and in the age of the animals used in the investigations. Many authors have in fact given a protein-free diet for a short period to induce anaemia (Ghitis et al., 1963b; Ito et al., 1964; Reissmann, 1964a; 1964b), whereas the present study involved maintenance on low protein diets for a longer duration. The present experiments themselves clearly indicated that the effect on the bone marrow was dependent on the duration of diet. Moreover, an extreme shortage in protein intake must, at some stage, restrict the metabolism of bone marrow as well as that of other organs, although with a limited protein intake it is possible that the control mechanism is able, through erythropoietin, to stimulate the bone marrow sufficiently to more than compensate for the limitations in protein supply: thus a protein-free diet could induce hypoplasia but a low protein diet hyperplasia of the bone marrow. Another difference in the present investigation was that weaned, hooded rats were used and they were very much younger and smaller than the rats used by Ito et al. (1964) and Reissmann (1964a; 1964b), than the monkeys studied by Ghitis et al. (1963b) and than the dogs of Woodruff et al. (1970). Many of the young rats in this study were unable to survive the most severe effects of protein

deficiency at week 8 on the 2 NDpCal% diet and the mortality at this time was high (see Chapter 1, Section 4, of this Part).

Comparison of results of investigations into the effects of protein malnutrition in man with those performed in experimental animals is made very difficult by the presence of many other factors in human subjects experiencing protein malnutrition, since this malnutrition is so often accompanied by mineral or vitamin deficiency or by infection, or by a combination of these factors (Scrimshaw et al., 1955; Woodruff, 1955; 1961; 1969; Trowell and Simpkiss, 1957; Walt et al., 1957; Lubby et al., 1960). These other factors can generally be eliminated in the strictly controlled conditions appropriate to animal studies. Nevertheless, the results of some human studies have some similarity with the findings of the present experiments.

Woodruff (1955) carried out research into protein deficiency on three groups of patients : (a) pregnant women, (b) those in early childhood, and, (c) older children and adults; and found that the following were characteristic of all groups: (1) The anaemia was orthochromatic and normocytic when judged by MCHC and MCV. (2) The red cells were much thinner and macrocytic when judged by MCT and MCD. (3) Microscopic examination of bone marrow films showed that erythropoiesis was active

and that the cells were larger than normal, although not so large as those of the megaloblastic series. (4) Nearly all the patients had hepatosplenomegaly and various parasites. (5) The response to a balanced diet of rich protein was good in the first two groups (pregnant women and early childhood), but the condition of the third group appeared to be more chronic. Pereira and Baker (1966) found that 61% (58 of 95) of a group of kwashiorkor patients had normoblastic bone marrow while the remainder had megaloblastic bone marrow. Twenty four of those with megaloblastic bone marrow had low levels of serum folate and two had a low level of serum vitamin B_{12}, and these factors may have been responsible for the bone marrow condition rather than the protein deficiency per se. The remaining eleven had apparently normal levels of serum B_{12} and folate, but the authors suggested that apparently normal folate levels may have resulted from faulty assay. In contrast, Ghitis et al. (1963a) consistently found erythroid hypoplasia in children with malnutrition (kwashiorkor and marasmus). With subsequent protein feeding there was an increased production of normoblasts, and in most cases erythroid hyperplasia occurred. They postulated that the anaemia of kwashiorkor is primarily due to the protein deficiency resulting in a decreased production of red cell precursors.

The most interesting question posed by the present

results is in relation to the cause of the bone marrow activity. The most logical answer is that the hyperplasia reflected an increase in the stimulating factor, which was most likely to be erythropoietin since only the erythroid series was found to increase in activity. Such an explanation, however, would appear to conflict with some of the other observations in the present study, such as the persistence of the anaemia. Some further information concerning the hormone erythropoietin is thus necessary to understand the complete mechanism. For this reason further experiments were planned to measure directly, if possible, effects of protein deficiency on the level of erythropoietin in plasma and these experiments are discussed in Part 5. Also, since the hyperplasia of the bone marrow was observed to be unable to prevent anaemia arising, a study was made of the quality of the erythrocytes in the circulation, with reference to their rate of haemolysis, and this aspect is discussed in Part 4. It is hoped that these investigations, combined with those already performed, may shed further light on the roles of erythropoietin and haemolysis in the anaemia of protein deficiency.

CHAPTER 3 EFFECTS OF PROTEIN DEFICIENT DIETS ON SERUM PROTEINS

EFFECTS OF PROTEIN DEFICIENT DIETS ON SERUM PROTEINS

The intention of the experiments reported in this chapter was to investigate whether some important serum protein fractions, specifically albumin, globulin and transferrin, were affected when rats were fed with a protein deficient diet, and to examine the time course of any changes that occurred. One particular aim was to assess the time at which the serum protein concentrations fell to their minimum values. It was also of interest to study whether the changes in serum proteins could be correlated with the degree of anaemia (see Chapter 2) and with the clinical characteristics (see Chapter 1) of the rats on the protein deficient diets.

Groups of weaned rats were fed with various low protein value diets, namely 2, 3 and 5 NDpCal%, and with 10 NDpCal% control diet. Some animals from each group were killed by anaesthesia (ether) at weeks 2, 4, 8, 12, 16, 20 and 24. Blood was taken from the heart, allowed to clot, then separated and the serum was stored at -20°C until analyses could be performed. Total serum protein, serum albumin and serum iron were measured by micromethods, while serum transferrin was assessed by an immunodiffusion method (see Part 2).

The results for total serum protein, albumin and globulin (Section 1) will be assessed and discussed

separately from those for serum transferrin and serum iron (Section 2).

SECTION 1. Total Serum Protein, Serum Albumin and Serum Globulin Concentrations

Many reports have indicated that hypoproteinaemia, particularly hypoalbuminaemia, is consistently associated with kwashiorkor. Gamma globulin concentration, on the other hand, is usually normal, or sometimes increased, in association with this nutritional syndrome (Woodruff, 1955; Trowell, 1960; Waterlow et al., 1960). There is still a lack of unanimity in the assessments of changes in the individual protein fractions, especially in those of α, β, and γ globulin. These inconsistencies may be related to the effects of infections which are frequently found in protein deficiency in children, or due to the choice of unsuitable control groups for comparison with the patients, owing to differences in race or age, for instance (reviewed by Edozien, 1960). These problems should be eliminated by the use of rats to demonstrate the changes associated with protein-energy malnutrition.

RESULTS

1. TOTAL SERUM PROTEIN

The total serum protein concentrations in the rats fed on diets of various protein contents and for various durations are shown in Table 1 and Figure 1. There appeared, in general, to be a reduction in the total

TABLE 1

Total serum protein concentrations (g/100 ml) of rats fed on diets providing different protein values, at various durations of diet.

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
2	5.43***	5.30***	-	7.14
	0.09	0.22	-	0.17
	(6)	(5)	-	(9)
4	4.90***	4.87***	6.37***	7.10
	0.13	0.17	0.08	0.12
	(7)	(9)	(7)	(11)
8	4.74***	5.32***	5.64***	7.21
	0.30	0.17	0.28	0.07
	(8)	(10)	(7)	(11)
12	5.48***	5.64***	6.86**	7.44
	0.20	0.23	0.18	0.09
	(9)	(9)	(8)	(13)
16	-	6.11***	7.44	7.75
	-	0.12	0.14	0.08
	-	(10)	(7)	(12)

TABLE 1 (continued)

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
20	-	6.28***	7.11	7.44
	-	0.15	0.15	0.07
	-	(12)	(8)	(11)
24	-	6.40***	7.39	7.39
	-	0.23	0.13	0.06
	-	(7)	(7)	(7)

Significance of differences from control diet indicated by

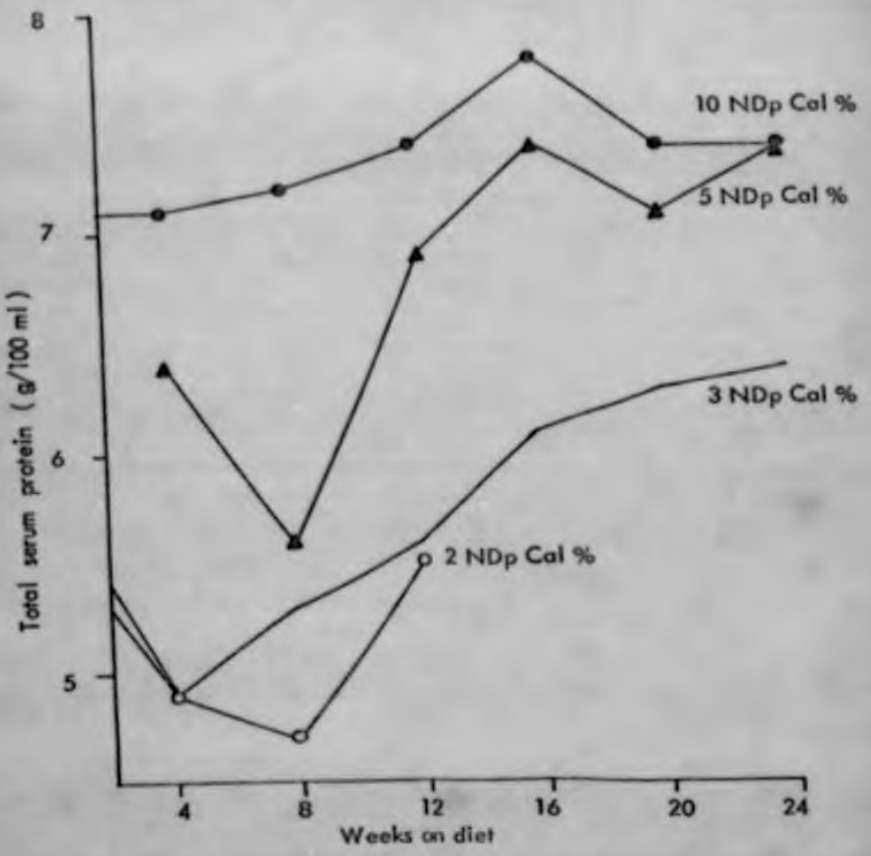
* for $p < 0.05$

** for $p < 0.01$

*** for $p < 0.001$

FIGURE I

Total serum protein levels of rats fed on diet of different protein concentrations.



serum protein level on each of the low protein diets, and this reduction was greatest at 4 or 8 weeks on each diet.

RATS FED ON 2 NDpCal% DIET

The total serum protein level of the rats fed on this diet was appreciably below that of the control group throughout the experiment (2 NDpCal% diet continued to 12 weeks only) and the differences were highly significant ($p < 0.001$, except at week 2). Moreover, the level on the 2 NDpCal% diet was significantly below ($p < 0.05$ at each week) that of the rats on the 5 NDpCal% diet, although differences from the 3 NDpCal% diet were not significant ($p > 0.2$). The greatest reduction in serum protein level, compared with the control diet, was observed at week 8, after which the extent of the reduction appeared to lessen, although not significantly so ($p > 0.1$) by week 12. It should be mentioned that the mortality rate of rats on the 2 NDpCal% diet was high (see Chapter 1, Section 4 of this Part) and that this in itself could lead to an apparent recovery in serum protein level at the longer durations of the diet if the animals with the lowest serum protein levels were those which tended to die.

RATS FED ON 3 NDpCal% DIET

The serum protein level was again much lower ($p < 0.001$), except at week 2) than that of the control group throughout the period of the experiment (diet continued to 24 weeks), and also below that of the 5 NDpCal% diet rats ($p < 0.01$ generally). The largest reduction in

serum protein on this diet was found at week 4, beyond which the level tended to rise towards the control level although significant recovery ($p < 0.001$) was not found until week 20. Nevertheless, complete recovery was not achieved even by week 24.

RATS FED ON 5 NDpCal% DIET

There was once again a significant reduction ($p < 0.01$) in the total serum protein level for rats on this diet for the first 12 weeks, but beyond that time the difference from the control rats was no longer significant ($p > 0.05$). The maximum effect of the diet was at week 8, and significant recovery ($p < 0.05$) from this effect was found at week 12 with near complete recovery to the control level by week 24. It thus appeared that the 5 NDpCal% diet was insufficient to maintain the normal total serum protein level during the growing period (first 12 weeks) but became just adequate to maintain a normal level in adult animals.

COMPARISON OF LOW PROTEIN DIETS

The greatest reductions in total serum protein level were found on the diet with lowest protein content (2 NDpCal%), and the recovery in later weeks was slower and less complete on the 3 NDpCal% diet than on 5 NDpCal%. The extent of the reduction in total serum protein level

on the three low protein diets correlated well with the deficit in dietary protein content at weeks 4, 8 and 12 ($r = 0.89$, $p < 0.01$; $r = 0.86$, $p < 0.01$; $r = 0.75$; $p < 0.01$; respectively), when the dietary protein deficiencies had their greatest influence.

7. SERUM ALBUMIN

The effects of dietary protein content and duration on the serum albumin level are indicated by the results in Table 2 and Figure 2. Generally, it appeared that the serum albumin level in rats fed on each low protein diet was lower than that of rats fed on control diet. The greatest reduction seemed to be at week 4 or 8 on each diet.

RATS FED ON 2 MDpCal% DIET

The serum albumin level was significantly reduced ($p < 0.001$) when compared with the group receiving control diet, and also significantly reduced ($p < 0.01$) below the level for the rats on 5 MDpCal% diet, throughout the whole experiment (12 weeks). A significant difference ($p < 0.05$) from the rats fed on 3 MDpCal% diet also was observed at week 8. The greatest reductions in serum albumin level were found at weeks 4 and 8. Later, the serum albumin level appeared to improve, although the recovery was not significant ($p > 0.1$) by week 12. The previous comment

on the three low protein diets correlated well with the deficit in dietary protein content at weeks 4, 8 and 12 ($r = 0.89$, $p < 0.01$; $r = 0.86$, $p < 0.01$; $r = 0.75$; $p < 0.01$; respectively), when the dietary protein deficiencies had their greatest influence.

2. SERUM ALBUMIN

The effects of dietary protein content and duration on the serum albumin level are indicated by the results in Table 2 and Figure 2. Generally, it appeared that the serum albumin level in rats fed on each low protein diet was lower than that of rats fed on control diet. The greatest reduction seemed to be at week 4 or 8 on each diet.

RATS FED ON 2 NDpCal% DIET

The serum albumin level was significantly reduced ($p < 0.001$) when compared with the group receiving control diet, and also significantly reduced ($p < 0.01$) below the level for the rats on 5 NDpCal% diet, throughout the whole experiment (12 weeks). A significant difference ($p < 0.05$) from the rats fed on 3 NDpCal% diet also was observed at week 8. The greatest reductions in serum albumin level were found at weeks 4 and 8. Later, the serum albumin level appeared to improve, although the recovery was not significant ($p > 0.1$) by week 12. The previous comment

TABLE 2

Serum albumin concentrations (g/100 ml) of rats fed on diets providing different protein values, at various durations of diet.

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
2	2.74***	2.51***	-	4.08
	0.12	0.16	-	0.07
	(6)	(5)	-	(9)
4	1.87***	2.06***	3.35***	4.02
	0.19	0.15	0.23	0.08
	(7)	(9)	(7)	(11)
8	1.94***	2.45***	3.19***	4.19
	0.19	0.15	0.23	0.08
	(8)	(10)	(7)	(11)
12	2.38***	2.82***	3.69**	4.17
	0.20	0.17	0.16	0.06
	(9)	(9)	(8)	(13)
16	-	3.29***	4.25	4.47
	-	0.11	0.11	0.07
	-	(10)	(7)	(12)

TABLE 2 (continued)

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
20	-	3.25###***	3.89	4.18
	-	0.07	0.11	0.07
	-	(12)	(8)	(11)
24	-	3.13###	4.18	4.25
	-	0.17	0.12	0.04
	-	(7)	(7)	(7)

Significance of differences from control diet indicated by

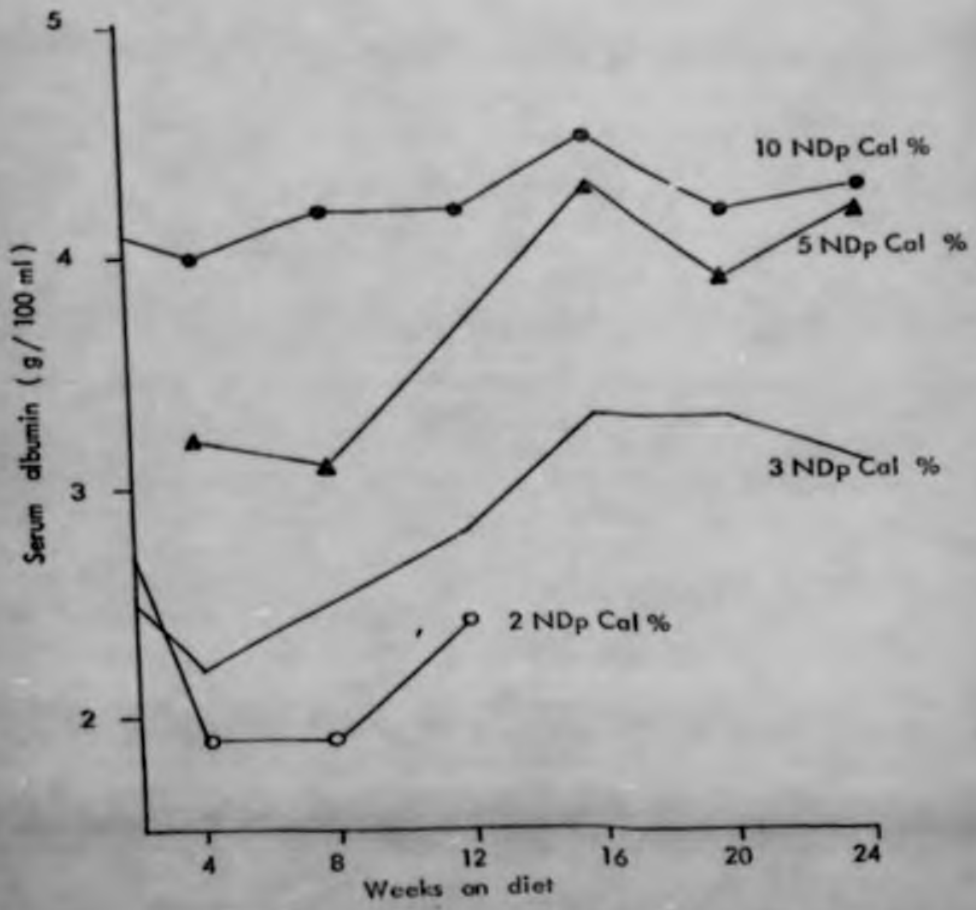
* for $p < 0.05$

** for $p < 0.01$

*** for $p < 0.001$

FIGURE 2

Serum albumin levels of rats fed on different protein concentrations.



on the possible effect of mortality on the apparent recovery (see page 184) is again relevant.

RATS FED ON 3 NDpCal% DIET

For this group, the serum albumin was significantly reduced ($p < 0.001$) when compared with that of rats fed on control diet, and also significantly reduced ($p < 0.02$) in comparison with the level for the rats fed on 5 NDpCal% diet, throughout the experimental period (24 weeks). The limited protein intake on this diet had its maximum effect in lowering the serum albumin level at week 4, beyond this period the level tended to rise and a significant recovery ($p < 0.05$) was observed from week 12 onwards. Complete recovery to the normal level was not attained, even by week 24.

RATS FED ON 5 NDpCal% DIET

There was a significant reduction ($p < 0.01$) in serum albumin level on the 5 NDpCal% diet, compared with the control diet, for the first 12 weeks, but during the remaining 12 weeks the difference was no longer significant ($p > 0.05$). The maximum effect of the diet was noticed at week 8 and significant recovery ($p < 0.05$) was seen during weeks 16 to 24. This pattern indicated that the 5 NDpCal% diet was not sufficient to maintain the normal serum albumin level during the growing period (approximately

the first 8 weeks) but become just adequate to maintain a nearly normal level when the rats came of age.

COMPARISON OF LOW PROTEIN DIETS

The serum albumin level was found to be reduced to the greatest extent in the rats on the diet with the lowest protein content (2 NDpCal%). Moreover, the recovery was slower and less complete on the 3 NDpCal% diet than on 5 NDpCal% diet. The reduction in serum albumin level on the three low protein diets correlated well with the deficiency of protein concentration in the diet at weeks 4, 8 and 12 ($r = 0.89$, $p < 0.01$; $r = 0.88$, $p < 0.01$; $r = 0.81$, $p < 0.01$; respectively).

3. SERUM GLOBULIN

Table 3 and Figure 3 display the serum globulin levels of rats fed on diets of the various protein values and for various durations.

In general, the serum globulin levels of the rats fed on the low protein diets appeared to be low compared with the control values. There was little clear evidence of correlation between the protein content of the diet and the serum globulin level (correlation coefficients insignificant except at week 12 when $r = 0.37$, $p < 0.05$). Although this does not eliminate the possibility that the change in serum globulin on a low protein diet may be

dependent on the dietary protein content, the differences between the three low protein diets appeared to show no systemic pattern and these diets (2, 3 and 5 NDpCal%) were thus grouped together.

The greatest reduction in serum globulin level for the grouped low protein diets, compared with the control diet, was found at week 8 (control level = 3.03 ± 0.06 g/100ml, low protein diets grouped together = 2.73 ± 0.09). At this time and at week 12, the serum globulin level of the rats on the low protein diets (grouped) was significantly below ($p < 0.05$) the control level. By week 20 or 24 the serum globulin level on the low protein diets appeared to have recovered to near the control level.

TABLE 3

Serum globulin concentrations (g/100 ml) of rats fed on diets providing different protein values, at various durations of diet.

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
2	2.69 0.04 (6)	2.59 0.15 (6)	- - -	3.06 0.15 (9)
4	3.03 0.11 (7)	2.72 0.13 (7)	3.01 0.11 (7)	3.09 0.09 (11)
8	2.80 0.14 (8)	2.88 0.09 (10)	2.46 0.23 (7)	3.03 0.06 (11)
12	3.10 0.04 (9)	2.81 0.13 (9)	3.17 0.17 (8)	3.27 0.05 (13)
16	- - -	2.82 0.12 (10)	3.19 0.09 (7)	3.28 0.07 (12)

TABLE 3 (continued)

([illegible] values ± S.E.M.)

Time on diets (weeks)	Diet in ND$_2$Calf			
	2	3	5	10
20	-	3.02	3.21	3.26
	-	0.14	0.08	0.05
	-	(12)	(8)	(11)
24	-	3.27	3.21	3.14
	-	0.09	0.04	0.03
	-	(7)	(7)	(7)

Significance of differences from control diet indicated by

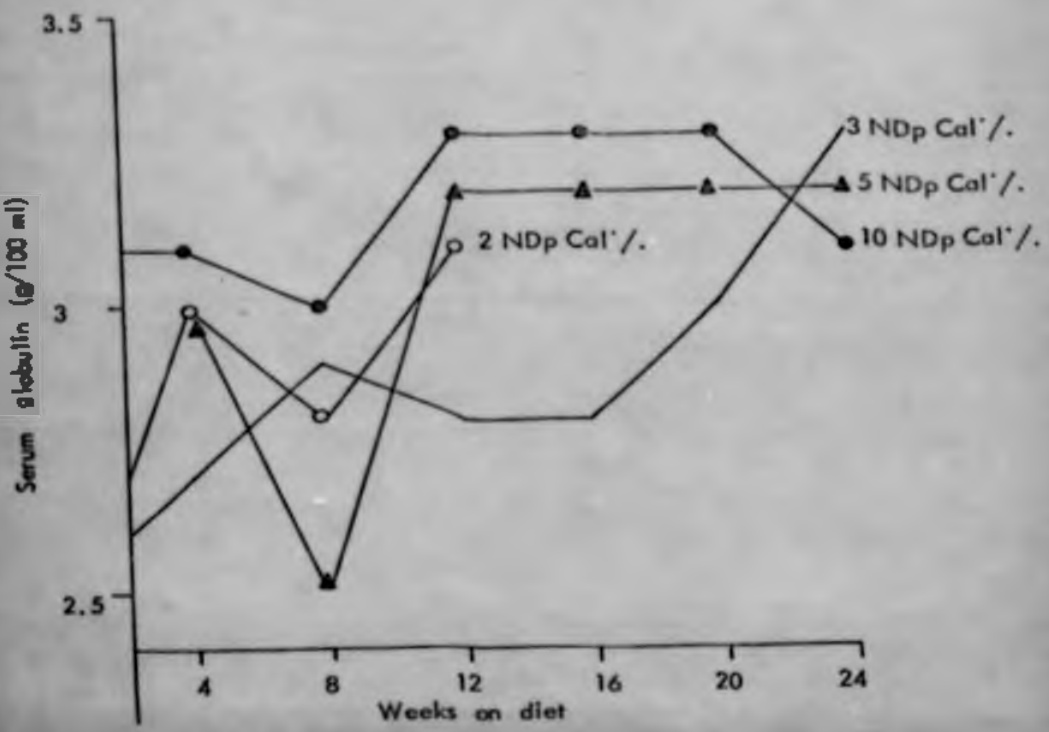
* for $p < 0.05$

** for $p < 0.01$

*** for $p < 0.001$

FIGURE 3

Serum globulin levels of rats fed on diets of different protein concentrations.



DISCUSSION

It was observed in the present study that the total serum protein concentration was reduced in rats maintained on a low protein diet. This reduction was attributable to a great extent to depletion of albumin and to a much lesser extent to depletion of globulin. At week 8, when the severest reductions in each serum protein were found, the serum albumin level of the rats on 2 NDpCal% diet had fallen 54 ± 5% below the control level (for rats on 10 NDpCal% diet), that for the 3 NDpCal% diet had fallen 42 ± 4% and that for the 5 NDpCal% diet had fallen 24 ± 6%. In contrast, at the same stage of protein deficiency, the serum globulin level for the low protein diets (grouped together as indicated on page 192)had fallen only 10 ± 3% below the corresponding control level. For the 2 NDpCal% diet, the overall reduction at week 8 in total serum protein concentration by 2.47 ± 0.31 g/100 ml from the control level was accounted for by a drop of 2.25 ± 0.21 g/100 ml in serum albumin and a fall of only 0.23 ± 0.15 g/100 ml in serum globulin. At other durations of diet a similar pattern was found with the fall in serum albumin representing the major proportion of the drop in total serum protein and the reduction in serum globulin being relatively minor.

Similar changes have been found in protein deficiency in man. Trowell et al. (1954) found that a low serum protein

concentration was closely associated with kwashiorkor, an observation which has since been frequently confirmed, as also has the fact that serum albumin in particular is reduced (Gitlin et al., 1958; Masawe and Kwabwego-Atenyi, 1973). The hypoalbuminaemia is probably not the result of an inability of the liver to synthesise albumin but appears instead to be due to a lack of sufficient quantities of the necessary substrates at the site of synthesis. Gitlin et al. (1958) studied some aspects of albumin metabolism in kwashiorkor by using ^{131}I-labelled albumin as a tracer. During the period of hypoproteinaemia, the half-life for catabolism and excretion of albumin was found to be the same as that measured after serum albumin had risen to a normal level during recovery, and it was thus concluded that hypoalbuminaemia was due to a decrease in synthesis and was not related to an increased loss or destruction of albumin. This result was later confirmed by Cohen and Hansen (1962), who studied the metabolism of albumin and of gamma globulin in kwashiorkor. These workers found that there was a reduction of about 50% in total body albumin in kwashiorkor, with the reduction proportionally greater in the extravascular than in the intravascular pool. In protein-depleted children the average rate of albumin synthesis was only one-third of that found after recovery, but albumin synthesis appeared to increase promptly in response to an adequate protein

intake. The fractional rate of breakdown of albumin was low during protein depletion and increased during recovery, so there was no evidence that hypercatabolism contributed to the hypoalbuminaemia of kwashiorkor. Many other workers have confirmed that serum albumin concentration rises very promptly when kwashiorkor patients are given an adequate supply of protein (Anderson and Altman, 1951; Dean and Schwartz, 1953; Carr and Gelfand, 1957; Senecal, 1958; Cohen and Hansen, 1962), indicating that the capacity of the liver for albumin synthesis has not been impaired.

With the exception of the γ-globulin fraction, the liver is the main site of synthesis of the serum proteins. Since hepatic disease usually results in a reduction in serum albumin concentration but an increase in concentration of γ-globulin with, therefore, a net lowering of the albumin/globulin (A/G) ratio, attention has naturally been focussed on the liver as the most likely cause of the marked depression of the A/G ratio regularly observed in kwashiorkor. Hyperbilirubinaemia is not frequently found in kwashiorkor (Waterlow, 1948; Kinnear and Pretorius, 1956), however, and liver function tests, including the sensitive bromsulphthalein retention test, have provided evidence of a severe limitation of hepatic function only in less than 30% of cases (Waterlow, 1948). Consequently, liver damage does not satisfactorily account for the low A/G ratio so characteristic of kwashiorkor, although possibly it is

a factor in some cases.

In kwashiorkor there is a qualitative and a quantitative deficiency of amino acids (Westall et al., 1958; Edozien et al., 1960). The sulphur-containing amino acids mainly provided by animal proteins are inevitably very deficient in the diet of kwashiorkor patients, since animal protein intake, such as from milk and meat, is usually severely limited. More sulphur and sulphur-containing amino acids are found in albumin and beta globulin than in alpha-2 and gamma globulin (Edsall, 1947). Thus, in kwashiorkor, the pattern of amino acid deficiency may be of importance in determining the relative proportions of the serum proteins, with a deficiency of sulphur-containing amino acids effectively limiting the synthesis of albumin and beta globulin. Whitehead and Dean (1964a; 1964b), and Grimble and Whitehead (1969) observed that the beginning of the fall in serum albumin concentration in kwashiorkor was correlated with the appearance of a distorted serum amino acid pattern, and considered that this indicated a possible cause and effect relationship. This view has subsequently been confirmed by Kirsch et al. (1969), who perfused normal rat liver preparations with serum from well-nourished and from protein-malnourished rats. When the latter perfusate was used, albumin synthesis was markedly reduced; but, when the abnormal serum amino acid pattern was corrected by the addition of the branched-

chain amino acids, valine, isoleucine and leucine, albumin synthesis increased significantly. It was concluded that the principle cause of the lowering of the synthesis rate of albumin during protein depletion was the diminished availability of these amino acids in plasma. There is also strong evidence that tryptophan has an important role in the regulation of albumin synthesis. Rothschild et al. (1969) found a large increase in albumin synthesis rate in addition of tryptophan to the fluid perfusing the liver of fasting rabbits, while Mori et al. (1967) and Allen et al. (1969) have suggested that the regulatory role of tryptophan may be related to the low level of tryptophanyl transfer RNA normally present in the liver cells. An imbalance in serum amino acids is thus considered to be a possible cause for the severe reduction in albumin synthesis in kwashiorkor, but it is not yet clear which amino acids are effectively limiting the albumin synthesis in this condition.

The present experiments also demonstrated a reduced serum albumin level and a depression of the A/G ratio in protein deficient conditions. At week 8, the A/G ratio for the rats on control diet (10 NDpCal%) was 1.38 ± 0.04, for the 5 NDpCal% diet the ratio was slighly depressed to 1.30 ± 0.15, and the ratios for the 3 NDpCal% and 2 NDpCal% diets were markedly depressed to 0.85 ± 0.06 and 0.69 ± 0.08, respectively.

An alterative explanation of the much greater reduction in serum albumin than in serum globulin in protein deficient conditions, both in the present investigations and in general, could be that the globulin fractions may be include proteins that are physiologically less " labile " than serum albumin, which appears to a large extent to represent storage protein. It is known that plasma contains immune antibodies, transport proteins and several enzymes, these mostly being in the globulin fractions (Hughes, 1954; Wroblewski, 1959). It is thought that the globulins have more specific physiological roles than does albumin and for this reason the serum globulin levels may be regulated within closer limits than the albumin level. The synthesis of albumin might thus be more sensitive to dietary protein intake and be able to be diminished to a great extent in conditions of protein deficiency, while the synthesis of most of the globulin fractions is still maintained. Cohen and Hanson (1962) have reported that, in contrast to albumin, the distribution and turnover of gamma globulin are relatively unaffected by the state of nutrition. These authors observed that in kwashiorkor with infection the γ-globulin synthesis rate was three times higher than in uninfected children, and Woodruff (1955) found that serum γ-globulin concentration could rise appreciably above the normal range in this condition. That protein-depleted subjects are able to

produce large amounts of γ-globulin suggested that the γ-globulin forming cells make better use of the available amino acids than do other protein synthesising systems. The synthesis of other biologically important proteins may, as a result, be seriously restricted, accounting for the clinical manifestations of kwashiorkor which are often precipitated by infection. While there are many reports that the serum immunoglobulin concentrations (IgG, IgM and IgA) tend to be normal in protein-energy malnutrition (Kent et al., 1969; McFarlane et al., 1970a,b; Smythe et al., 1971). Aref et al. (1970) found, in contrast, that children over one year of age with protein-energy malnutrition presented an abnormal distribution of the individual immunoglobulins, with the IgG level tending to be high, IgM very low and IgA variable.

Of these alternative explanations for the reduced A/G ratio in protein deficiency, the latter view that serum globulins are generally less " labile " and more strictly regulated than albumin appears to be the more tenable since a reduced A/G ratio was observed in the present investigations when the dietary protein was of animal origin, as casein, as well as in kwashiorkor patients when animal protein intake is usually very limited (Edozien, 1960). The relative proportions of the amino acids in the diet in these two cases presumably differed considerably, so the finding of similar reductions in the A/G ratio would

be unlikely if albumin synthesis were controlled only by the serum amino acid distribution.

The extent of the reductions in total serum protein and serum albumin varied appreciably with duration of diet. The reductions in serum globulin appeared to follow a similar pattern to those in total protein and albumin but the changes were only small and comparisons with duration of diet are thus of very doubtful statistical significance. Total serum protein and albumin were both considerably reduced below the control levels after only two weeks on the low protein diets (Figure 1 and 2) and the extent of these reductions became greater at weeks 4 and 8 but then became smaller beyond week 8. The largest differences in total protein and in albumin between the rats on low protein diet and those on control diet were found to be at week 8 for the 2 NDpCal% and 5 NDpCal% diets and at week 4 for the 3 NDpCal% diet, with the differences between weeks 4 and 8 generally not significant. Serum globulin was also reduced by the greatest amount at week 8 for the low protein diets (grouped together). The initial period when these protein concentrations in serum were falling progressively probably represented the time necessary for protein stores in the body to become depleted and for the serum albumin to be utilised and probably did not reflect an increasing protein demand by the animals, although these two possibilities cannot be clearly separated using the results of

the present experiments. The recovery of the total serum protein and serum albumin concentration towards the control levels in the later period beyond week 8, in contrast, appeared to reflect a decreasing protein demand by the animals. Mortality may also have had some influence on the apparent recovery of protein concentrations for the rats on the 2 NDpCal% diet: the mortality rate was high between weeks 5 and 8 on this diet (see Chapter 1) and, since the rats with lowest serum protein concentrations were probably the most likely to die, the mortality could in itself have led to an observed increase in serum proteins without being indicative of real recovery in the individual animals. For the other low protein diets, however, the mortality rate was low and could not account for the observed recovery. Thus, the recovery must have been a real effect on the 3 NDpCal% and 5 NDpCal% diets and was therefore probably a real effect on the 2 NDpCal% diet also. By week 16, total serum protein and serum albumin and globulin concentrations in the rats on 5 NDpCal% diet had each recovered to near the control levels, indicating that this diet provided an almost adequate protein intake once the rats had reached adulthood, although protein intake had clearly been inadequate during the growing period. Considerable recovery in these serum protein concentrations was also seen in the rats on 3 NDpCal% diet, yet nevertheless serum albumin remained 26 ± 4 % below control level, and

total serum protein 13 ± 3 % below, even at a diet duration of 24 weeks. The protein intake of the rats on 3 NDpCal% diet was not adequate even in adulthood. Rats were not maintained on 2 NDpCal% diet for longer than 12 weeks, owing to the high mortality rate for this diet, so the extent of recovery was not observed for this diet.

The pattern of these changes in serum proteins indicated that the full protein intake provided by the 10 NDpCal% control diet was necessary for maintenance of normal serum protein levels during the growing period. All the low protein diets were inadequate during this period. By the time the rats had reached adulthood, however, their protein demand was less and near normal serum protein levels could be attained on the 5 NDpCal% diet. The 3 NDpCal% diet, and presumably the 2 NDpCal% diet, remained inadequate even for adult rats.

The changes in serum proteins clearly showed a generally similar pattern to those found for the degree of anaemia and for other clinical characteristics, such as body weight, fur loss, behaviour and mortality rate. The reductions in total serum protein and in serum albumin concentrations were greatest in the rats fed on the diet providing the lowest amount of protein (2 NDpCal%), while the anaemia was severest on this diet (see Chapter 2) and the clinical manifestations were also most apparent (see Chapter 1). Moreover, the serum protein concentrations generally showed

their greatest reductions at week 8 and this corresponded closely to the time of severest anaemia, maximum percentage weight deficit, worst fur loss and highest mortality rate. With each of these variables showing such a similar pattern, it becomes almost impossible, however, to separately assess which factor might be responsible for the degree of anaemia and for the various clinical characteristics. The changes in all factors are related to a common cause, the limited protein content of the diet, but it is difficult to distinguish changes which are directly related to the reduced protein substrate supply from those which may be directly related to some more specific factor which itself could be related to the protein substrate supply. Using the data for all four diets (2, 3, 5 and 10 NDpCal%) and for the three weeks (4, 8, 12) when measurements were made for rats on each diet, it was found that total serum protein correlated well with the degree of anaemia, as measured both by haemoglobin level ($r = 0.90$, $p < 0.001$) and by PCV ($r = 0.92$, $p < 0.001$), and with the rate of growth ($r = 0.77$, $p < 0.01$), but did not correlate significantly with the mortality rate ($r = -0.52$, $p > 0.05$). Serum albumin correlated slightly better with haemoglobin, PCV and growth rate ($p < 0.001$ in each case) but again not with mortality ($r = -0.53$, $p > 0.05$); whereas serum globulin did not correlate significantly with any of these variables ($p > 0.2$ in each case). Both serum protein and

serum albumin were thus good indicators of the prognosis for growth in protein deficient conditions, but neither would provide a reliable guide to the likelihood of mortality. Serum globulin as a whole did not appear to be a useful indicator either for growth or for mortality, but the individual globulin fractions might be more valuable in this regard.

In the present study, globulin was not separated into its different electrophoretic fractions as the band separation was not clearly defined. Assessment was made of the changes in serum transferrin (a β-globulin), however, and these are discussed in Section 2. The clinical state of a child with protein-energy malnutrition is reflected by the serum transferrin concentration and this provides one of the most accurate biochemical tests for the assessment of the disease. Reduced survival is usually correlated with decreasing transferrin concentration (Neale et al., 1967; Antia et al., 1968; McFarlane et al., 1969; 1970), and measurement of transferrin is thus a useful aid to prognosis. In addition to having bacteriostatic properties, transferrin may be closely linked with the immune systems of the body as it is synthesised by cells of the lymphoid series (Soltys and Brody, 1970) as well as by the liver.

Complement, which comprises a whole series of serum globulins mainly with beta-electrophoretic mobilities and which is also an important agent for bacterial control,

has also been reported to be frequently reduced in children with protein-energy malnutrition (Smythe et al.,1971). Additionally, it has been observed that the cell-mediated immune response is generally impaired in protein-energy malnutrition (Smythe et al. 1971). This impairment might be due to atrophy of the thymus and other lymphatic organs, probably resulting from raised plasma-glucocorticoid levels. Sbarra et al. (1971) found that, in malnutrition, phagocytosis appeared to be impaired and observed a reduction in the amounts of the two important white-cell enzymes involved in the phagocytic process, firstly myeloperoxidase (an iron-containing enzyme) and secondly nicotinamide adenine dinucleotide phosphate reduced oxidase. This effect of malnutrition on phagocytosis has yet to be confirmed by further investigation. In the malnourished subject, one of these immune mechanisms may be impaired, or a combination of more than one, with the degree of malnutrition and the presence or absence of infection determining the extent to which these immuno-deficiencies exist. The frequent occurence of defects in the immune system during protein-energy malnutrition may explain the prevalence of infection in kwashiorkor.

Another important function of the plasma proteins, particularly of plasma albumin, is to control the distribution of fluid within the body and advanced conditions of protein-energy malnutrition are frequently complicated by oedema, although it is not yet fully understood how this

abnormality is brought about. There have been many reports that the albumin level in plasma is of importance in the genesis of clinical oedema and that serum albumin concentrations below 2.5g/100 ml are usually associated with oedema (Bruckman et al., 1930; Bruckman and Peters, 1930; Peters et al., 1931; 1932; Payne and Peters, 1932), yet the role of plasma proteins in the causation of oedema remains unclear. Starling originally proposed that physicochemical alterations, caused by the low plasma protein levels, could explain the development of the oedema under protein deficient conditions, but it is now realised that his hypothesis fails to explain various established properties of the oedema of protein malnutrition. For instance, kwashiorkor children shed their oedema during treatment long before any significant increase occurs in the serum protein concentrations. Although Trowell et al. (1954) stated that hypoalbuminaemia has often been observed in severely oedematous patients, what has not been stressed is that marked hypoalbuminaemia may occur in the absence of clinical oedema. Moreover, in the present study, none of the rats with hypoalbuminaemia had oedema and there was only a small difference in body fluid between the low protein groups and the control group, a difference which appeared in fact to be related more to the size of the protein deficient rats than to the protein deficiency per se (refer to Chapter 1 of this Part). It is thus apparent

that other factors, in addition to the plasma protein concentration, must be significant in the aetiology of oedema in kwashiorkor, particularly in explaining the variability in degree and the distribution of the oedema in the body. Some of these other factors have been discussed in the section on body fluid (see Chapter 1, Section 2 of this Part).

SECTION 2. Serum Transferrin and Serum Iron Concentrations

It has been suggested that the anaemia of kwashiorkor results partly from transferrin deficiency and is secondary to the associated hypoproteinaemia (Scrimshaw and Behar, 1961; Antia et al., 1968). Confirming this, there have been reports of a preferential synthesis of transferrin over other plasma proteins on refeeding malnourished infants with a high protein diet (Adam and Scrugg, 1965).

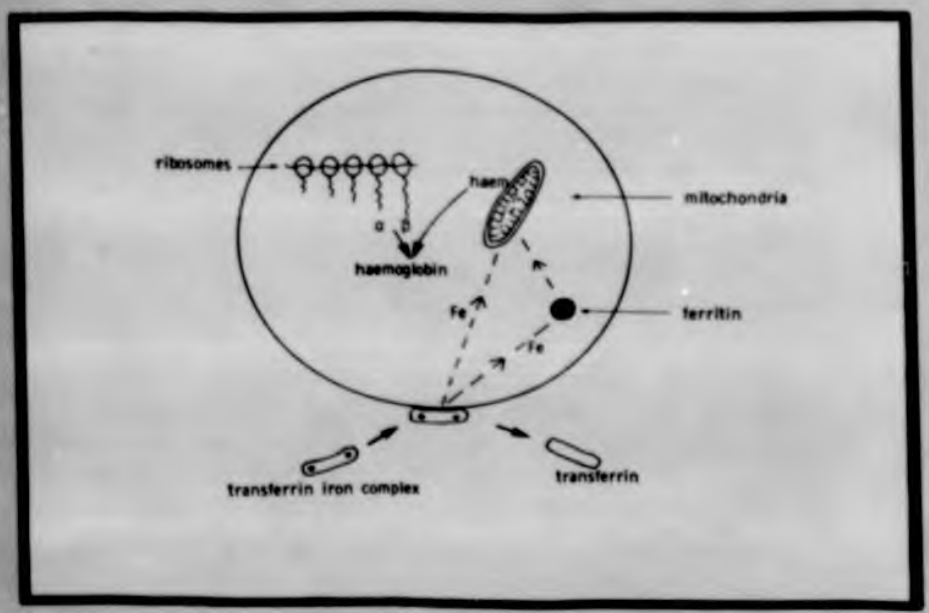
Iron is picked up by a specific transport protein in the serum once it has been absorbed across the intestinal epithelium (Hagberg, 1953; Ramsay, 1958; Tuenbull and Giblett, 1961; Jassermann et al., 1965; Bowman, 1968; Sinniah and Neill, 1968). The majority of serum iron is bound to this specific iron-transport protein, which is a β-globulin of glycoprotein or mucoprotein form (Roberts et al., 1966) and has been variously named as transferrin, siderophillin and iron-binding protein. The liver is probably the principal site of synthesis of transferrin (Gitlin and Blasucci, 1969) although other sites, for instance lymphocytes in peripheral blood, have also been recognised (Soltys and Brody, 1970). The iron-binding protein appears to be a true carrier with no enzymatic or metabolic functions per se, since it transports iron from one region to another without being taken up or used in any appreciable quantity by the receptor tissues (Laurell, 1952; Paoletti,

1957). It has been demonstrated that transferrin has other functions related to the inhibition of bacterial, viral and fungal growth, but these are probably consequences of its ability to bind iron and so inhibit growth by iron deficiency (Martin and Jandl, 1960; Martin, 1962; Esterly et al., 1967; Caroline et al., 1969). Lactoferrin is another iron-binding protein, which has been detected in trace amounts in various body secretions and in neutrophils and which may also have a role in infection resistance. Lactoferrin will reversibly bind two molecules of iron with a greater affinity than transferrin (Masson et al., 1969).

Transferrin itself is responsible for the great majority of iron transport in serum. Iron from the serum is selectively deposited in certain tissues, principally in the bone marrow for haemoglobin synthesis. Much smaller quantities are deposited in body stores, excreted or utilised for cell metabolism throughout the body. Transferrin, with bound iron, enters the bone marrow and then must become attached to specific receptor sites on the surface of developing red cells in order that the iron can be released to these red cells, in the manner shown in Figure 4. Since free transferrin binds less well to these receptor sites than does the transferrin-iron complex, it is released from the cell surface by the arrival of the next transferrin-iron molecule.

FIGURE 4

Proposed mechanisms of incorporation of iron from plasma into haemoglobin in developing cells.
(Taken from Hoffbrand, 1972)



It is generally recognised that there are difficulties in the assessment on a clinical basis alone of the degree of malnutrtion and of its prognosis in kwashiorkor, so numerous attempts have been made to find suitable biochemical tests to provide a solution to these problems (Waterlow, 1960). Evidence has been presented by McFarlane et al. (1969) that serum transferrin provides a more accurate reference for the assessment of severity and of prognosis in protein-energy malnutrition than do the methods proposed by other authors (Trowell, 1948; Waterlow, 1950; Dean and Schwartz, 1953; Kinnear, 1956; Scrimshaw et al., 1956; Whitehead, 1964).

Serum transferrin concentrations were measured in rats suffering from protein-energy malnutrition in order to assess the role of decreased transferrin levels in the aetiology of the anaemia in this condition, and to estimate the value of transferrin measurement as an indicator of severity and of prognosis. Serum iron concentrations were also measured for comparison.

MEASUREMENT OF SERUM TRANSFERRIN

Serum transferrin concentrations have usually been determined by measurement of total iron binding capacity (TIBC). Recently, however, Van de Heul et al. (1971) have stated that the TIBC method for determining transferrin may be misleading, since iron not only binds to transferrin

but also is distributed over the other serum components. It is likely, therefore, that TIBC is not identical with transferrin content, and these authors found that normal human serum transferrin concentration was 20% lower, in general, when determined immunochemically than would have been expected from the total iron -binding capacity.

The immunochemical determination of transferrin is simple, reliable and specific. The principle of the immunodiffusion method employed in this study is that transferrin (antigen) molecules can diffuse freely from the well into the agar gel, containing specific transferrin antibodies, until each comes into contact with an antibody molecule and reacts to form a virtually immobile complex. The resulting precipitin ring is made up of all such antigen-antibody complexes and has a diameter related to the concentration of the antigen assayed. The method used was a modification of the method of Mancini et al. (1965).

RESULTS

a. SERUM TRANSFERRIN

The effect observed on the serum transferrin concentration of maintaining rats on low protein diet (2 NDpCal%) and on control diet (10 NDpCal%) for various durations is presented in Table 4 and Figure 5. The standard rat transferrin and rat transferrin antiserum were, unfortunately, only available in small quantities, so this study was limited to weeks 4, 8 and 12 and to these two diets.

The mean serum transferrin concentration of the rats fed on the low protein diet (2 NDpCal%) were lower than those of the control group, but a significant reduction was observed only at weeks 4 and 8 ($p < 0.01$ in each case). The greatest effect of the low protein diet on the serum transferrin level was found at week 8. When the diet was continued to week 12 the serum transferrin rose significantly ($p < 0.05$ and $p < 0.01$, respectively) above its level at weeks 4 and 8, and recovered to a level only marginally below ($p > 0.1$) the control.

TABLE 4

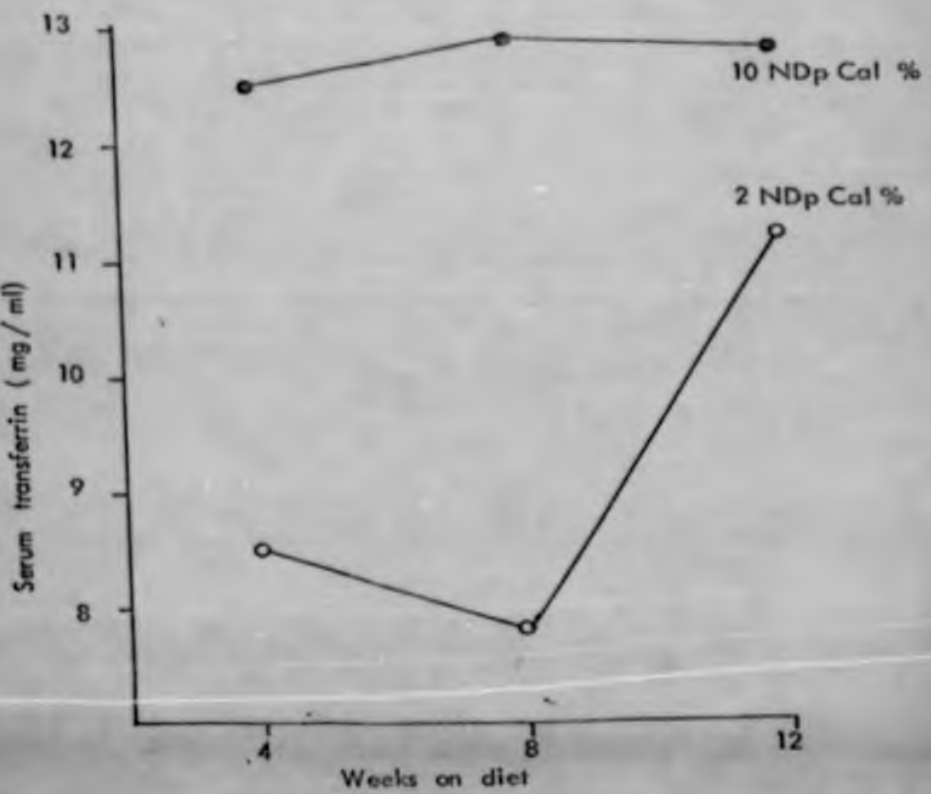
Serum transferrin concentration (mg/ml) of rats fed on low protein diet (2 NDpCal%) and on control diet (10 NDpCal%), for various durations.

Diets in NDpCal%		Duration (weeks)		
		4	8	12
2	Mean ± S.E.M. Number	8.50** 0.88 6	7.85** 0.93 6	11.19 0.58 7
10	Mean ± S.E.M. Number	12.50 0.70 6	12.90 0.75 6	12.76 0.82 7

** - Indicates values that are significantly different ($p < 0.01$) from the control value at that particular time.

FIGURE 5

Serum transferrin levels of rats fed on low protein diet (2 NDp Cal %) and control diet (10 NDp Cal %)



b. SERUM IRON

Table 5 and Figure 6 show the effect on serum iron concentration of maintaining rats on the low protein diets (2, 3 and 5 MDpCal%) and on the control diet (10 MDpCal%) for various durations.

The serum iron levels of the rats fed on the low protein diets were lower than those of control group, but the differences were only significant at weeks 4 and 8 ($p < 0.02$ at each week, for the differences between all the low protein diets together and the control group). There were no significant variations in the serum iron level of the control group throughout the experimental period (2 to 24 weeks), whereas the serum iron levels on the low protein diets were lowest at week 4 and then tended to rise towards the normal level. A significant recovery ($p < 0.05$), from the 4 week minimum, in the serum iron levels on the low protein diets (grouped together) was observed by week 12, and this recovery was maintained or further improved in the subsequent weeks. Complete recovery, however, was not apparent even at week 24. There was some indication that the extent of the reduction in serum iron level was correlated with the deficit in the protein content of the diet: this correlation was significant at week 4 and 8 (correlation coefficient $r = 0.38$, $p < 0.05$; $r = 0.40$, $p < 0.02$; respectively).

TABLE 5

Serum iron concentrations (µg/100 ml) of rats fed on low protein diets (2, 3 and 5 NDpCal%) and on control diet (10 NDpCal%), for various durations.

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCal%			
	2	3	5	10
2	208 14 (6)	212 26 (5)	– – –	221 13 (9)
4	181 10 (7)	174 12 (9)	179 16 (7)	212 14 (11)
8	193 16 (8)	187 16 (10)	203 13 (7)	228 10 (13)
12	194 15 (9)	205 10 (9)	207 13 (8)	216 11 (13)
16	– – –	219 9 (10)	212 12 (12)	228 9 (12)

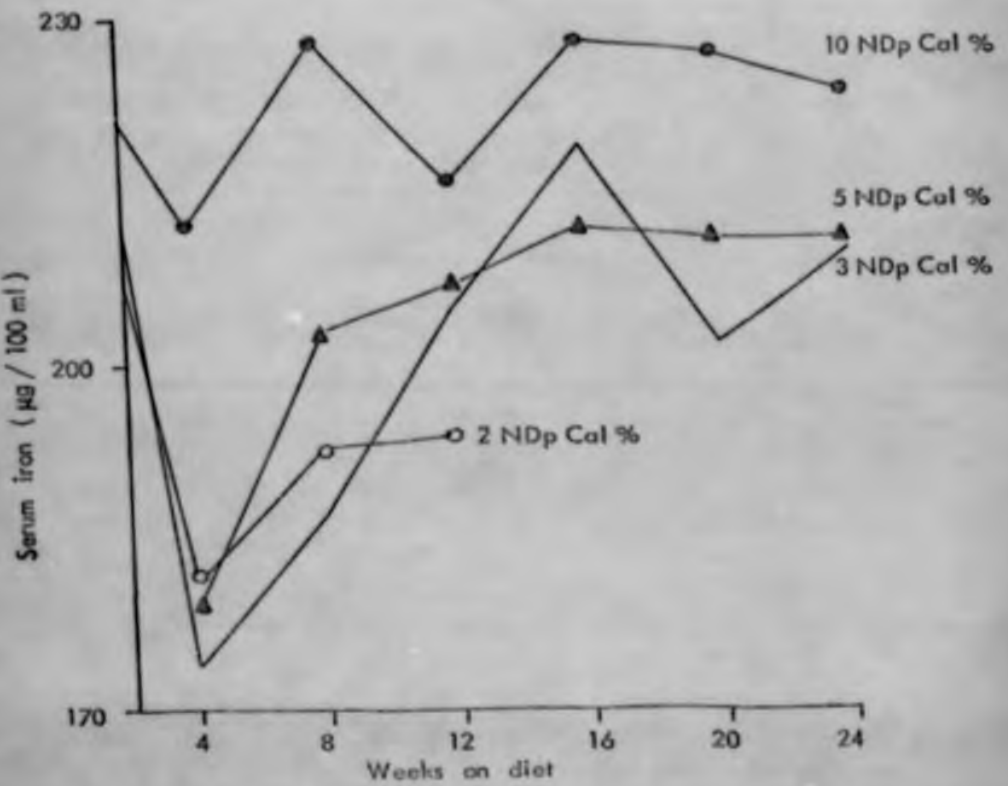
TABLE 5 (continued)

(Mean values ± S.E.M.)

Time on diets (weeks)	Diet in NDpCa%			
	2	3	5	10
20	-	202	211	227
	-	7	8	7
	-	(12)	(8)	(12)
24	-	210	211	224
	-	12	13	13
	-	(7)	(7)	(7)

FIGURE 6

Change in serum iron levels of rats fed on diets containing different amounts of protein



Discussion

Many types of anaemia associated with kwashiorkor have been reported by various research workers, one of these being the hypochromic and/or microcytic type. Serum transferrin has been reported to be diminished in kwashiorkor (Scrimshaw and Behar, 1961; Antia et al., 1968; El-Hawary et al., 1969; McFarlane et al., 1969, 1970), a result confirmed by Gabr et al. (1971), who also found that haemoglobin, serum iron and iron-binding capacity were low in all cases of kwashiorkor. For this reason, there have been suggestions that transferrin deficiency, secondary to the associated hypoproteinaemia, is partly responsible for the anaemia of kwashiorkor (Scrimshaw and Behar, 1961; Antia et al., 1968).

In the present study, the pattern shown by the reductions in serum transferrin concentration of rats fed on low protein diet (2 NDpCal%) was similar to that shown by the haematological data (Chapter 2 of this Part) and by some of the biochemical tests. The serum transferrin levels at weeks 4, 8 and 12 of the rats fed on 2 NDpCal% and on control diet correlated well with the haemoglobin levels ($r = 0.97$, $p < 0.01$) and with the packed cell volume ($r = 0.98$, $p < 0.001$). There were also, however, good correlations between the reductions in transferrin level and those in total protein ($r = 0.95$, $p < 0.01$) and

in serum albumin ($r = 0.93$, $p < 0.01$). It thus appeared that serum transferrin and serum albumin were affected by protein-energy malnutrition in a similar way, although perhaps not to the same extent since serum transferrin fell 39 ± 8 % below control at week 8 on the 2 NDpCal% diet whereas serum albumin was reduced somewhat more severely (by 54 ± 5 %). Since this parallelism was found between transferrin and albumin, it was difficult to establish which of these, if either, was the cause of the changes in haematological data. It was not possible to distinguish, by statistical analysis, between limitation of haemoglobin production by shortage of protein supplied by serum albumin and limitation by shortage of iron supplied by serum transferrin to the bone marrow.

The observation that none of the protein deficient rats exhibited the hypochromic and/or microcytic type of anaemia (refer to Chapter 2 of this Part), however, did suggest that the anaemia was not caused by a restricted iron supply due to the decreased serum transferrin level. The reductions in serum transferrin were reflected to some extent by decreases in serum iron (correlation coefficient, $r = 0.86$, $p < 0.05$), but the latter fell only 15 ± 8 % below control at week 8 on the 2 NDpCal% diet compared with a drop of 39 ± 8 % in serum transferrin at this time. The mean cell haemoglobin concentration of erythrocytes, however, appeared to be unaffected by

the reductions in serum transferrin, as there was no significant correlation between these variables ($r = -0.59$, $p > 0.1$), further implying that red cell production had not been restricted by a shortage of iron supplied to the bone marrow. Previously, Antia et al. (1968) found little correlation between the serum transferrin (siderophillin) level and the packed cell volume in kwashiorkor, and therefore considered that other factors were probably more relevant to the anaemia. This view was subsequently confirmed by Gabr et al. (1971), who found that the relationship between serum transferrin and haematological data was not so apparent as had been suggested at one time. The good correlation between serum transferrin and PCV found in the present study might have been related to the much more consistent nature of the diet, leading to a higher correlation between serum transferrin and total serum protein than that usually found in human subjects with protein-energy malnutrition. Masawe and Rwabwogo-Atenyi (1973) have recently claimed that the serum transferrin level provides the best screening test for distinguishing between anaemia due to iron deficiency and that resulting from kwashiorkor, in view of their observations that the transferrin level was uniformly raised in a group of patients with the former type of anaemia but uniformly diminished in the latter.

It is not possible to be certain from the results

of the present experiments alone what role the reductions in serum transferrin concentration had in the aetiology of the anaemia. Since no hypochromic and/or microcytic red blood cells were found and since the mean cell haemoglobin concentration was unaffected, it is considered probable that the decreases in serum transferrin played little part in the development of anaemia in the protein deficient rats.

McFarlane et al. (1969) have stated that serum transferrin levels were closely associated with the nutritional conditions of a group of children suffering from protein-energy malnutrition. A serum transferrin of less than 0.45 mg/ml appeared to be indicative of severe protein malnutrition, with values below 0.30 mg/ml implying a poor prognosis. All the children who died had had greatly depressed transferrin values when first seen, while an increase in the serum transferrin concentration during treatment invariably indicated a good prognosis. These authors concluded that, in every clinical group, the serum transferrin provided an accurate assessment of the true nutritional state and appeared to provide a reliable measure both of severity and of the response to treatment in patients with protein-energy malnutrition (McFarlane, 1969).

The greatest reduction in serum transferrin in the protein deficient rats (fed on 2 NDoCal% diet), in the

present study, was found at week 8 and thus coincided with the period of highest mortality in these rats (see Chapter 1, Section 4 of this Part). By week 12, when there were no further deaths, serum transferrin was observed to have risen close to the control level (Figure 5). The surviving animals were also seen to have improved in general appearance, in haematological values and in biochemical data by this time. Serum transferrin, therefore, appeared to give a good general indication of the severity of protein-energy malnutrition and this was confirmed by the close correlations between serum transferrin and total serum protein ($r = 0.95$, $p < 0.01$), serum albumin ($r = 0.93$, $p < 0.01$), haemoglobin ($r = 0.97$, $p < 0.01$) and PCV ($r = 0.98$, $p < 0.001$). The mortality rate also correlated with the serum transferrin concentration ($r = -0.89$, $p < 0.02$), whereas it did not correlate significantly either with total serum protein ($r = -0.75$, $p > 0.05$) or with serum albumin ($r = -0.70$, $p > 0.1$) in this group of protein deficient rats, fed on 2 MDpCal% diet, and their corresponding control animals. Thus, serum transferrin appeared to be a more reliable indicator of prognosis than total serum protein or serum albumin.

It is a more difficult problem to assess whether there is any causal relationship between the reductions in serum transferrin in protein deficiency and the elevated mortality rate. There appears to be some evidence for

such a direct effect in protein-energy malnutrition in man. Serum transferrin is responsible for the transport of iron in the body and, under normal conditions, only negligible concentrations of iron are found free in the circulation. The in vitro studies of Shade (1963; 1966) demonstrated a bacteriostatic effect of serum transferrin, in that iron-requiring pathologic bacteria, such as Staphylococcus aureous, Shigella paradysenteriae, and Pseudomonas aeruginosa, would grow more readily in sera containing an excess of free iron, resulting from over-saturation of the available transferrin, than in sera containing all the iron bound to transferrin. It is commonly found, in protein-energy malnutrition, that children at death have acute bacterial infections, and Soltys and Brody (1970) remarked that the occurrence of gram negative sepsis, in conditions where the concentration of free transferrin is diminished, implies that this iron-binding protein may be a component of an auxillary antibody-globulin system. McFarlane et al. (1970a) observed that many of the children died immediately after treatment had started, and suggested that, in children with severe kwashiorkor and low serum transferrin levels, any increase in free-circulating iron might encourage bacterial infections. Rogers (1967) has also indicated that bacterial growth would be promoted by the available free iron, resulting in extensive infection and finally

in death. For this reason it may be necessary to reconsider the appropriate time for providing iron therapy in such cases (McFarlane et al., 1970a). In addition, the haemosiderosis, which is so often found in kwashiorkor at autopsy, may be a further result of the low serum transferrin and increased free circulating iron.

The relevance of these results, of studies of protein-energy malnutrition in man, to the present animal experiments, however, is uncertain. The rats were maintained under more strictly controlled and consistent conditions and there were no signs of infection in the animals that died during the imposed protein deficiency. Nevertheless, there was a good correlation between serum transferrin and mortality rate. Partial correlation coefficients were calculated in an attempt to separate the influences of serum transferrin and of serum albumin on the mortality rate. The partial correlation coefficient between serum transferrin and mortality, excluding the influence of serum albumin, was once again significant ($r = -0.90$, $p < 0.05$); whereas that between serum albumin and mortality, excluding the influence of serum transferrin, was of no statistical significance ($r = 0.73$, $p > 0.1$) and in any case appeared to be more in the direction of positive than of negative correlation. This significant partial correlation between serum transferrin and mortality is more suggestive of a causal relationship, since the effects of one other factor have been eliminated in its calculation; but, with many other

factors possibly influencing the mortality rate in protein deficiency, the existence of a direct causal relationship must remain in doubt.

In the protein deficient rats, fed on 2 NDpCal% diet, there was an appreciable drop in serum transferrin but a relatively small drop in the total concentration of serum globulins (see Section 1 of this Chapter). It is interesting to assess the changes in the serum globulin fractions other than transferrin and the overall the remaining globulins, principally α- and γ-globulins, is simply estimated by subtraction of the serum transferrin concentration from the total globulin concentration. At week 8, the rats on control diet (10 NDpCal%) had a mean serum globulin concentration of 3.03 ± 0.06 g/100 ml while the average serum transferrin level was equivalent to 1.29 ± 0.07 g/100ml, indicating a contribution of 1.74 ± 0.09 g/100 ml from the remaining globulins. For the 2 NDpCal% diet, in contrast, the mean serum globulin concentration of the rats was 2.80 ± 0.14 g/100 ml whereas the serum transferrin was 0.79 ± 0.09 g/100 ml implying a contribution of 2.01 ± 0.17 g/100 ml from the remaining globulins. Thus, these remaining globulins tended to rise rather than to fall in the protein deficient rats, although the change was not in fact statistically significant ($p > 0.1$). Nevertheless, this result need not necessarily indicate that none of the α- and γ-globulins were reduced in

protein deficient conditions, only that the total for all these globulins was little affected. Other investigators have also found that the serum proteins most influenced by protein deficiency are albumin and the β-globulins, of which transferrin is the main component, while the α- and γ-globulins are virtually unaffected (Woodruff, 1955; Cohen and Hansen, 1962).

CONCLUSION TO PART 1

Protein deficiency in these rats was observed to cause a restriction in growth, some loss of fur, reductions in the serum proteins and the onset of anaemia. When the protein deficiency was very severe, a high mortality rate was also found. There were no clinical signs of oedema in the protein deficient animals, and only a very small increase in body fluid percentage was detectable. These rats thus showed the characteristics of the marasmic type of protein-energy malnutrition more than of the kwashiorkor type.

The magnitude of the effect of protein deficiency on each of the measured characteristics was found to depend both on the protein content of the diet on which the rats were maintained and on the duration of the diet. Whenever the effect was great enough for the different low protein diets to be compared by statistical analysis, it was seen that the diet of lowest protein content (2 NDpCal%) induced the biggest changes from the control level, while the 3 NDpCal% and 5 NDpCal% caused progressively smaller differences from control. In addition, the severest effects of protein deficiency, for each of the low protein diets, were invariably observed to occur at or near week 8, which corresponded approximately to the end of the growing period. This pattern exhibited by the changes induced by

protein deficiency was very similar whichever characteristic of the rats was assayed, whether it was body weight, mortality rate, haemoglobin concentration, packed cell volume, normoblast count in bone marrow, serum albumin concentration, total serum protein, serum transferrin or serum iron. Beyond the minimum level near week 8 on each low protein diet, there was usually a gradual recovery towards the control level, and in the case of the 5 NDpCal% diet most variables had approached close to the control by the end of the experimental period (24 weeks). This recovery was considered to reflect a reduced protein demand by the rats once they had reached adulthood. Figures 7-12 show the changes of some of the most important variables during the first 12 weeks of maintaince on the 2 NDpCal% diet and on control diet (10 NDpCal%) as an illustration of these patterns.

The 5 NDpCal% diet appeared to provide an almost adequate protein supply for adult rats, but insufficient for rats during their growing period. The 2 NDpCal% and 3 NDpCal% diets, on the other hand, could not supply enough protein to the animals at any stage of the diet, although the greatest effects again occurred in the growing period.

Protein deficiency resulted in an appreciably fall in serum albumin concentration, while serum globulin was relatively little affected. The albumin/globulin ratio at week 8 in the rats on 2 NDpCal% diet was thus only

half its control value. This pattern is characteristic of protein-energy malnutrition. Although the serum globulins were generally unaffected by the protein-deficiency, there was found to be a large reduction in serum transferrin level in the rats fed on 2 NDpCal% diet. A good correlation was found between serum transferrin and the degree of anaemia and also between transferrin and mortality rate, while neither total serum protein nor serum globulin showed a significant correlation with the latter. Serum transferrin appeared, therefore, to be a valuable indicator of the severity of protein-energy malnutrition as well as a more reliable guide to prognosis than either total serum protein or serum albumin.

Anaemia developed in the protein deficient rats, but was of mild or moderate degree and of normochromic normocytic type. The bone marrow exhibited erythroid hyperplasia, except for the observations at week 2, but the reticulocyte count in peripheral blood was within the normal range, or even somewhat below normal. The reason for this ineffective erythropoiesis was not apparent from these measurements, however. The reduction in serum transferrin was considered not to be an important role in the aetiology of the anaemia as no red cells of hypochromic or microcytic type could be detected. Further investigations were thus planned, with comparisons to be made at week 8 between rats fed on 2 NDpCal% diet and those

on control diet to maximise the effects of the protein deficiency, in an attempt to answer two important questions posed by the results of this part of the study:

(a). Why did bone marrow show erythroid hyperplasia in the protein deficient rats?

(b). What prevented this erythropoiesis from being effective?

FIGURE 7

Comparison of Hb values of rats fed on control diet (10 NDp Cal %) and protein deficient diet (2 NDp Cal %)

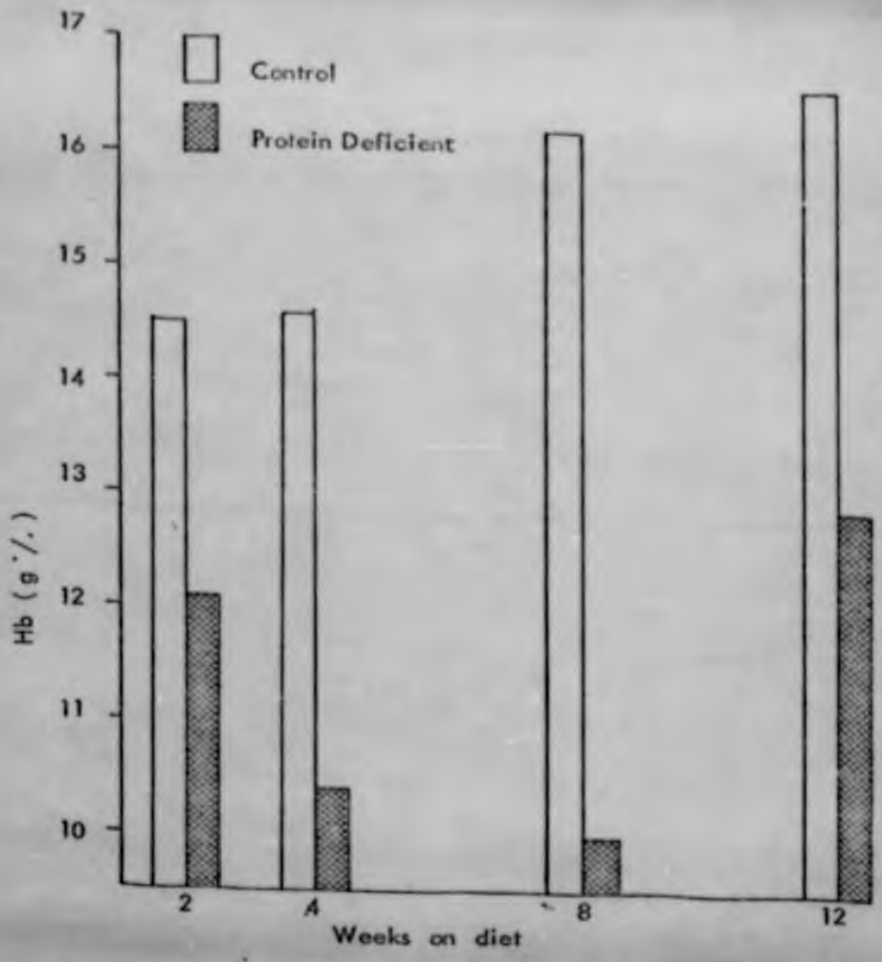


FIGURE 8

Comparison of PCV values of rats fed on control diet (10 NDp Cal /.) and protein deficient diet (2 NDp Cal /.)

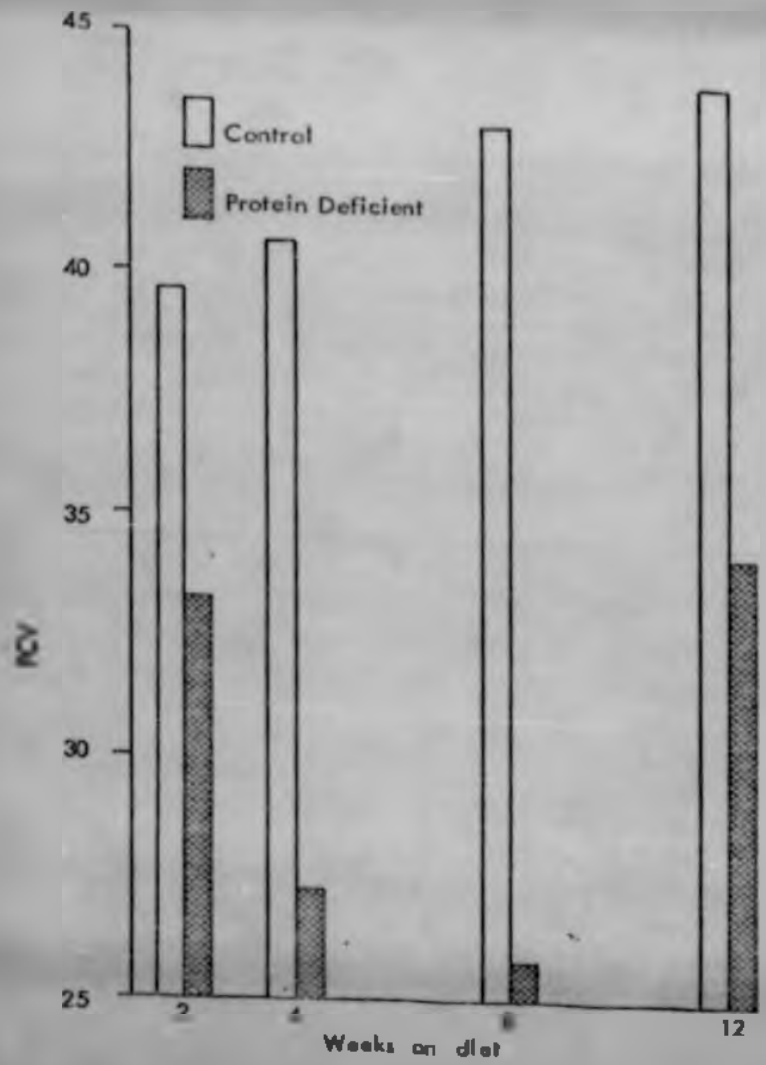


FIGURE 9

Comparison of normoblast counts of rats fed on control diet (10 NDpCal %) and protein deficient diet (2 NDpCal %)

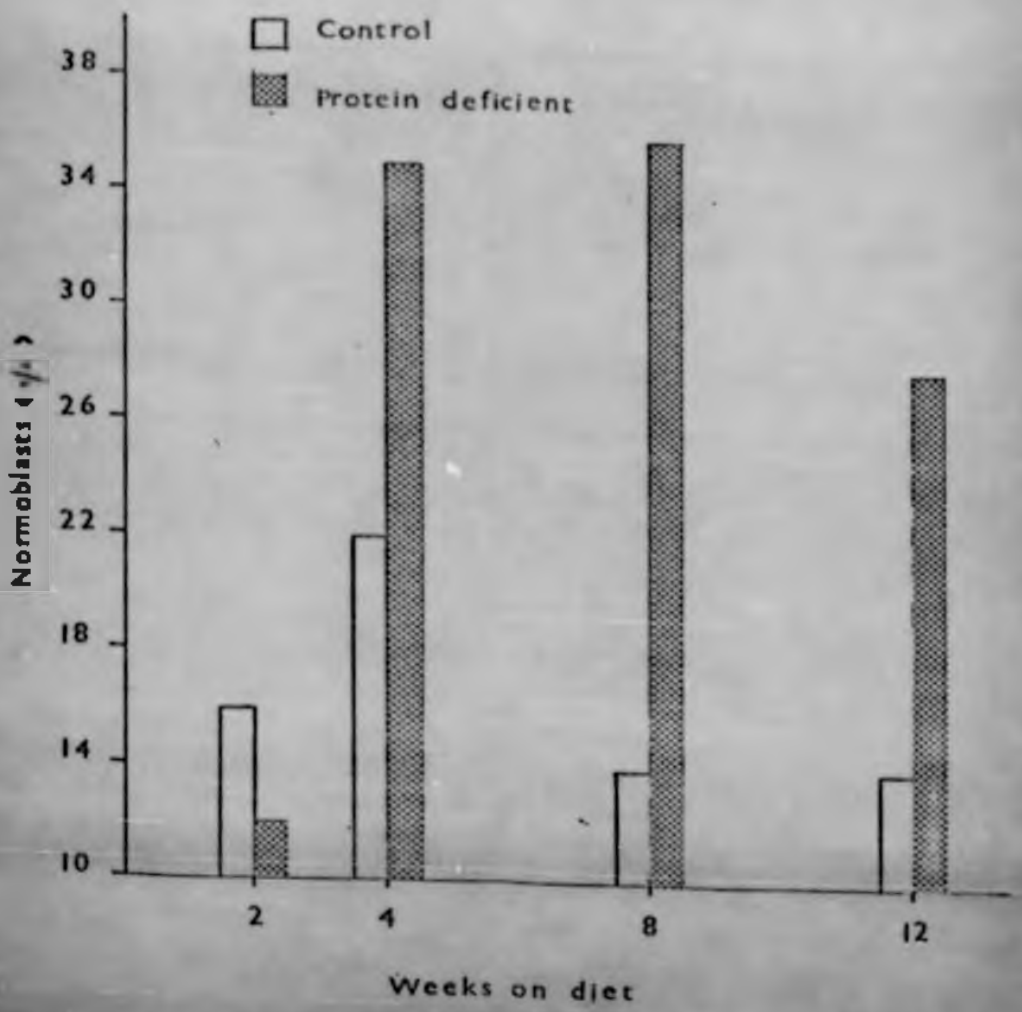


FIGURE 10

Total serum protein levels of rats fed on control diet (10 NDp Cal %) and protein deficient diet (2 NDp Cal %)

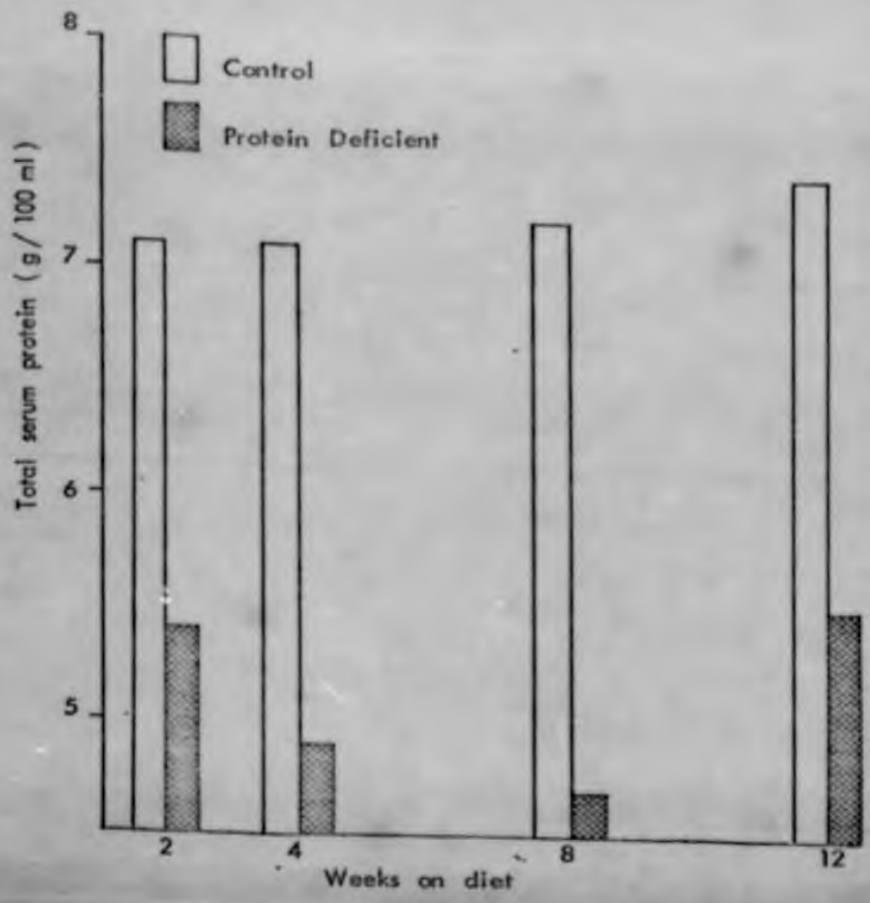


FIGURE 11

Serum albumin levels of rats fed on control diet (10 NDp Cal %) and protein deficient diet (2 NDp Cal %)

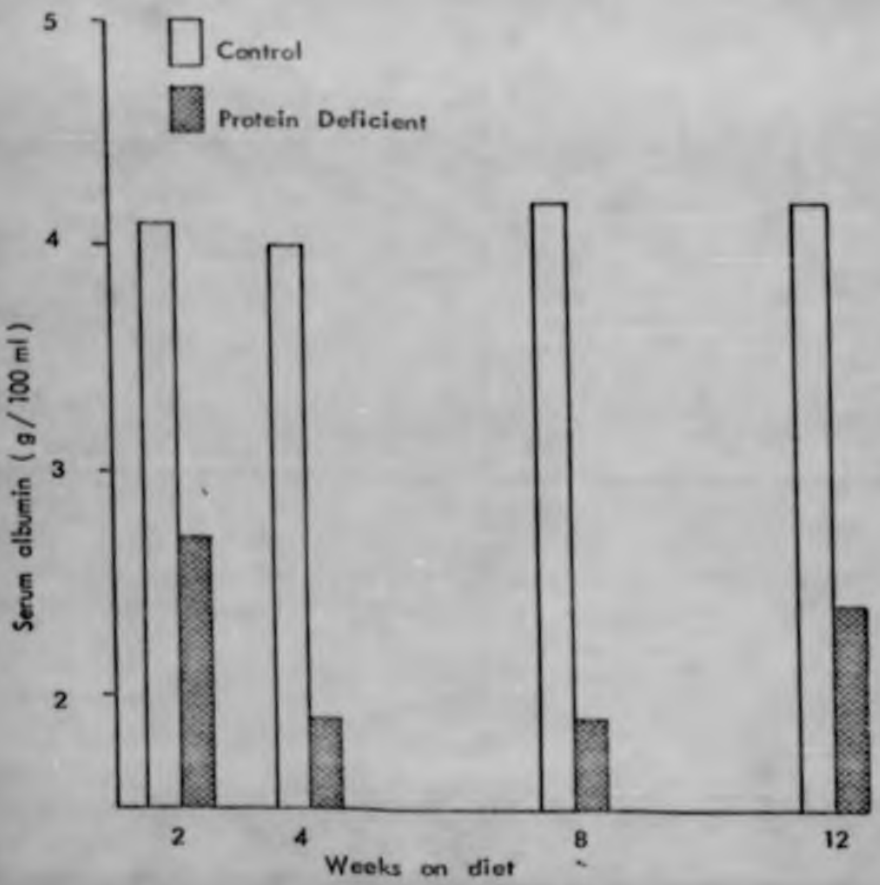
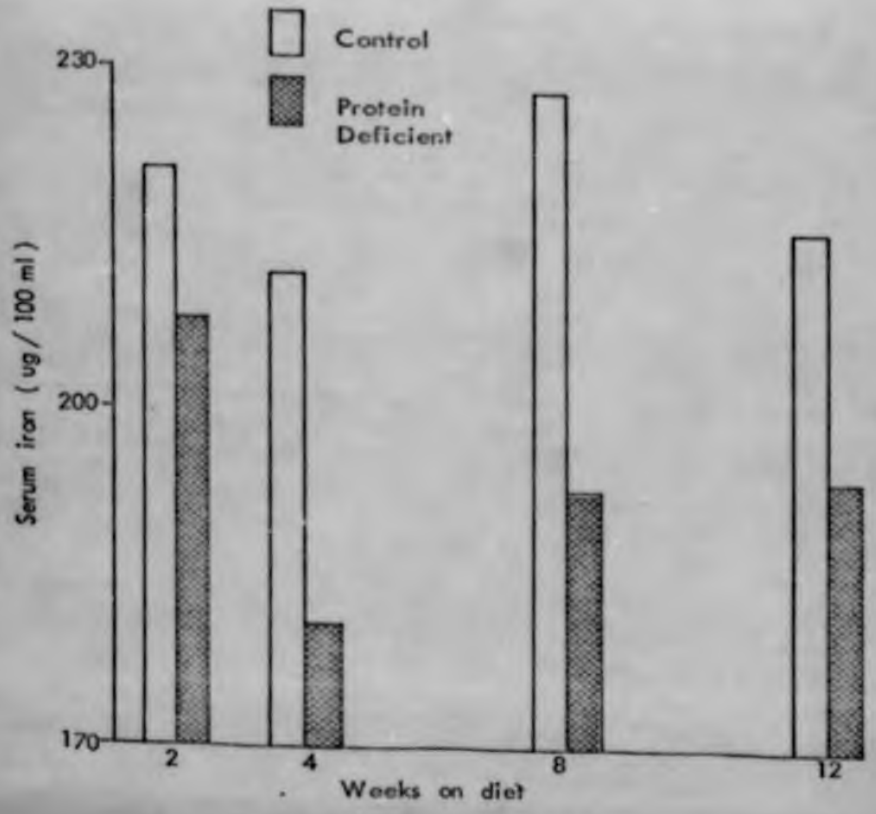


FIGURE 12

Serum iron levels of rats fed on control diet (10 NDp Cal %) and low protein diet (2 NDp Cal %)



PART 4

HAEMOLYSIS AS A POSSIBLE CAUSE OF ANAEMIA INDUCED BY PROTEIN DEFICIENCY

PART 4

[illegible]

INTRODUCTION

Woodruff (1951; 1955) carried out research work on protein-energy malnutrition with anaemia in Nigeria and found that the majority of patients had hepatosplenomegaly. He suggested that the anaemia might be caused by impairment of the liver and, using Schumm's test, observed a haemolytic tendency in some cases. In an experiment carried out by Lanzkowsky et al.(1967) on patients with protein-energy malnutrition (kwashiorkor and marasmus), erythrocytes from some patients were found to have shortened survival half-time ($T\frac{1}{2}$) values, both when auto-transfused and when injected into normal controls. Moreover, the $T\frac{1}{2}$ value of erythrocytes from normal donors appeared to be reduced when injected into these patients. They concluded from these observations that this shortened erythrocyte survival time in protein-energy malnutrition appeared to be due to both corpuscular and extra-corpuscular factors. They also concluded that protein depletion was probably mainly responsible for this shortened survival, since a considerable improvement occurred with protein feeding and this improvement in erythrocyte survival occurred even on a protein diet of low iron content and without

haematinics or vitamin supplements.

It is the purpose of this experiment to assess whether haemolysis is a cause of anaemia and to determine the mechanism of this haemolysis arising during protein deficiency.

The experimental procedures adopted were as follows:-

1. Measurement of erythrocyte life span.
2. Study of osmotic fragility of erythrocytes.
3. Electron microscopic study of erythrocytes.

CHAPTER 1 ERYTHROCYTE LIFE SPAN OF CONTROL RATS AND OF THOSE WITH PROTEIN-ENERGY MALNUTRITION

CHAPTER 3

[illegible] IN CONTROL RATS AND OF THOSE WITH PROTEIN-[illegible] MALNUTRITION

INTRODUCTION

Dacie (1960) stated that the essential feature of a haemolytic anaemia is a reduction in the life span of the patient's erythrocytes. Thus a quantitative measurement of erythrocyte life span is a necessary feature of any study of erythropoiesis. Under normal circumstances the mean cell life span (MCL) of erythrocytes is approximately 120 days in man (Ebaugh et al., 1953; Berlin et al., 1957; Garby, 1962). Measurement of this mean cell life is useful in order to distinguish between an anaemia due to failure of the marrow to respond to an increased demand, which would be within the ability of a normally functioning marrow, and an anaemia resulting from an excessive demand. The normal marrow can expand its production of red cells about six-fold. Thus, haemolysis in which the MCL is more than 20 days (in humans) should not lead to anaemia (this is a compensated haemolysis). When the MCL is 15 days or less, however, anaemia is inevitable (uncompensated haemolysis), but a disappropriate anaemia suggests a degree of marrow incapacity (Crosby and Akeroyed, 1952).

At present, much valuable information is derived from erythrocyte survival studies using ^{51}Cr and the technique

is so satisfactory and widely applicable that the method is the one most commonly employed. The data available at present indicate that the chromium label enters the cell as chromate ion, changes its valency and becomes firmly bound to haemoglobin, preferentially to the β-chains of the globin moiety (Pearson and Vertrees, 1961; Heisterkamp and Ebaugh, 1962; Malcolm et al., 1963; Pearson, 1963;1966). In comparative studies of erythrocyte life span, it is customary to give the half-life ($T\frac{1}{2}$) of the cells which is the time in days when 50% of the labelled cells have been removed from the peripheral blood.

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EXPERIMENTAL PROCEDURE FOR ERYTHROCYTE SURVIVAL STUDY

1. The preparation of blood from control rats and from those with protein-energy malnutrition

Ten rats from each of the groups fed on diets of 2 and 10 NDpCal% were killed after 8 weeks on the diet, their blood being drawn by heart puncture. The blood from the 2 NDpCal% diet rats was pooled into one sterile bottle and the blood from the 10 NDpCal% diet rats into another, each bottle containing acid citrate dextrose solution (ACD) as an anticoagulant. The haematocrit was measured for each group.

2. Radioisotope labelling

A 10 ml aliquot from each bottle was incubated at 20°C for 1 hour with ^{51}Cr-labelled sodium chromate ($Na_2{}^{51}Cr\ O_4$) of activity 100–200 μCi/ml. This suspension was gently agitated every 15 minutes by means of a glass rod. After incubation, the red cells were washed three times, by addition of sterile isotonic saline at 37°C and centrifugation at 1,500 g for 5 minutes, to remove traces of unadsorbed ^{51}Cr. The labelled cells were then reconstituted to their original haematocrit concentration, using isotonic saline. Intact receiver rats fed on control diet were then injected, via the saphenic vein, with 1 ml of the labelled erythrocyte suspension.

3. Measurement of radioactivity in blood samples

The rats were bled from the tail vein at specific interval thereafter, at 1 hour and at 2, 4, 7 and 14 days. The radioactive ^{51}Cr content of these blood samples was measured in a well-type scintillation counter, counting the 1 hour sample as a reference standard with each of the subsequent samples.

4. Calculation of erythrocyte survival

Erythrocyte survival was calculated from the formula

$$\% \text{ RBC survival (day t)} = 100 \times \frac{\text{sample radioactivity (day t)}}{\text{sample radioactivity (1 h)}}$$

^{51}Cr-survival curves were drawn by plotting the blood radioactivity as a function of time on semilogarithmic graph paper. The $T_{\frac{1}{2}}$ ^{51}Cr was obtained from this curve, expressed in terms of days. No correction was made for elution of ^{51}Cr from the erythrocytes.

TABLE 1

In vivo survival in control receiver rats of ^{51}Cr-labelled erythrocytes from rats fed on a low protein diet (2 NDpCal %) and on control diet (10 NDpCal%)

Time	Percentage survival in receiver rats on control diet (Mean ± S.E.M.)		
	Erythrocytes from 2 NDpCal % diet rats	Erythrocytes from 10 NDpCal % diet rats	p value
1 hour	100.0	100.0	-
2 days	77.7 ± 0.6	83.0 ± 1.4	$p<0.01$
4 days	63.8 ± 3.4	72.0 ± 2.1	$p<0.05$
7 days	52.2 ± 0.9	59.6 ± 0.5	$p<0.001$
14 days	27.6 ± 0.2	34.5 ± 0.9	$p<0.0001$

Results for 6 and 5 receiver rats, respectively, for the ^{51}Cr-labelled erythrocytes from the rats on the 2 NDpCal % and 10 NDpCal % diets

FIGURE 1

^{51}Cr red - cell survival curves for control rat (●——●) and protein - calorie malnourished rat (o——o)

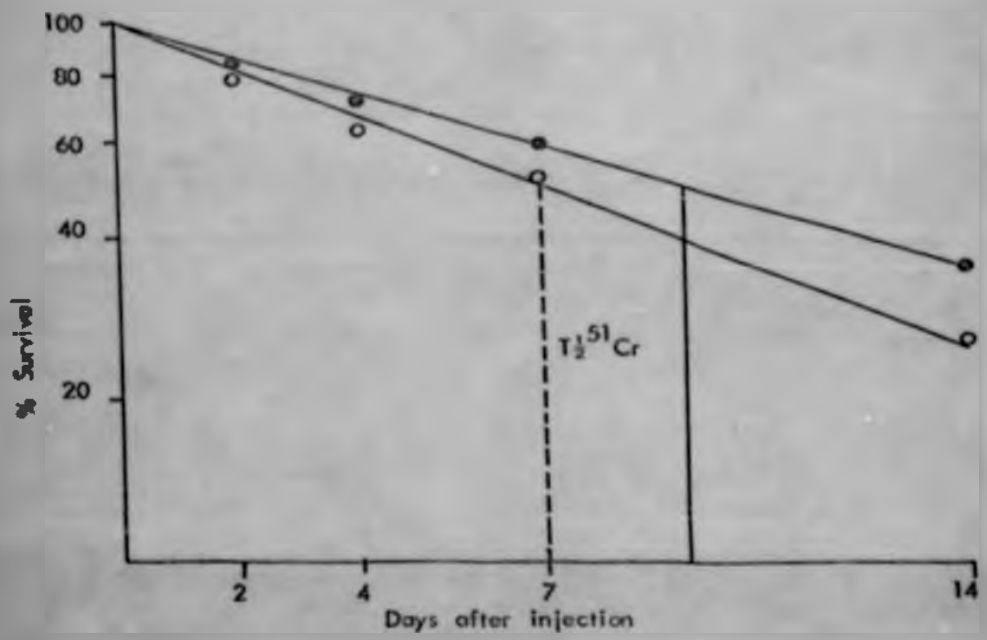


TABLE 2

Half - time survival in control receiver rats of ^{51}Cr-labelled erythrocytes from rats fed on low protein diet (2 NDpCal%) and on control diet (10 NDpCal %)

Diet (in NDpCal %) of donor rats	Number of rat	$T_{\frac{1}{2}}$ ^{51}Cr (days)
2	1	7.2
	2	7.8
	3	7.6
	4	7.5
	5	7.5
	6	7.4
	Mean ± S.E.M.	7.5 ± 0.1
10	1	9.5
	2	9.9
	3	9.1
	4	9.4
	5	9.3
	Mean ± S.E.M.	9.4 ± 0.1***

*** = Significant difference with $p < 0.001$

Table 1 displays the survival percentages, in receiver rats on control diet, of ^{51}Cr-labelled erythrocytes obtained from one group of rats fed on low protein diet (2 NDpCal%) and from a second group on control diet (10 NDpCal%). The erythrocytes of rats fed on low protein diet disappeared more rapidly from the circulation of the control receiver rats than did the cells from the control animals. This more rapid disappearance of the red cells from the rats on low protein diet was apparent throughout the experimental period, judging by the lower survival percentages at each time (Table 1).

Typical survival curves for a control rat and a protein deficient rat are shown in Figure 1. The mean survival time of erythrocytes from rats fed the low protein diet was significantly shortened ($p < 0.001$) compared with that of red cells from those animals receiving the control diet. Table 2 shows the individual $T\frac{1}{2}$ ^{51}Cr values in the receiver rats and the mean values of 7.5 ± 0.1 days for red cells from the low protein group and 9.4 ± 0.1 days for red cells from the control group, the difference (in time) being almost 2 days.

DISCUSSION

The present study showed that erythrocytes from rats fed on a low protein diet had a survival time, when injected into control rats, that was significantly below the survival time for red cells from control animals. This result was similar to that obtained by Delmonte et al. (1964) who found a $T_{\frac{1}{2}}$ value of about 6 days for erythrocytes from a group of protein deficient rats as compared to 9 days for those from control animals. The present results and those of Delmonte et al. (1964) are thus consistent with the view that a cause of haemolysis in the anaemia arising from protein deficiency is a structural defect in the erythrocytes. This intracorpuscular factor might, however, be accompanied by extracorpuscular factors in the protein deficient rats contributing to the haemolysis in these animals but not in the control receiver rats. Woodruff et al. (1970) measured erythrocyte life span in dogs, by using ^{51}Cr, and also found that the erythrocyte life span of malnourished dogs, with the associated anaemia, was shorter than that of well-nourished ones, but in their case the difference was not significant and they concluded that haemolysis was not a major factor in the anaemia.

It is of interest to assess to what extent the increased haemolysis in the protein deficient rats can account for the onset of anaemia in these animals. If no

other factors, such as a change in rate of release of erythrocytes from bone marrow, were operating in these animals, the more rapid rate of haemolysis would tend to reduce the red cell count in the blood in proportion to the degree of shortening of the mean red cell life span and thus reduce the haemoglobin level in a similar proportion. In a normal animal these changes would usually induce a stimulus for an increased production rate of red blood cells in bone marrow, but this effect will be disregarded in the first instance for the sake of simplicity. The reduction in mean red cell life span should be paralleled approximately by the reduction in survival half-time measured by the ^{51}Cr method, which showed a reduction of 20% (from 9.4 to 7.5 days) at week 8 of the 2 MDpCal% diet compared with the control (10 MDpCal%) diet : this represents the change in life span due to intracorpuscular factors alone since the $T\frac{1}{2}$ ^{51}Cr values were measured in control receiver rats. The effect of this faster haemolysis would be to reduce the red cell count, PCV and haemoglobin level by about 20%. It was observed, however, that the extent of the reduction in each of these variables at week 8 on the 2 MDpCal% diet was about twice as great : red cell count dropped by 39% (from 7.52 to 4.61 million/mm^3), PCV fell by 40% (from 43.0 to 25.7%) and Hb. decreased by 38% (from 16.4 to 10.1 g/100 ml)(see Part 3, Chapter 2).

Haemolysis due to the intracorpuscular factors could thus account for no more than about half of the observed reductions in red cell count, PCV and haemoglobin level. Moreover, the relatively small reduction in life span for the red cells from the rats on 2 NDpCal% diet could readily be compensated by increased red cell production if the bone marrow were normal.

The anaemia of the rats on 2 NDpCal% diet at week 8 should normally induce an erythropoietic stimulation of the bone marrow and produce hyperplasia (see also Part 5). Hyperplasia of the bone marrow was observed in these animals and it appeared from the measured myeloid : erythroid ratios (see Part 3, Chapter 2) at week 8, of 1.1:1 for the rats on 2 NDpCal% diet and 3.5:1 for the rats on control diet, that the bone marrow of these protein deficient rats was producing erythroid elements at approximately three times the normal rate. Without any counteracting factors, this increased production of erythrocytes would tend to raise the red cell count about threefold. There must therefore have been a strong counterbalancing factor to cancel out this increased rate of production and produce the resultant fall in red cell count on the 2 NDpCal% diet. The increased haemolysis rate due to the intracorpuscular factors could not be account for the observed fall in red cell count even in the absence of increased production, so clearly

could only account for only a small proportion of counterbalancing reduction in the presence of increased red cell production. Thus the observed increase in haemolysis appears to be only a minor factor in the anaemia resulting from protein deficiency in the present study.

The question then arises as to what are the main factors responsible for the anaemia of protein deficiency. The present experiments did not themselves rule out the possibility that haemolysis was affected by extra-corpuscular factors operative in the protein deficient animals, since measurements of haemolysis rate were performed in receiver rats fed on control diet. Very few workers have studied the importance of extracorpuscular factors to haemolysis in protein deficiency, but some observations have been made in human subjects with kwashiorkor by Lanskowsky et al. (1967). Although they found some evidence that survival of normal erythrocytes was reduced when these were transfused into kwashiorkor subjects, they also observed that there was no significant difference in the rate of haemolysis, as judged by $T\frac{1}{2}$ ^{51}Cr values, of erythrocytes from kwashiorkor subjects when they were auto-transfused rather than transfused into normal subjects. It is the latter observation that is relevant to the present study since it implies that measurement of $T\frac{1}{2}$ ^{51}Cr values in normal receiver subjects

provides a good indication of haemolysis rate in the protein deficient donors. Extracorpuscular factors appeared to have little additional effect on the survival of protein deficient erythrocytes which were already subject to increased haemolysis due to structural defects or other intracorpuscular factors. Belmonte et al. (1964) also suggested that the defective composition of protein deficient serum might be less effective than normal serum in protecting erythrocytes against chemical and mechanical trauma, but they concluded that the available evidence pointed to an intracorpuscular factor, namely a structural defect, as the principal cause of haemolysis in protein deficiency anaemia. It is thus anticipated that extracorpuscular factors should be less important than intracorpuscular factors in relation to their effects on haemolysis of red blood cells in protein deficient rats and therefore that haemolysis due to both types of factor should represent only a secondary cause of the anaemia of protein deficiency in the present investigation.

Thus the major cause of anaemia in protein deficiency appears to be some form of restriction in the supply of red blood cells to the circulation rather than an increased rate of destruction of these cells. Since the bone marrow of the protein deficient rats, at week 8 on 2 NDpCal% diet, was found to exhibit hyperplasia, it is considered that the principal cause of the anaemia in these animals was

related to a limitation either in the later stages of red cell production in the bone marrow or in the release of red cells from the marrow. This aspect will be discussed in more detail in a later section (in Part 5, Chapter 4).

The more rapid haemolysis in protein deficient rats appears to reflect a structural defect in the erythrocytes and it is of interest to consider the probable nature of such defects arising on a protein deficient diet. In order to assess probable defects in the structure, it is necessary first to examine the normal structure of the erythrocyte, and particularly of the erythrocyte membrane. Protein and lipid are the main constituents of the red cell membrane and the former could be affected directly by protein deficiency while abnormalities in lipid content have in fact been observed in kwashiorkor (Coward, 1971). There is also some evidence of an effect of protein deficiency on the osmotic fragility of erythrocytes, with a decreased fragility found in children with kwashiorkor (Lanzkowsky et al., 1967), and such changes are presumably related to changes in membrane structure. Abnormalities in haemolysis rate and in osmotic fragility may both be related to a common cause in the form of a structural defect in the erythrocyte membrane, and, for this reason, both these aspects will be discussed together in the next chapter after analysis of the results of osmotic fragility tests.

CHAPTER 2 OSMOTIC FRAGILITY OF ERYTHROCYTES FROM CONTROL RATS AND FROM THOSE WITH PROTEIN-ENERGY MALNUTRITION

CHAPTER 2

OSMOTIC FRAGILITY OF ERYTHROCYTES FROM CONTROL RATS AND FROM THOSE WITH PROTEIN-ENERGY MALNUTRITION

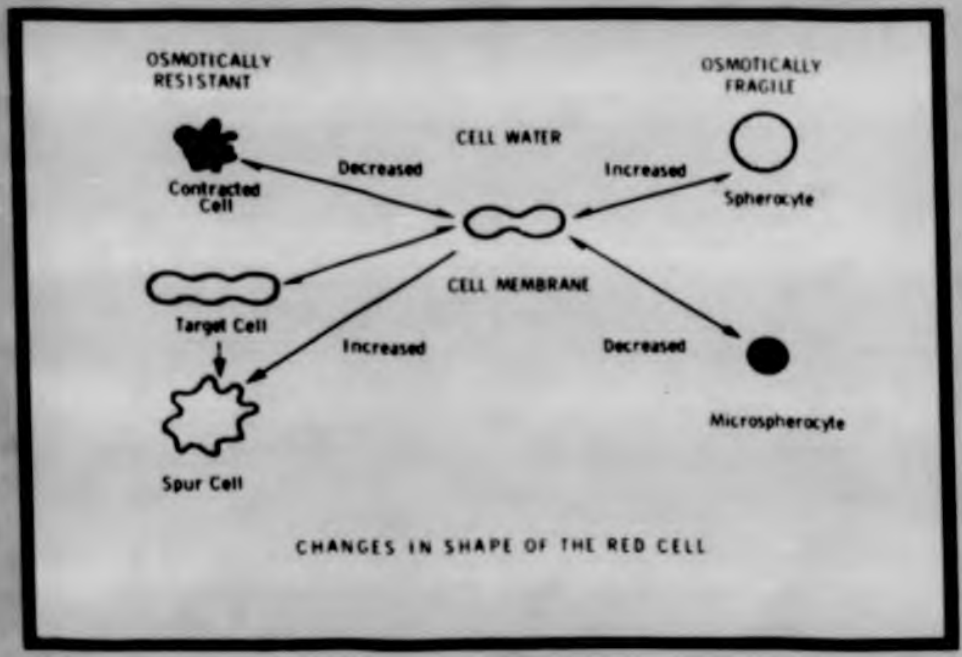
INTRODUCTION

The osmotic fragility test provides information on the structure of the erythrocyte membrane. It gives an indication of the surface area to volume ratio, in that fragility tends to increase as the cell becomes spherical. The lipid content of the membrane plays an essential role in the control of red cell shape (Murphy, 1962; Smith et al., 1964; Sibler et al., 1966; Norum and Gjone, 1967; Cooper and Jandl, 1968; Diamond, 1968; Gjone et al., 1968; Jaffe and Gottfried, 1968; Cooper, 1969; Cooper and Jandl, 1969a; McBride and Jacob, 1970) : increased amounts of lipid can lead to an extension in the total area of cell membrane, thereby increasing the surface area to volume ratio. The extra membrane produces folds in the cells surface, which can be either regular as in target cells (Murphy, 1962; Cooper and Jandl, 1968) or irregular as in spur cells (Smith et al., 1964; Sibler et al., 1966; Grahn et al., 1968; Tchernia et al., 1968; Cooper, 1969; McBride and Jacob, 1970). Alternatively, a loss of membrane during circulation (as may occur in patients with extensive burns, for example) can lead to a decrease in the ratio of surface area to volume, with consequent sphering of

the cell (Ham et al., 1948; Rand, 1964). Such changes are illustrated in Figure 1 (taken from Gordon-Smith, 1972). There are no synthetic pathways for the replacement of lost membrane in the mature red cell, but some of the membrane lipids are in equilibrium with lipids in the surrounding plasma (Murphy, 1962).

FIGURE 1

Changes in shape and osmotic fragility produced by changes in volume and surface area.



Experimental Procedure of Fragility Test

Reagents

A series of hypotonic solutions equivalent to 0.90, 0.75, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10% NaCl was prepared by dilution of 1% NaCl solution with distilled water.

Method

0.05 ml of heparinised blood was added to 10 ml of each of the series of hypotonic solutions and mixed by inverting several times. The tubes were allowed to stand at room temperature for 30 minutes, then re-mixed and centrifuged at 1,500 *g* for 5 minutes. The extent of haemolysis was assessed from optical density measurements at 540 nm with a photometer (Dacie and Lewis, 1970).

Results

Osmotic fragility tests performed on red blood cells from rats fed on low protein diet (2 NDpCal%) and on control diet (10 NDpCal%) provided the results shown in Table 1 and the fragility curves in Figure 2. The extent of haemolysis of red blood cells from the protein deficient rats was significantly less than of those from the control rats in the hypotonic solutions of NaCl concentration 0.55, 0.50 and 0.45 g/100 ml (p values shown in Table 1).

It was clear that the fragility of red blood cells (RBC) of rats fed on low protein diet was decreased (increased erythrocyte osmotic resistance). This is in agreement with the work of Lanskowsky (1967), who found that there was a significant increase in erythrocyte osmotic resistance, and in thermal resistance, in some cases of protein malnutrition and that an improvement occurred in these parameters following protein feeding without haematinics.

TABLE 1

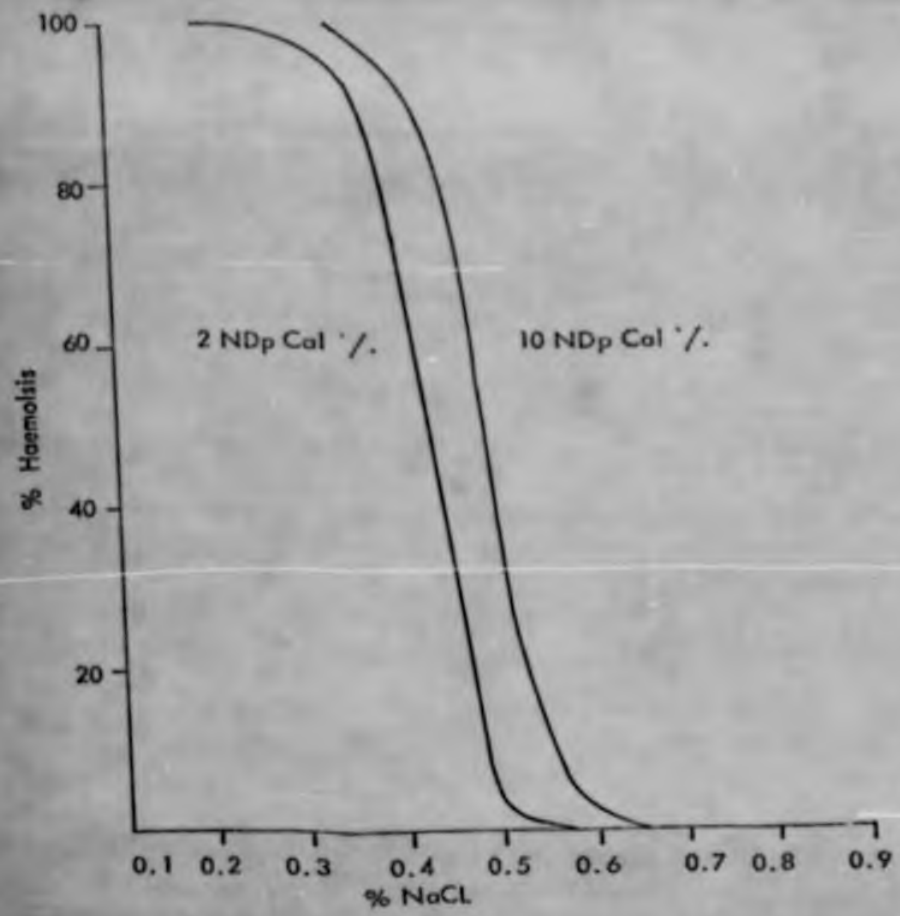
Osmotic fragility test on rats fed on low protein diet (2 NDpCal %) and on control diet (10 NDpCal %)

Tube No.	Concentration (NaCl, g/100ml)	Mean haemolysis of RBC (%) (Mean ± S.E.M.)		p value
		Low protein diet	Control diet	
1	0.90	-	-	-
2	0.75	-	-	-
3	0.65	-	-	-
4	0.60	-	1.9 ± 1.3	$p > 0.1$
5	0.55	-	13.5 ± 4.9	$p < 0.02$
6	0.50	4.0 ± 1.5	46.5 ± 8.7	$p < 0.001$
7	0.45	38.3 ± 8.8	83.4 ± 6.3	$p < 0.002$
8	0.40	73.9 ± 8.3	95.1 ± 3.4	$p > 0.05$
9	0.35	94.4 ± 2.9	100.0 ± 0.0	$p > 0.05$
10	0.30	98.0 ± 2.0	100.0 ± 0.0	$p > 0.1$
11	0.20	100.0 ± 0.0	100.0 ± 0.0	-
12	0.10	100.0 ± 0.0	100.0 ± 0.0	-

N.B. - = no haemolysis
Number of rats in each group was 7.

FIGURE 2

The mean of Osmotic-fragility curves of rats fed on control diet (10 NDp Cal %) and those fed on low protein diet (2 NDp Cal %)



DISCUSSION

This observation of a decrease in the osmotic fragility of the erythrocytes from the protein deficient rats indicates that these cells should not rupture in the circulation and thus implies that extravascular haemolysis must have been responsible for the shortening of erythrocytes survival time observed in Chapter 1 of this part.

Changes in osmotic fragility of red cells should reflect changes in the erythrocyte membrane, which is comprised principally of lipid and protein. Lipids, in particular phospholipids, are essential components of membrane systems in erythrocytes as well as in all biological membranes. There have been many reports of abnormalities in lipid metabolism during protein deficiency in man, with observations of reductions in serum lipid concentrations (Schwartz and Dean, 1957; Flores et al., 1970) and in α- and β-lipoproteins (Gravioto et al., 1959; Monckeberg, 1968) as well as frequent findings of fatty infiltration of the liver (Schwartz and Dean, 1957; Macdonald et al., 1963; Lewis et al., 1964; Monckeberg, 1966; Flores et al., 1967; Truswell et al., 1969). Moreover, changes in erythrocyte membrane have been described in several conditions in association with defects in lipid metabolism : the

phospholipid composition has been found to be abnormal in a number of liver diseases (Nye and Marinetti, 1967; Boon et al., 1969), in various lipidoses (Hooghwinkel et al., 1969) and in the rare syndrome of abetalipoproteinaemia or acanthocytosis (Phillips, 1962; Ways et al., 1963). More recently, Coward (1971) has reported an increase in phospholipid composition, principally in lecithin content, of erythrocyte membranes in Ugandan children with kwashiorkor. A decreased osmotic fragility of the erythrocytes has been observed in association with a high lecithin content in hepatitis and obstructive jaundice (Pitcher and Williams, 1963) as well as in kwashiorkor (Lanzkowsky et al., 1967), and it has been speculated that these two factors may be interrelated.

Van Deenen and de Gier (1964) reported that the lipid composition of the mature human erythrocyte comprises phospholipids, free cholesterol and glycolipids. Although the precise structure of the red cell membrane is not yet clearly established, Davson and Danielli (1943) have suggested that it is composed of two layers of lipid molecules, which are arranged so that their non-polar hydrocarbon chains lie towards the centre of the membrane while the charged, polar, and therefore hydrophilic, parts of the molecule point outwards into the extracellular fluid or inwards into the intracellular (Figure 3). This configuration can account for many of the membrane

FIGURE 3

The Structure of the Red-Cell Membrane.
(After Davson and Danielli, 1943)

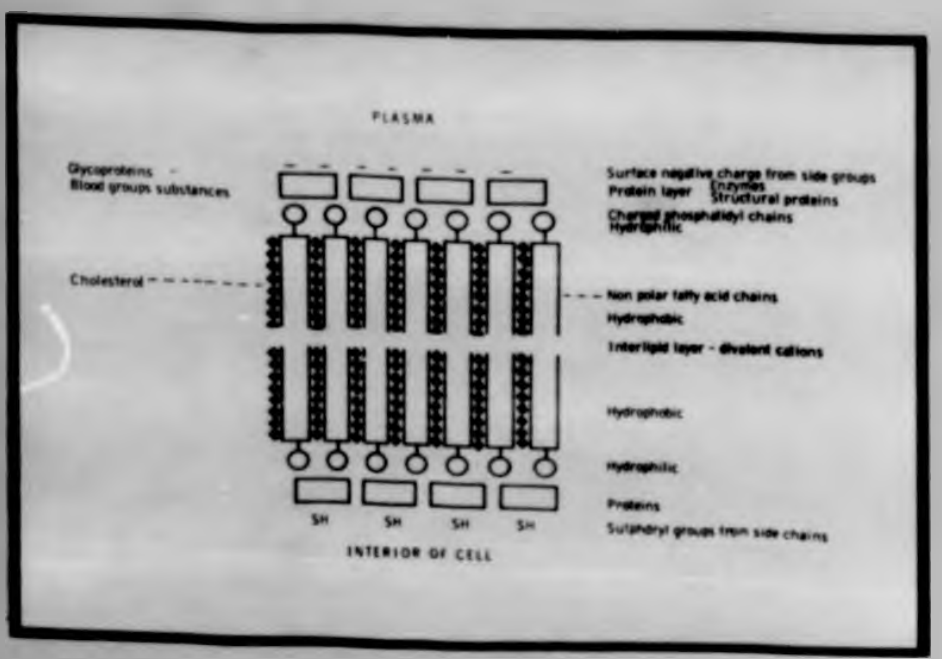
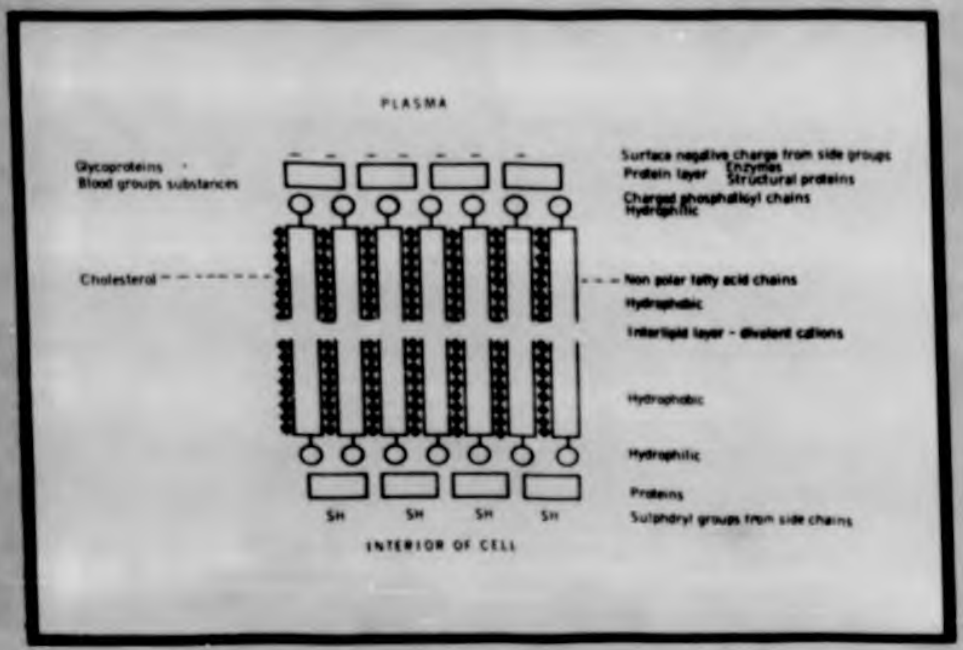


FIGURE 3

The Structure of the Red-Cell Membrane.
(After Davson and Danielli, 1943)



properties and is strongly supported by electron microscopic studies and X-ray diffraction studies. It is also quite consistent with the characteristics of lipid associations observed at interfaces or in bulk lipid-water systems (van Deenen, 1965).

Ways and Dong (1965) found that young erythrocytes are normal in appearance and in their phospholipid distribution in abetalipoproteinaemia but that abnormalities in both these aspects develop during the circulation of these erythrocytes. The findings in this disease indicate that the lipid composition of plasma may influence the proportion of phospholipids in individual erythrocytes through a process of exchange. Simon and Ways (1964), Ways and Simon (1964) reported that in this disease there was an abnormality in shape and a shortened in vivo survival of these erythrocytes. There is evidence that many of the lipids of the red cell membrane may be exchanged with those in the plasma (Murphy, 1962; Gjone et al., 1968; Norum and Gjone, 1967) and the surface area to red cell volume ratio is thus partly controlled by the lipid constituents of the plasma. The concentrations of cholesterol and phospholipids (lecithin) in plasma, and the ratio between these, may cause changes in red cell shape and thereby lead to a shortening of red cell survival time (Cooper, 1969).

Target cells and acanthocytes (or spur cells), both

of which are associated with an increased red cell membrane area to volume ratio, represent the two main abnormalities of erythrocyte morphology found in liver diseases such as infectious hepatitis, cirrhosis, obstructive jaundice and severe hepatocellular disease, and also in other diseases, including abetalipoproteinaemia and lecithin-cholesterol acyltransferase deficiency. These erythrocyte abnormalities have been found to be associated with changes in the cholesterol and phospholipid (lecithin) contents of the red cell membrane, as summarised in Table 2 (Dacie, 1968; Gordon-Smith, 1972). In many cases these changes were associated with changes in plasma levels of these lipids, but increases in red cell membrane cholesterol and lecithin have also been observed to occur without changes in plasma lipids in some patients with liver disease. Nye and Marinetti (1967) suggested that erythrocyte lecithin content could be correlated with plasma lecithin, although 4 out of 11 of their patients with high lecithin concentration in the red cells had a decreased, rather than increased, plasma concentration. Moreover, Boon et al. (1969) found that 2 of his 4 patients, with high red cell lecithin, had low serum lecithin concentration, while the serum values were high in the other 2.

An association between changes in erythrocyte membrane lipids and changes in plasma lipids in protein deficiency, however, has not been established. Woodruff (1951)

TABLE 2

Changes in Red-Cell Lipids in Plasma Lipid Disorders

Disease	Cholesterol	Lecithin	Cholesterol Lecithin Ratio	Cell shape
Infectious hepatitis	Increased	Increased	Reduced	Target cells
Cirrhosis	Increased	Increased	Reduced	Target cells
Obstructive jaundice	Increased	Increased	Reduced	Target cells
Severe hepatocellular disease	Increased	Normal	Increased	Acanthocytes
Abetalipoproteinaemia	Increased or normal	Reduced	Increased	Acanthocytes
Lecithin–cholesterol acyltransferase deficiency	Increased	Increased	Reduced	Target cells

reported that the anaemia associated with protein deficiency, in Nigerian women during pregnancy, was characterised by an increase in the diameter of the red cell and a reduction in thickness. This finding indicated that the ratio of surface area to volume of the red cells was increased, a result which has been confirmed by Coward (1971) during studies on patients with untreated kwashiorkor. Moreover, Coward (1971) observed that the increased surface area was associated with an elevated lecithin content of the erythrocyte membrane. Flores et al. (1970) have measured plasma lipid levels in children with kwashiorkor and, in contrast, found these to be low, especially for the triglycerides. Plasma phospholipids were also significantly reduced, but the greatest change observed was in the low-density lipoprotein fraction (of density < 1.063). The raised lecithin content of the erythrocyte membrane in protein deficiency thus does not appear to be related to corresponding changes in serum lecithin. The cause of the changes in red cell lipids remains unclear. Perhaps these changes in the erythrocytes are related to changes in the metabolism of phospholipids in the liver or in bone marrow.

Defects in the lipid composition of the red cell membrane and their effects on membrane morphology are better understood than alterations in protein content, owing to the difficulties in isolating membrane proteins

without denaturing them. It is known, however, that protein constitutes about 60% of the membrane and that several structural proteins are present. Moreover there is some evidence for the presence of a contractile protein which may be important in maintaining the shape of the red cell (Rocenthal et al., 1970). It has been suggested that an abnormality of protein, rather than of lipid, is the fundamental defect in some hereditary disorders of the erythrocyte. Hereditary spherocytosis is a particular example of such a disorder, where only minor abnormalities in lipids have been found (Jacob and Karnovsky, 1967), but where the membrane protein has been reported to be qualitatively abnormal (Jacob et al., 1972). Membrane proteins extracted from normal erythrocytes, by dialysis of ghosts in low ionic strength media, are found to aggregate and align into microfilaments when ionic strength is reconstituted, especially in the presence of ATP and Mg^{++} (Marchesi and Steers, 1968). In contrast, membrane proteins from hereditary spherocytosis red cells appear unable to aggregate or are found to aggregate to a much lesser extent (Jacob, 1974). Analogous microfilamentous proteins have been demonstrated in a great number of different cell types, including slime moulds, nerve cells and blood platelets, and these filaments have been found to be critical to normal cell shape, plasticity and motility in each case (Adelman et al., 1968). This general

pattern is consistent, therefore, with the finding that defects in this type of microfilamentous protein in the red cell membrane underlie the abnormal shape, plasticity and survival of hereditary spherocytes.

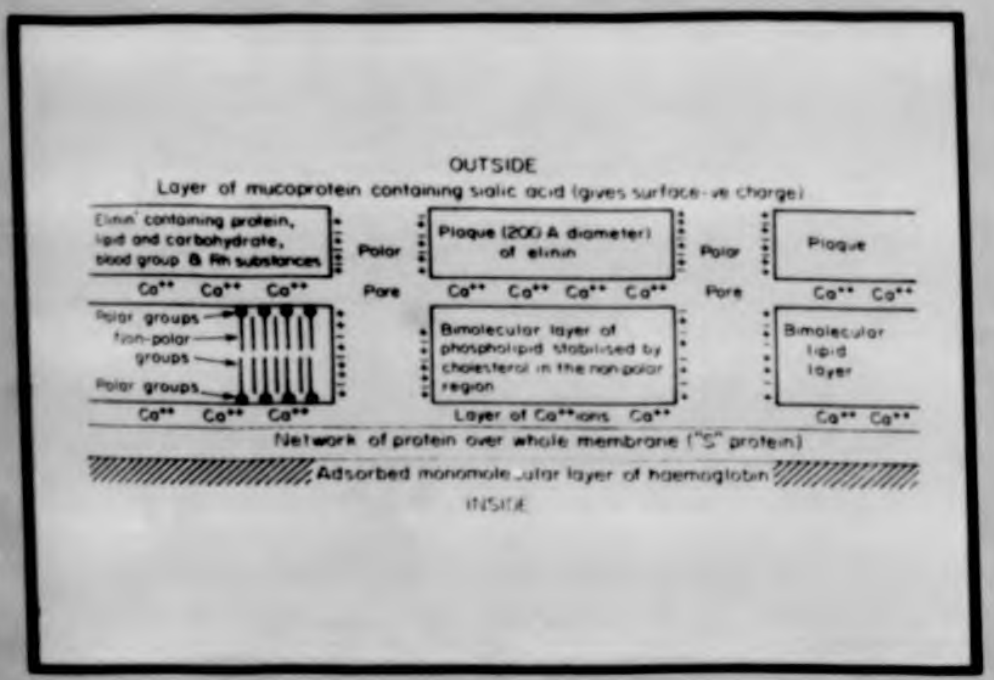
Rega et al. (1966) have reported that one of the most important characteristics of the human red cell membrane protein is that it is a glycoprotein containing hexoses, hexosamines, fucose and the total complement of sialic acid residues present in red cell ghosts. Several workers (Eylar et al., 1962; Glaeser, 1963; Seaman and Uhlenbruck, 1963) have presented evidence that the sialic acid is primarily responsible for the highly acidic nature of the red cell surface and that it is the main determinant of the erythrocyte's electrophoretic properties. The sialic acid contributes a negative charge which is localized in the glycoprotein of the exterior surface of the intact erythrocyte (Winzler, 1969). Red cells are normally kept apart by virtue of their surface charge, which produces the cell's zeta potential and is dictated chiefly by the sialic acid residues (Pollack et al., 1965). If a decrease in their negative surface charge occurs as a result of antibody building or following enzymatic treatment (Marikovsky and Danon, 1969), or if the dielectric constant of the medium is raised by an agent such as dextran, then the electrostatic repulsive forces between the cells are diminished and agglutination tends to occur. Any change

in the balance between the zeta potential and forces favouring cellular adhesion may result in agglutination and consequently removal of the affected cells from the circulation by the spleen or liver (Jandl, 1964).

Whittam (1958) has proposed a model of the red cell membrane incorporating these features and other experimental data (Figure 4). In this model, the external surface is regarded as consisting of a glycoprotein (mucoprotein) layer containing sialic acid, which gives the red cell its negative surface charge. Under this layer are plaques of elinin, which is a conglomerate of protein, carbohydrate and lipid and incorporates the blood group activity. Beneath these is a biomolecular layer of lipid, lined on its inner and outer surfaces by calcium ions; and beneath this is an inner layer of protein that separates the haemoglobin-enzyme content of the interior from the cell membrane. Penetrating the membrane are depicted polar pores, possibly protein lined and with positive charges, allowing ready access of water and anions such as HCO_3^- and Cl^- to the cell's interior, but restricting access of Na^+ and K^+. Kavanau (1966) has also constructed a model, for biological membranes in general but also appropriate for the erythrocyte membrane, based on the concept that the lipid phase exists in the form of globular micelles structured between the inner and outer layer of protein (Sjostrand, 1963; Lucy and

FIGURE 4

Whittam model of red cell membrane, which includes an outer mucoprotein layer, plaques, bimolecular leaflet of phospholipid, and a layer of protein adjacent to the haemoglobin. The pores might be lined with protein so as to give a net positive charge (from Whittam, 1958).



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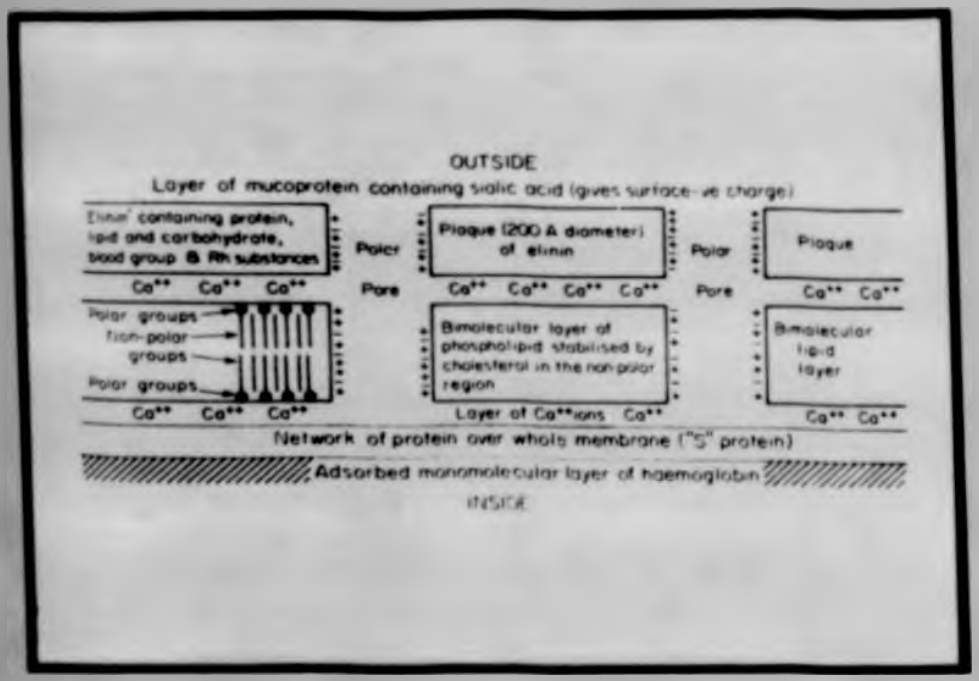
Whittam model of red cell membrane, which includes an outer mucoprotein layer, plaques, bimolecular leaflet of phospholipid, and a layer of protein adjacent to the haemoglobin. The pores might be lined with protein so as to give a net positive charge (from Whittam, 1958).

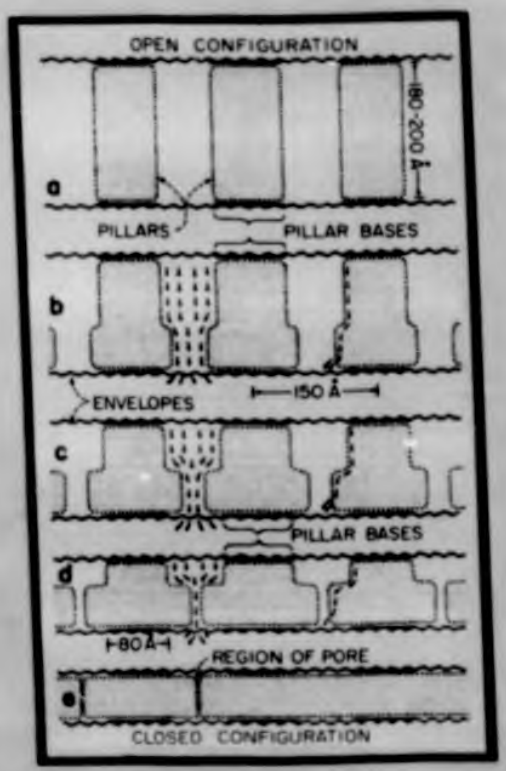


Glauert, 1964; Lucy, 1968). These globular micelles are considered to be dynamic, undergoing a transformation from pillars (pores open) to discs (pores closed)(Figure 5). Such a transformation could explain many membrane functions, including diffusion, active transport, contraction and expansion, coalescence and fragmentation.

The red cell membrane is thus known to have a complex structure which allows it to perform its many different functions. In a protein deficient condition, it may not be possible for a perfect membrane structure to be assembled owing to a shortage of some of the required substrates or an absence of some of the necessary enzymes. The reduced protein supply to the bone marrow may have a direct effect on the protein composition of the membrane, or alternatively may have an indirect influence on the structure through deficiencies in the enzyme complement. Changes in lecithin content have already been reported (Coward, 1971), and, although no abnormalities in protein composition have yet been established, such changes might not be unexpected in protein deficiency. Alterations in serum concentrations of total protein, albumin and transferrin (see Part 3, Chapter 3) were found in the present investigations, while other workers have reported changes in serum lipids (Flores et al., 1970) and it has been suggested that serum lipids may have some influence on circulating erythrocytes through a process of exchange.

FIGURE 5

A highly diagrammatic cross-sectional representation of the postulated gross geometrical changes of micellar form that occur in the transformation from the open to the closed configuration of a region of a biological membrane (from Kavanau, 1966).



The precise mechanism by which protein deficiency causes a modification in erythrocyte membrane structure has not yet been established, but it seems clear that such a modification in the membrane does occur in this condition.

Changes in the membrane structure have a direct effect on the osmotic fragility of the membrane and the observation of a reduction in fragility during protein deficiency is consistent with the view that the membrane has a higher lecithin content in this condition. Effects of structural changes on the survival of circulating red cells are related to the ability of cells to deform when they have to pass through the capillaries and the even narrower channels between the splenic pulp cords and the sinusoids. Destruction of erythrocytes in the spleen can result from changes in the red cell membrane causing rigidity, from changes in the red cell shape restricting its ability to deform, or from alterations to the small vessels preventing passage of normal red cells even when deformed. Changes in the cell membrane or in cell shape usually lead to destruction of all affected cells in the spleen, whereas alterations to small blood vessels can cause destruction of red cells within the circulation itself, producing microangiopathic haemolytic anaemia. The ability of the cell to deform is also dependent on the fluid nature of the red cell contents. In this way, haemoglobin precipitation (in sickle cell disease) or haemoglobin denaturation (Heinz body formation)

results in a hold up of cells in the narrow vascular channels of the spleen and, consequently, in an increased rate of haemolysis (Gordon-Smith, 1972; Jacob, 1974). No abnormalities in red cell shape were uncovered in the present study, neither during the measurements of erythrocyte size (see Part 3, Chapter 2) nor during electron microscopic examination (see Part 4, Chapter 3), and it is thus considered that a more rigid red cell membrane represents the most probable cause of the more rapid haemolysis in the protein deficient rats. Increased rigidity of the membrane could be explained by a higher lecithin content, but the present study provided no evidence either for or against this as erythrocyte lipids were not assayed. The increased haemolysis and the reduced fragility of red cells from the protein deficient rats are likely to be related to the same type of defects in the structure of the red cell membrane.

Changes in the membrane structure may have some effect on the transport of materials across the membrane and Coward (1971) has observed a reduction in the passive permeability of the membrane both to glycerol and to thiourea in kwashiorkor. Such changes during protein deficiency might possibly also affect oxygen transport and thus have a further deleterious effect in addition to the anaemia itself, but it has not in fact been clearly established yet whether membrane permeability has a major

effect on oxygen movement into the erythrocyte (Gibson et al., 1955; Staub et al., 1961) or not (Kreuzer and Yabr, 1966).

CHAPTER 3 AN ELECTRON MICROSCOPIC STUDY OF THE EFFECTS OF PROTEIN DEFICIENCY ON ERYTHROCYTE STRUCTURE

CHAPTER 3

AN ELECTRON MICROSCOPIC STUDY OF THE EFFECTS OF PROTEIN DEFICIENCY ON ERYTHROCYTE STRUCTURE

The erythrocytes of rats fed on low protein diet (2 NDpCal%) and on control diet (10 NDpCal%) were studied by electron microscopy, in the hope that this might provide additional information unobtainable by optical microscope observations as the latter were unable to discriminate between the red cells of rats on low protein diet and those of control rats (refer to Part 3, Chapter 2).

Observations by an electron microscope might provide information on the nature of the structural defects in the erythrocyte membrane which are considered to be the cause both of the shortened survival time (Chapter 1 of this Part) and of the reduced osmotic fragility (Chapter 2 of this part) of erythrocytes from protein deficient rats (on 2 NDpCal% diet). Structural defects in the membrane might be observed whether these are related to differences in the protein components or to changes in lecithin content as suggested by Coward (1971). There have been no other reports of observations by electron microscopy of erythrocyte structure in protein deficiency, but any abnormalities found in this way might provide an insight into the relation between the structural defects and the observations on haemolysis and osmotic fragility.

PREPARATION OF ERYTHROCYTES FOR [illegible]

MATERIALS

1. Buffer solution

Sodium cacodylate ($(CH_3)_2AsO_2Na.3H_2O$)	21.4 g
Distilled water	500 ml

2. Solution for fixation

a. 3% glutaraldehyde fixative

Buffer solution	33 ml
25% Glutaraldehyde	12 ml
Distilled water	55 ml

Adjust to pH 7.4 (using 1M HCl or 1M NaOH).

b. 3% glutaraldehyde with Ruthenium Red (0.01%) fixative

Buffer solution	33 ml
Ruthenium Red (0.1%)	10 ml
25% Glutaraldehyde	12 ml

Adjust to pH 7.4.

N.B. Precautions taken in preparation of Ruthenium Red solution as detailed by Luft (1966).

3. Buffer for washing

Buffer solution	165 ml
Distilled water	335 ml

Adjust to pH 7.4 (using 1M HCl or 1M NaOH).

4. Post fixative solution

a. 1% OsO_4 fixative

OsO_4	0.2 g
Distilled water	13.4 ml
Buffer solution	6.6 ml

b. 1% OsO_4 with 0.01% Ruthenium Red

OsO_4	0.1 g
Distilled water	5.7 ml
Buffer solution	3.3 ml
0.1% Ruthenium Red	1.0 ml

In each case, the osmium tetroxide was allowed to dissolve in water overnight, before addition of buffer solution and adjustment to pH 7.4.

5. 2% agar

6. Ethanol at various concentrations (10% to 100%)

7. 0.5% uranyl acetate

8. Toluene

9. Araldite embedding medium (Durcupan ACM Fluka)

Epoxy resin	10 ml
964 Hardener	10 ml
Dibutyl phthalate	0.15 ml

These were mixed well, then 964 Accelerator (0.35 ml) was added, with further mixing. The mixture was degassed, then used on the same day or stored in a deep freeze until required.

Note : Each solution was adjust to pH 7.4 as this corresponds to the normal pH of erythrocytes (Altman, 1961).

METHOD

Step 1. Fixation

Three rats from each group (2 EDpCal% and control diet) were killed by cervical fracture, since the use of anaesthetic drugs might affect the ultrastructure of the red blood cells. Immediately after death, the blood from their hearts was collected into heparinised tubes, which were gently agitated before separation of the red cells from plasma by centrifugation.

The packed red cells from each rat were then divided into two portions. 3% glutaraldehyde fixative (approximately 3 volumes) was added to one portion, whereas the fixative solution (3 volumes) of 3% glutaraldehyde with Ruthenium Red (0.01%) was added to the other. One hour was allowed for complete fixation to occur. Ruthenium Red preferentially stains the membranes, specifically their polysaccharide components.

Step 2. Washing

The red cells in each tube were washed in four changes of solution, each for 15 minutes, with buffer for

washing, then allowed to stand for a minimum of 6 hours in a fifth change of solution. This process removed any excess glutaraldehyde.

Step 3. Post fixation

This was performed by addition of 1% OsO_4 fixative solution to the first tube and 1% OsO_4 with 0.01% Ruthenium Red to the second tube (that originally fixed with Ruthenium Red), and then leaving each mixture to stand for 30 minutes.

Step 4. Washing

Red cells in each tube were washed with two changes, each of 30 minutes, of buffer for washing to remove excess osmic acid.

Step 5. Agar pre-embedding

2% agar solution was mixed with the fixed red cells in a Pasteur pipette and allowed to set, then the resulting agar column was cut into small sections.

Step 6. Dehydration and staining

Progressively increasing concentrations of ethanol were used to dehydrate the agar sections, using nine changes of solution as follows :

Concentration of ethanol	Time (minutes)
10%	10
10% ethanol with 0.5% uranyl acetate	30
20%	10
40%	10
60%	10
70%	10
80%	10
90%	10
100%	10

N.B. Staining by uranyl acetate was performed simultaneously with the second of these dehydration stages. This stain is selective for nucleic acids, but also stains protein to a lesser extent.

Step 7. Transitional solvent work

The ethanol was then replaced by toluene, as the latter is easily miscible with araldite and thus assists its penetration into cells. Two changes of toluene, each of 10 minutes, were used to effect this replacement.

Step 8. Embedding

Araldite mixture was chosen for the final embedding. This was carried out by soaking the red cell sections for 30 minutes at 60°C (in warming cabinet) in

each of two changes of araldite medium, leaving overnight in a third araldite bath (with rotation) to ensure even penetration throughout the tissue, and finally encapsulating the specimens with a fourth araldite mix in Beem capsules by heating to 60°C for 72 h.

Step 9. Sectioning

An ultramicrotome (Reichert "Om U2") was used to cut 50–80 nm sections, which were mounted on copper grids. Some sections were stained with lead citrate for 5 minutes, using the method of Venable and Coggeshall (1965). Lead preparations stain a number of cellular components, while their most important characteristic for the present investigation is that they make cell membranes appear crisply defined. Sections were examined on an electron microscope (A.E.I.E.M. 801 or Zeiss E.M. 9A).

RESULTS AND CO[illegible]

Electron micrographs of erythrocytes from rats fed on low protein diet (2 NDpCal%) and on control diet are shown at various magnifications in Figures 1, 2 and 3.

No evidence of fragmentation of the erythrocytes from the protein deficient rats (on 2 NDpCal% diet) was found during the observations by electron microscopy. Also particular attention was paid to the membrane structure when the electron micrographs were examined, but no abnormalities in structure or differences from the erythrocytes of control rats (on 10 NDpCal% diet) could be detected. This type of examination should demonstrate any gross defects in membrane structure. It is considered, however, that some chemical defects in the composition of the membrane might result in only minor, and thus undetectable, modifications in the physical structure yet nevertheless have a profound influence on other properties of the erythrocyte, such as survival time and osmotic fragility. The present observations should not be regarded as implying rejection of the view that there was a structural abnormality in the erythrocyte membrane of the protein deficient rats, only as indicating that any abnormality present must have been below the resolution of the method adopted.

FIGURE 1

Electron micrographs (x 50,000) of erythrocytes of rats fed on :- (a). Control diet, (b). Low protein diet.

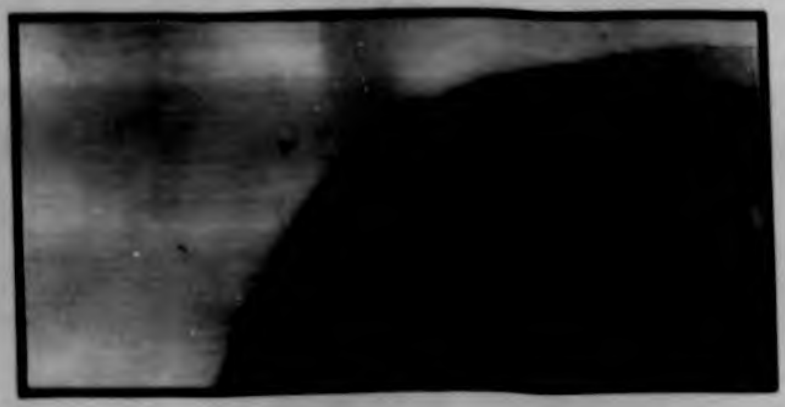
(a)

(b)

FIGURE 2

Electron micrographs (x 50,000) of erythrocytes fixed with Ruthenium Red of rats fed on :-
(a). Control diet, (b). Low protein diet.

(a)

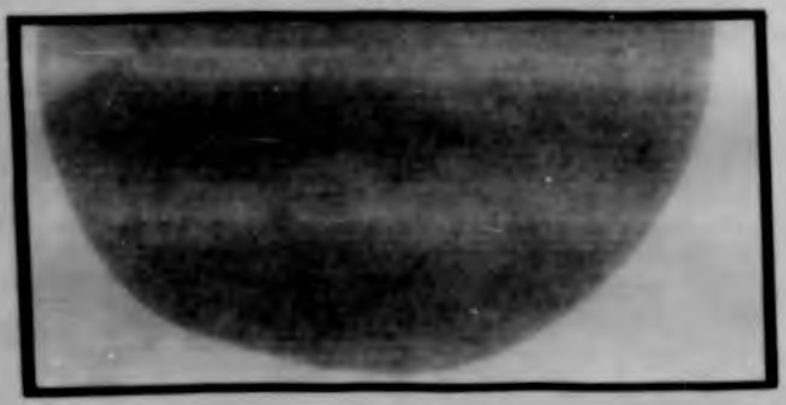


(b)

FIGURE 3

Electron micrographs (x 32,000) of erythrocytes fixed with Ruthenium Red of rats fed on :-
(a). Control diet, (b). Low protein diet.

(a)



(b)

CONCLUSIONS FROM PART A

The three main observations made in this part of the study were as follows :

1. Survival, in control receiver rats, of erythrocytes from rats fed on low protein diet (2 NDpCal%) was significantly shortened compared with survival of erythrocytes from rats on control diet (10 NDpCal%).

2. Erythrocytes of rats fed on low protein diet (2 NDpCal%) showed a significant decrease in osmotic fragility (increased resistance to haemolysis).

3. No abnormalities could be detected in the red blood cells of rats fed on low protein diet (2 NDpCal%) during observations by electron microscopy.

The observed decrease in fragility of the erythrocytes during protein deficiency would not be consistent with rupture of the cells in the circulation, so extravascular haemolysis is indicated as the principal cause of the shortened survival of these erythrocytes. It is suggested that there may have been a reduction in the deformability (increase in rigidity) of erythrocytes in rats fed on a low protein diet, with such a change resulting in a delayed passage through the reticuloendothelial system and thus allowing haemolysis and phagocytosis to occur. Although no abnormalities of the erythrocytes were apparent under the electron microscope, it is

thought that a structural defect, possibly related to an increase in membrane lecithin content (Coward, 1971), was responsible for the shortened survival of red cells from the protein deficient rats as the survival times were measured in receiver rats on control diet. Increased haemolysis, however, does not appear to be the principal cause of the anaemia in the protein deficient rats, as the shortening of erythrocyte survival time was inadequate to account for the observed reductions in red cell count, packed cell volume and haemoglobin level.

PART 5

ERYTHROPOIETIN LEVELS IN ANAEMIA INDUCED BY PROTEIN DEFICIENCY

CHAPTER 1 THE PURPOSE OF THE STUDY AND A REVIEW OF CURRENT KNOWLEDGE ABOUT ERYTHROPOIETIN.

THE PURPOSE OF THE STUDY

Anaemia is a major clinical manifestation of protein-energy malnutrition, as mentioned previously (refer to Part 3), and is often relatively refractory to treatment, thus presenting particular problems in this respect. The mechanism(s) of anaemia resulting from protein deficiency is still unknown. There is now, however, evidence that erythropoietin is normally the most important factor in the regulation of erythropoiesis and, consequently, in the maintenance of a relatively constant red cell mass in the circulation. An erythropoietic stimulus is required to ensure both that under physiological conditions the rate of production of new erythrocytes is equal to their rate of destruction, and that an increased production rate occurs in response to anoxia, high altitude, haemorrhage or haemolysis. There is strong evidence that erythropoietin provides this stimulus (Lewis, 1972).

Erythropoietin is a glycoprotein, with protein as its most prominent component but also containing hexoses, hexosamines and sialic acid. The purest available erythropoietin preparation that has been analysed chemically is from anaemic sheep plasma (Step V-450 units) and has been found to consist of approximately 71% protein and 29% carbohydrate (hexosamine and sialic acid)(Goldwasser et al., 1962). For human plasma erythropoietin, Kuratowska et al.

(1962)have reported a constituency of 85.8% protein and 14.2% carbohydrate; and erythropoietin, with activity 100-200 units/mg, obtained from human urine has been observed to contain 17-18 amino acids (Lowy and Knighly, 1968). Since the major component of erythropoietin is protein, it is possible that protein deficiency might directly influence the erythropoietin level, but this need not be the case as protein usage in erythropoietin production should represent only a very small proportion of the whole body protein usage. The available evidence in regard to the effects of protein deficiency on erythropoietin is scanty and often incomplete owing to a lack of a suitable erythropoietin standard for purposes of comparison, of a really effective indicator and of a sensitive method for the measurement of erythropoietin.

Murthy (1965) and El Ridi (1963) have both estimated erythropoietin levels in kwashiorkor and marasmus, Murthy using reticulocyte response induced in starved rats while El Ridi used the incorporation of ^{59}Fe into red cells of starved rats. The reticulocyte response is not suitable as an indicator, however, since it may also be induced in the normal animal by some non-specific stimuli (Seip, 1953). In addition, difficulties arise in the starved rat assay owing to its sensitivity to factors in the blood unrelated to the amount of erythropoietin, including such factors as protein contained in the injected materials. Realistic

interpretation of results became possible only with the use of a purified fraction or of suitable controls for these non-specific factors (Gordon and Weintraub, 1962). McKenzie et al.(1967) have measured erythropoietin levels in kwashiorkor by using ^{59}Fe incorporation into erythrocytes of polycythaemic mice and this remains the best method available at the present time. One disadvantage with their results was that they were not expressed in International Erythropoietin Standard Units, making comparison with other results impossible. Fortunately, an International Erythropoietin Standard is now available and the method has been improved in sensitivity sufficiently to allow normal, and even subnormal levels, of urinary excretion of erythropoietin to be convincingly demonstrated (Finne, 1965; Alexanian, 1966; Van Dyke et al., 1966; Adamson and Finch, 1968). Nevertheless, the technique is still not sensitive enough to measure normal plasma levels, although erythropoietin can be readily detected if the level is increased to three times the normal.

The purpose of this part is to attempt to evaluate any change in the pathophysiological erythropoietin mechanism resulting from protein deficiency. Since erythropoietin is considered to be the major factor controlling the red cell mass in the circulation and thereby normally counteracting the development of anaemia, any change in the erythropoietin system could have a

fundamental significance in the onset of anaemia under condition of protein shortage.

Earlier work, which had led to the understanding from which the present experiments were developed, will first be reviewed.

1. Background work on erythropoietin.
2. Fundamental stimulus of erythropoiesis.
3. Site(s) of erythropoietin production.
4. Mechanism of action of erythropoietin.
5. Influence of protein deficiency on erythropoietin and on erythrocyte production.
6. Relation of erythropoietin secretion to clinical anaemias.

CURRENT KNOWLEDGE ABOUT ERYTHROPOIETIN

1. BACKGROUND WORK ON ERYTHROPOIETIN

About a hundred years ago, Bizzozero (1868) and Neumann (1868) recognised that bone marrow was the site of blood cell formation and about twenty years ago the constancy of the circulating red cell mass was noted by Grant and Root (1952). However, the requirements for the maintenance of a stable red cell concentration by regulation of the rate of erythropoiesis were unknown.

It was observed that there was an increase in red cell production at high altitude where there was a reduced oxygen supply (Bert, 1882; Bancroft et al., 1923; Grant and Root, 1952) and it was therefore suggested that a deficiency in the oxygen supply to the bone marrow acted as a direct stimulus to red cell production. This was confirmed experimentally by subjection of animals to hypoxia, a subsequent increase in erythropoiesis being noted (Gordon and Kleinberg, 1937; Stickney et al., 1943; Altland and Highman, 1951). An increase in erythropoiesis resulted from atmospheric or anaemic hypoxia, whereas hyperoxia and plethora, created by the transfusion of red cells, caused a reduction in erythropoiesis (Krumbhaar and Chanutin, 1922; Campbell, 1926; 1927; Reinhard et al., 1944; Tinsley et al., 1949). Experimental measurement, by Grant and Root (1947; 1952), of marrow oxygen concentration did not substantiate

the suggestion that this regulated the rate of erythropoiesis.

Carnot and Deflandre's original hypothesis, in 1906, was that erythropoiesis was not controlled by direct deoxygenation of the bone marrow but instead by a humoral mechanism, a factor of which was elaborated outside the marrow and secreted into the blood in response to hypoxia. This suggestion resulted from a demonstration of an erythropoietic effect in a normal rabbit when injected with plasma from donor rabbits rendered anaemic by bleeding. This hypothesis began to receive a great deal of attention and, in 1950, Reissmann verified these findings by exposure to air at low oxygen tension of one member of a pair of parabiotic rats while the other partner breathed normal air. Erythropoietic stimulation resulting in polycythaemia occurred in both animals, indicating the passage of a humoral erythropoietic substance (humoral factor) from one partner to the other. Interest in this erythropoietic substance was stimulated by the work of Erslev (1953), who demonstrated an erythropoietic effect in an animal injected with large volumes of anaemic plasma, and by the work of Stohlman et al. (1954), who studied a patient with a patent ductus arteriosus and reversed blood flow, a situation in which hypoxia (and cyanosis) occurred in the lower half of the body while the upper half was oxygenated normally. The

latter investigators found that erythropoietic hyperplasia developed not only in the bone marrow of the hypoxic area but also in the normally oxygenated areas of the marrow. They concluded that erythropoiesis was not controlled by the local marrow oxygen tension but rather by a humoral factor produced below the diaphragm. With the increase in experimental sophistication, there were shortly many reports confirming Carnot and Deflandre's hypothesis. Various workers injected large amounts of plasma from anaemic animals into normal animals and observed an increase in reticulocyte count (Eralev, 1953; Bornook et al., 1954; Gordon et al., 1954; Hodgson and Toha, 1954; Gray and Eralev, 1957) and Plzak et al. (1955) found an increase in ^{59}Fe incorporation into red cells. The plasma factor that increased erythropoiesis was termed haemopoietin by Carnot and Deflandre in 1906. As work proceeded, however, it appeared that this circulating factor was exclusively involved in red cell production (White et al., 1960) and it became referred to as the erythropoietic stimulating factor (ESF) or erythropoietin (EP), a name first suggested by Bonsdorff and Jalavisto in 1948.

The application of radioisotope technology to this field twenty years ago (Plzak et al., 1955) simplified erythropoietin assay and increased accuracy. Experimentation was broadened as an adequate quantitative end-point

was found for the estimation of erythropoiesis produced by this hormone. Studies of the chemical nature of erythropoietin, its metabolism, site of production, mechanism of action and its relation to physiological control of erythropoiesis were helped by the introduction of a quantitative assay. Erythropoietin became a means of obtaining much basic knowledge about erythropoiesis. There have been many reports indicating that erythropoietin is the main factor in the initiation and regulation of erythropoiesis and that it can control the process of differentiation itself (Jacobson et al., 1957; Alpen and Cranmore, 1959; Erslev, 1959; Lajtha et al., 1962; Krantz et al., 1963; Krantz and Goldwasser, 1965). Observations with this hormone have provided much of the available information regarding the regulation of erythropoiesis, although this information is still very incomplete.

2. FUNDAMENTAL STIMULUS OF ERYTHROPOIESIS

A direct correlation, within wide limits, between the erythropoietin level and the duration or degree of the hypoxic stimulus has been shown by studies of hypoxic hypoxia (Gurney et al., 1965; Siri et al., 1966; Carmena et al., 1967), while other workers have demonstrated a direct relationship between the degree of anaemia and the erythropoietin level in plasma and urine (Van Dyke et al., 1961; Eskuche and Hodgson, 1962;

Hammond et al., 1962; Hammond and Keighley, 1962; Gordon et al., 1964; Weintraub et al., 1964; Okcuoglu and Jones, 1966; Movassaghi et al., 1967). Grant and Root (1952) stated that tissue hypoxia acts as the basic stimulus to erythropoiesis and thus to a change in size of the erythrons. Many reports have confirmed that erythropoietin production or secretion was increased by local hypoxia of the kidney as a result of constriction of the renal artery (Takaku et al., 1962; Fisher et al., 1965; Matsumuto, 1965; Murphy et al., 1966; 1967a; 1967b; Fisher and Samuels, 1967; Fisher et al., 1967). The elevation of erythropoietin level in anaemia was found to be reversed, and erythropoiesis to be decreased, by hyperoxia (Jepson and Lowenstein, 1966; Linman and Pierre, 1968) and by plethora (Jacobson et al., 1957; Gurney et al., 1958; Adamson and Finch, 1966). From this evidence, it can be assumed that erythropoiesis is stimulated under conditions where oxygen supply is insufficient to meet oxygen demand and that it is allowed to subside when the supply exceeds the demand.

It has been noted that, irrespective of the oxygen supply, conditions that vary the oxygen body need also change the level of erythropoiesis (Fried et al., 1956; Jacobson et al., 1957). A decrease in metabolic rate, as seen with hypophysectomy (Crafts and Meineke, 1957; Evans et al., 1957; Meineke and Crafts, 1959), starvation

(Morgulis, 1923) or hypothyroidism (Crafts and Heineke, 1957; Evans et al., 1957; Heineke and Crafts, 1964), reduced the oxygen demand relative to a constant oxygen supply and was followed by reduced erythropoiesis (Bomford, 1938; Jacobson et al., 1959; 1960; Aschkenasy, 1963). Indirect evidence suggests that under these conditions erythropoietin levels are lower than normal and as a result lead to the onset of anaemia (McCarthey et al., 1959; Aschkenasy, 1963; Reissmann, 1964; Bozzini and Kofoed, 1966; Ito and Reissmann, 1966). In this way, the rate of oxygen supply and demand, at the site controlling erythropoietin production, appear to determine the level of erythropoiesis (Fried et al., 1957; Jacobson et al., 1957; 1959).

3. SITE(S) OF ERYTHROPOIETIN PRODUCTION

There have been numerous attempts to identify the site, or sites, at which the erythropoietic factor is produced. Excision of the spleen, the endocrine organs (thymus, thyroid, pituitary, adrenals, gonads, pancreas), stomach, intestinal tract and 90% of the liver did not abolish the ability to produce erythropoietin. It is impossible to remove some vital organs, such as lungs, brain, liver and heart, but extracts of these organs have not shown any erythropoietic activity. Studies of these tissues thus did not reveal the site of erythropoietin production. Evidence suggesting that the

kidney has an important role in erythropoiesis, and that it possibly represents the site of erythropoietin production, arose both from animal experiments by Jacobson et al. (1957) and from numerous clinical observations that anaemia often accompanies renal disease, with an inappropriate plasma erythropoietin response and polycythaemia both frequently being found to be associated with renal tumours and cysts (Stohlman, 1968).

It is now considered that the kidney is the major site of origin of the factor that results in the production of active erythropoietin and that it also represents the major site controlling the production of the hormone. Jacobson et al. (1957a; 1957b; 1960) first demonstrated that after bilateral nephrectomy rats and rabbits no longer showed markedly increased levels of plasma erythropoietin in response to bleeding or administration of cobalt, and that the increased erythropoietin levels which followed phlebotomy of rabbits fell to near normal after nephrectomy (Jacobson et al., 1957b). Control animals for these experiments consisted of ureter-ligated rats, which developed similar blood urea nitrogen levels but retained their ability to respond to cobalt or phlebotomy. Further experiments showed that nephrectomized rats responded only slightly to hypoxic hypoxia with a small increase in erythropoiesis

(Goldwasser et al., 1958), while nephrectomized mice showed a very slight response to a phenylhydrazine-induced anaemia (Jacobson et al., 1959). From these experiments it was estimated that about 10% of erythropoietin production was controlled by extrarenal sources (Jacobson et al., 1959; Jacobson, 1962). These results have been confirmed by many investigators and, moreover, Naets (1958a; 1958b; 1958c) has reported a large depression of erythropoiesis in nephrectomized dogs despite peritoneal dialysis to remove accumulated toxic products. No increase in serum erythropoietin levels, determined by assaying in fasted rats, was observed if the dogs were subjected to phlebotomy simultaneously with nephrectomy (Naets, 1959; 1960a; Naets and Heuse, 1964). Moreover, dogs showed a marked disappearance of erythroblasts (Naets, 1960b) and a decreased ^{59}Fe incorporation into circulating erythrocytes (Muirhead et al., 1968) following nephrectomy.

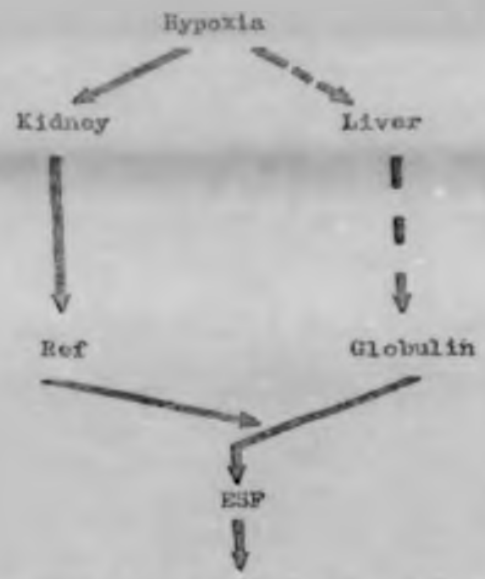
It appears that the kidney is the only site of erythropoietin production in some species of rodents and in dogs (Stohlman, 1968), but that a limited amount of extrarenal production can occur in some other species (Jacobson et al., 1959; Erslev, 1960; Nathan et al., 1964). Nephrectomy of rabbits was found to greatly reduce the plasma iron turnover rate and the

reticulocyte response to phlebotomy, but it did not abolish these effects (Erslev, 1958; 1960; 1964). Since these indices of erythropoiesis could be reduced still further by hyperoxia (Erslev, 1960) or by plethora (Erslev, 1964), it was suggested that a low level of erythropoietin-regulated erythropoiesis still persisted after nephrectomy. Moreover, studies of anephric man have clearly indicated that man also is able to support red cell production in the absence of his kidneys, although only at a reduced level, and that erythropoiesis can be increased and an increased level of erythropoietin can be demonstrated in response to hypoxaemia, augmented anaemia or androgen administration (Nathan et al., 1964; McCain, 1965; Naets and Wittek, 1968a; Mirand et al., 1968; 1969a; 1969b; Mirand and Murphy, 1969). In rat and baboon in the renoprival state, extrarenal production of erythropoietin has been demonstrated to occur in proportion to the severity of hypoxic stimulus (Fried et al., 1969; Mirand et al., 1969c). It is not yet known whether extrarenal sites of erythropoietin production are normally present and functional or whether they develop as an adaptive response in the period following nephrectomy.

Studies by Kuratowska et al. (1964) and Gordon et al. (1967) indicated that the kidney does not itself

produce intact erythropoietin but produces a factor, which is devoid of vasopressor or erythropoietic activity but which is capable of acting upon a component of normal plasma to produce active erythopoietin. Gordon et al. (1967) and Zanjani et al. (1967a) suggested that the renal factor, known as the renal erythropoietic factor (REF), behaves as an enzyme in its action upon a substrate present in normal plasma to produce erythropoietin. Numerous reports have confirmed that the biological activity of the material resulting from this reaction can be neutralised by an antibody to erythropoietin (Zanjani et al., 1968), although this antibody does not combine either with REF or with the plasma substrate (McDonald et al., 1969; Schooley et al., 1970). This should indicate that the factor generated was erythropoietin (EP). The site of synthesis of REF in the kidney has been investigated by Zanjani et al. (1967b) who observed REF to be in the light mitochrondrial fraction, with an equal distribution throughout the cortex and medulla of the kidney, while Wong et al. (1968) also found the primary location of REF to be in this fraction although smaller amounts were located in the microsomal fraction of kidneys from rats rendered hypoxic. It is suggested that REF is generated in the microsomal cell fractions and transported to the light mitochrondrial fraction for storage (Cantor et al., 1969).

Evidence has been obtained from timed studies and kinetic experiments with labelled amino acids, indicating that the increase in REF as a result of hypoxaemia precedes that of plasma erythropoietin by several hours (Gordon et al., 1967). The incorporation of labelled amino acid (^{14}C-isoleucine) into kidney proteins during hypoxaemic stimulation increased only at the onset of hypoxia, whilst their incorporation into liver and plasma proteins was consistently increased during hypoxaemia (Katz et al., 1960). Moreover, the time-course of the changes in incorporation into plasma proteins correlated with the time-course of the increases in erythropoietin titre in plasma. It was therefore proposed that the liver produces a factor which is converted into active erythropoietin (EP) by the enzymatic action of REF. A kidney (REF)-liver (serum substrate) axis is considered to be operative in the control of erythropoiesis, as shown by the following diagram (Gordon, 1959, Gordon et al., 1967; Gordon and Zanjani, 1971).



(Proposed scheme for the renal-hepatic axis involvement in the production of the ESF).

Other investigators have demonstrated that, under appropriate conditions, the kidney releases renal erythropoietic factor (REF) whose interaction with a plasma globulin results in the formation of active erythropoietin (Kuratowska et al., 1964; Kuratowska, 1968).

It is evident that the kidney is able to respond directly to hypoxia. After successful kidney transplantation in man, erythropoiesis is improved and erythropoietin titres (in plasma and urine) increase towards normal (Denny et al., 1966; Thompson and Denny, 1963) and may even

become elevated above normal levels during anaemic stress or local hypoxia due to vascular changes and the rejection phenomenon (Niles et al., 1965; Abecht et al., 1968; Mirand et al., 1969c). The denervated kidney is therefore capable of response, and further evidence of this has been provided by perfusion of isolated dog kidneys (in vivo and in vitro) with blood at normal and reduced oxygen tensions and with blood containing testosterone or cobalt (Pavlovic-Kentera et al., 1965; Fisher and Samuels, 1967; Fisher and Langston, 1968). No changes in erythropoietin titres were found when perfusing blood at normal oxygen tensions, while hypoxaemic blood was associated with significantly increased titres. When cobalt or testosterone (in dog kidneys pretreated with testosterone) were added to the perfusing blood at normal oxygen tension, increases in erythropoietin levels were induced, and in each case the effect was more marked when the perfusate was hypoxaemic. No significant damage was revealed on histological examination of the kidneys in some of the above studies (Fisher and Langston, 1967; 1968), where there was thus satisfactory evidence that the increased erythropoietin titres resulted from a direct stimulatory effect on the kidneys rather than from a release of damaged cells. Various conditions known to stimulate erythropoiesis and erythropoietin production, such as hypoxia, anaemia and cobalt and testosterone treatment, are accompanied in animals by increased amounts

of REF; in contract, hyperoxaemia and polycythaemia are accompanied by decreased amounts (Gordon et al., 1966; 1968; Zanjani et al., 1968). Since REF has been isolated from normal kidneys, it is presumed to be operative in the day-to-day regulation of erythropoiesis as well as during conditions of stress.

The site of extrarenal erythropoietin production has not been located, although as the result of several studies it is suggested that the liver is involved (Burke and Morse, 1962; Reissman and Nomura, 1962). Fried (1972) has reported that nephrectomized rats exposed to intense hypoxia produced sufficient erythropoietin to increase, detectably, their plasma erythropoietin titres, but if such animals were also subjected to 80% hepatectomy before being made hypoxic at 0.465 atmospheres extrarenal erythropoietin production was no longer detectable. Extrarenal erythropoietin production at 0.435 atmospheres was barely detectable in these partially hepatectomized animals and remained significantly lower than in the control nephrectomized animals. These results suggest that the liver plays an important role in the extrarenal production of erythropoietin.

4. MECHANISM OF ACTION OF ERYTHROPOIETIN

Alpen and Cranmore (1959a; 1959b) and Eraley (1960), first proposed that the initiation of normal red cell

differentiation from the primitive stem cell was closely associated with erythropoietin. The soundness of this view was convincingly demonstrated by the studies of Filmanowicz and Gurney (1961), Orlic et al. (1968) and Perretta and Tipapegui (1968) involving the administration of a single dose of erythropoietin to animals in which erythropoiesis had been virtually eliminated by hypertransfusion. The experiments of Filmanowicz and Gurney (1961) were performed in mice, animals in which the spleen is an important and active organ of haematopoiesis. The animals were made polycythaemic by hypertransfusion so that splenic erythropoiesis was eliminated, according to morphological and biological evidence. No change was apparently caused to the animals' haematopoietic system, since active erythropoiesis recommenced promptly at a fixed time interval after erythropoietin stimulation, irrespective of the duration of suppression. After the injection of erythropoietin, serial biopsy studies demonstrated the presence of proerythroblasts at 24 hours and maturation had proceeded to normoblasts, early reticulocytes and release of adult cells by 72 hours. Beginning with the appearance of the earliest recognisable cell of the erythrocyte series (where none was previously present), a wave of erythropoiesis swept through the spleen and progressed through the maturation stages to the formation of adult red cells. As soon as this group of cells had

matured and passed into the peripheral blood, the spleen was again left void of recognisable erythropoietic elements if no additional erythropoietin was given. These observations provide strong evidence that erythropoietin initiates the differentiation of a primative stem cell into recognisable red cell precursors and that normal maturation then follows with adequate nutrition. In the absence of recognisable red cell precursors it appears that the stem cells remain in a self-maintaining cycle, and in the presence of erythropoietin are always capable of responding by giving rise to red cells. There is a possibility that it is only at some specific stage in its cycle that the stem cell can respond to erythropoietin stimulation (DeGowin, 1967; Reisenman and Samarapoompichit, 1969).

Judging from the rate of growth of erythroid colonies and from the cell numbers at various points in the growth curve, O'Grady et al. (1968) concluded that these colonies could not have developed from single primitive stem cells but, more probably, from groups of committed cells, whose development was initiated in the absence of erythropoietin but then required erythropoietin for full development into recognisable erythroid cells. Bruce and McCulloch (1964), Stohlman (1967) and Stohlman et al. (1968) have suggested that two types of stem cells may exist, the most primitive being the mutipotential stem cell that can give rise to

any haematopoietic cell type, the other being a still unrecognisable cell which, however, is already irreversibly committed to one route of maturation (erythroid, myeloid or megakaryocyte). If this idea is true then erythropoietin would act on the committed cells, stimulating their differentiation into recognisable erythroid elements.

From the available experimental evidence, McCulloch (1970) has formulated a model for the cellular events in haemopoiesis (Figure 1). The pluripotential stem cell is shown in two physiological states, "rest" and "cycle", these being separated by a reversible transition with the transfer from "rest" to "cycle" referred to as "triggering". Cycling colony-forming cells experience self-renewal or may "decide" to follow a specific route to differentiation. The "decision" step is regarded as an irreversible change resulting in distinct classes of early differentiated cells. In the case of erythropoiesis, these early differentiated cells are referred to as erythropoietin-sensitive cells and there is evidence that they cycle continuously even when erythropoiesis is suppressed (Hodgson, 1967; Lajtha, 1967), but the production of mature differentiated cells from these cells requires the operation of a further control mechanism. Thus, having decided to enter a specific route, "action" is required for this "decision" to take effect. For erythropoietin-responsive cells to undergo a further irreversible transition, into

FIGURE 1

Model for control of proliferation and differentiation in haematopoietic system (from McCulloch, 1970).

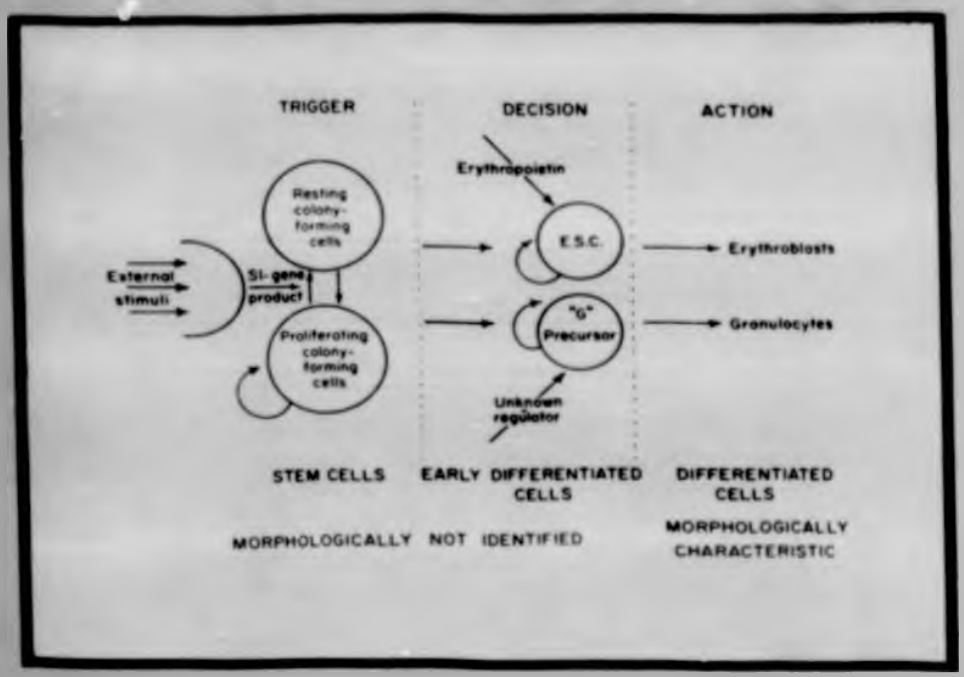
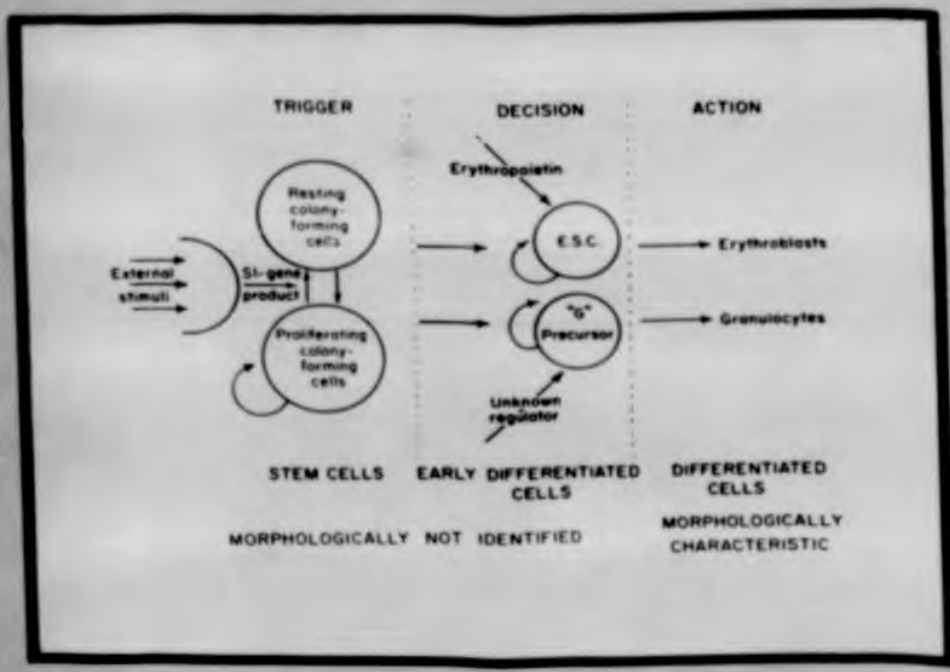


FIGURE 1

Model for control of proliferation and differentiation in haematopoietic system (from McCulloch, 1970).



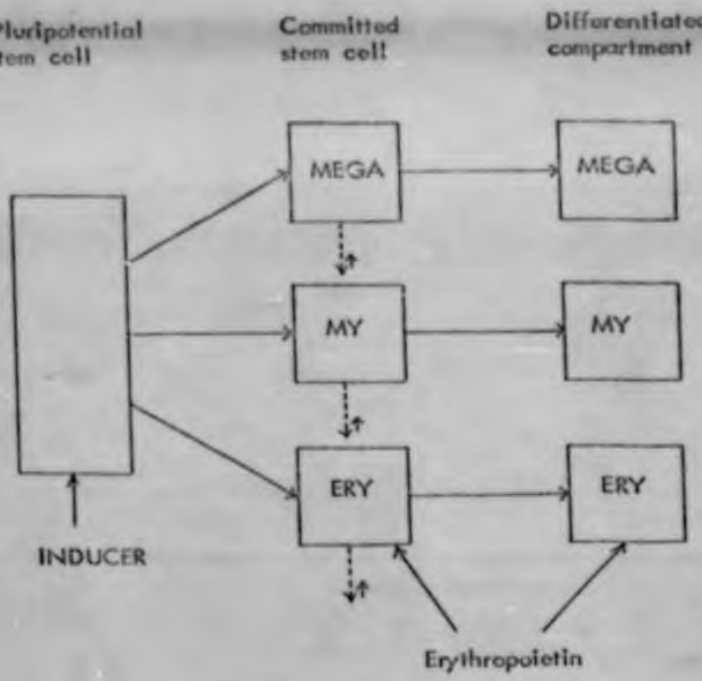
iron-incorporating erythroblasts, erythropoietin is needed. Three definite sites for the action of control mechanism are proposed in this model - the reversible "triggering" of resting stem cells into cycle, the "decision" of the stem cells to become differentiated, and "action" on this decision under the influence of specific regulators. Of all the suggested external regulators, however, only erythropoietin has been demonstrated with certainty, whereas genetic evidence has been invoked in order to postulate the existence of the Sl-gene product, which is apparently an external regulator of "triggering", and internal regulators controlled by the W and f loci.

There is agreement among those engaged in attempting to recognise different kinds of stem cell that additional studies and control are necessary, that no one interpretation can be regarded as final and, until it is possible to isolate and utilise pure stem cell populations, that many questions will remain unanswered.

Stohlman (1967) and Stohlman et al. (1968) proposed and developed the concept that there are two stem cell compartments, the first being pluripotential and capable of becoming one of three similar morphological types of committed stem cell, as shown in Figure 2. According to their hypothesis, most of the pluripotential stem cells do not participate in the cycle of development (Figure 3) until depletion of a committed stem cell compartment leads

FIGURE 2

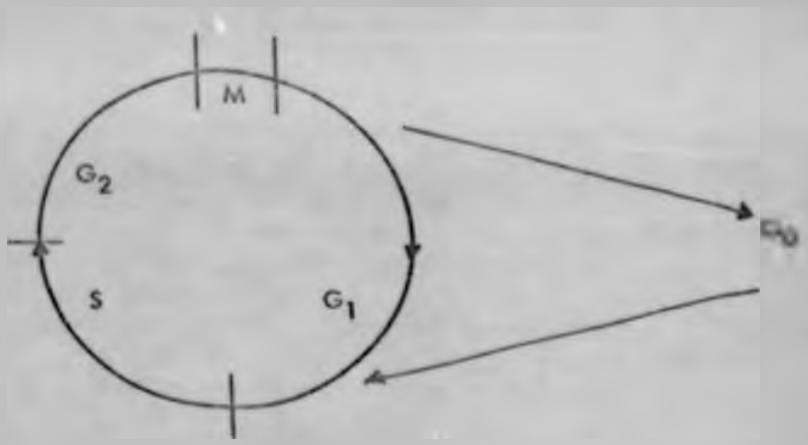
A CHEMATIC MODEL OF HAEMATOPOIESIS



MEGA = Megakaryocytic
MY = Myelocytic
ERY = Erythropoietic
Dashed line and cross indicate cell death (After Stohlman, 1967)

FIGURE 3

THE CELL CYCLE



G_1	Interval between mitosis and synthesis of DNA
S	Period of synthesis of DNA
G_2	Interval between synthesis of DNA and mitosis
M	Mitosis
G_0	Prolonged interphase with cell out of cycle

G_0 may be regarded as a variably long G_1

to its repletion from the pluripotential compartment. Against this view, Kubanek et al. (1968) demonstrated that, 24 hours after stimulation by large doses of erythropoietin, the splenic colony-forming units of donor mice were increased by a factor of 5 over the controls. They concluded that the stem cell participates in the response to erythropoietin without a depletion occurring.

The mechanism by which erythropoietin affects the committed stem cell has been the subject of much debate and discussion, especially as regards the relation of the regulatory mechanisms to the generative cycle and the means of preventing depopulation of this compartment. Lajtha (1964) has suggested that erythropoietin causes differentiation only of cells outside the cycle (G_0 in Figure 3) and triggers remaining cells into cycle. He also proposed that, once in cycle, cells are no longer dependent on this erythropoietin differentiation, thus preventing depopulation of the compartment. In contrast, Kratchmer (1966) used an analog computer to produce a model in which most of the committed stem cells are in active cycle, but, in the plan of Figure 3, they would have a variable G_1 phase. For erythropoietin to be effective in causing differentiation, it would have to be present during G_1 and a part of S. He further suggested that, since erythropoietin has a limited intracellular life span, in some cells erythropoietin would be present in G_1 but be metabolised before the cell entered

S thus preventing differentiation.

The two hypothesis of Lajtha (1964) and Kretchmer (1966) are clearly mutually exclusive, and subsequent experiments have helped to clarify which of these concepts is more realistic. Morse et al. (1970) used the drug hydroxyurea (OHU), which is a cytotoxic agent that kills cells in DNA synthesis (S phase), to investigate the relationship of erythropoietin differentiation to the generative cell cycle. When erythropoietin and OHU were given simultaneously to hypertransfused mice, a 20% reduction in erythroid response was observed compared with erythropoietin treated controls, indicating that committed stem cells are normally in cycle, even in hypertransfused animals, although possibly in a prolonged G_1. On increasing the interval between erythropoietin and OHU administration, an increasing kill was observed, implying that the ratio of synthesis time to total time for cycling was variable and controlled in some way by erythropoietin. A variable G_1 phase seemed to be the most likely explanation of these results. The conclusion was that erythropoietin was effective on cells in cycle suggesting that cells could be differentiated during S. Schooley (1966) found that one of the earliest response to erythropoietin was a shortening of G_1 and considered that "recruitment" of cells for differentiation is partly achieved in this way. The data provided by these studies were consistent with

Kretchmer's hypothesis, although it was not possible to deduce from these investigations whether erythropoietin would need to be present both in G_1 and in S. It appears, therefore, that the committed stem cell has a variable generation time due to a variable G_1. One effect of erythropoietin is a shortening of G_1, thus increasing the number of cells available for differentiation and inducing and erythroid response. It is not yet clear how depopulation of the compartment is avoided.

All these studies agree that the normal physiological function of erythropoietin is to initiate differentiation of the stem cell (multipotential or committed) into recognisable erythroid elements from which arise normal red cells with normal life spans. Under conditions of unusual stress when levels of erythropoietin may be greatly increased, however, it can also affect already differentiated cells and cause premature release to the peripheral blood of reticulocytes that are abnormally large, apparently because they have missed one or more mitotic divisions in their development. Such large reticulocytes are abnormal in that they have a significantly reduced life span and a changed cell membrane with enchanced anti-i activity (Hillman and Giblett, 1965; Card et al., 1969).

On perfusing isolated hind limbs of rabbits with control rabbit blood and with blood containing erthropoietin, Fisher et al. (1965) demonstrated a release of

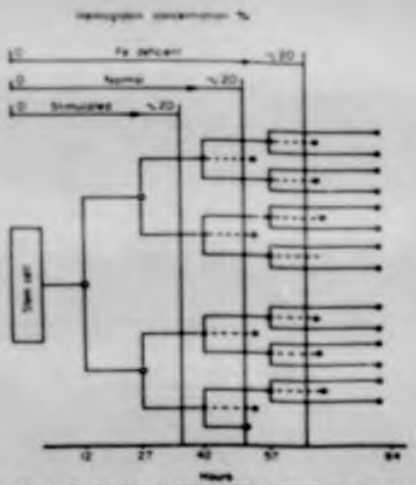
reticulocytes and of nucleated red cells from the marrow when the perfusate contained erythropoietin. Iron stores in the bone marrow were labelled prior to perfusion by injecting ^{59}Fe-labelled ferric citrate so that the release of nucleated red cells could be assessed from the ^{59}Fe content of the perfused blood. From the results of this and previous experiments (Fisher et al., 1964), these investigators deduced that erythropoietin affects the erythron in several other ways in addition to its effects on the stem cells. First, it can cause release of marrow reticulocytes, probably of mature reticulocytes from the marrow pool. Secondly, haemoglobin synthesis in existing normoblasts may be stimulated by erythropoietin, thus promoting the shortcut route for erythrocyte production with skipped mitotic divisions and possibly adding to the increased reticulocyte numbers. Thirdly, an increase in the absolute numbers of normoblasts in the marrow may be produced by erythropoietin stimulation.

Lajtha and Oliver (1960) and Stohlman (1967; 1968) have also proposed, in addition to its effect in differentiating the precursor cell, that erythropoietin affects the differentiated cells of the red cell series which are still capable of synthesising RNA, these including members of the series from proerythroblasts to early normoblasts. The most plausible mechanism for this would be initiation of haemoglobin synthesis by erythro-

poietin, with the subsequent rate of synthesis also governed by the availability of erythropoietin within the cell. A negative feedback system has been postulated to limit nucleic acid synthesis when a critical cytoplasmic haemoglobin concentration (CHC), about 20%, is attained (Stohlman, 1967; Stohlman et al., 1968). A model has been proposed by Stohlman et al. (1964; 1968) to incorporate these features (Figure 4). In this, the general time of differentiated erythroid precursors is considered to be fixed (Alpen and Cronmore, 1959a; 1959b; Nielsen et al., 1964), although the model would be relatively unaffected if small changes in generation time were caused by erythropoietin stimulation. It is suggested that intracellular haemoglobin concentration is responsible for shutting off nucleic acid synthesis and, ultimately, protein synthesis as well. Not only are more cells differentiated into the erythroid compartment but also the rate of haemoglobin synthesis is accelerated when there is an excess of erythropoietin, due to exogenous administration, severe anaemia or hypoxia, for example. With the proposed mechanism of control, acceleration of haemoglobin synthesis together with a fixed generation time would result in a cessation of nucleic acid synthesis earlier than normal, relative to the stage of differentiation, and consequently in a skipping of the terminal cell division and production of macrocytes. In contrast, a reduction in the rate of

FIGURE 4

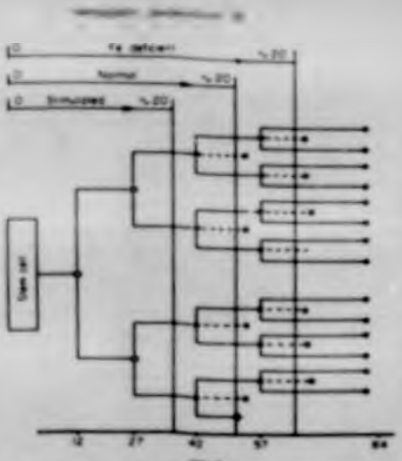
(After Stohlman et al., 1968).



A model of the kinetics of erythropoiesis in which it is proposed that when a cytoplasmic hemoglobin concentration of ~20% is achieved a negative feedback is initiated which shuts off DNA synthesis and division. The average generation time of the differentiated erythroid precursor is fixed so that changes in the rate of hemoglobin synthesis alter the numbers of divisions. Ineffective erythropoiesis is indicated in the lowest pathway. Reproduced with the permission of the N.Y. Acad. S...

FIGURE 4

(After Stohlman et al., 1968).



A model of the kinetics of erythropoiesis in which it is proposed that when a cytoplasmic hemoglobin concentration of ~20% is achieved a negative feedback is initiated which shuts off DNA synthesis and division. The average generation time of the differentiated erythroid precursor is fixed so that changes in the rate of hemoglobin synthesis alter the numbers of divisions. Ineffective erythropoiesis is indicated in the lowest pathway. Reproduced with the permission of the N.Y. Acad. Sci.

haemoglobin synthesis, even if in the presence of elevated erythropoietin, prolongs the period for nucleic acid synthesis with the result that additional cell divisions occur and microcytes are produced. This behaviour occurs in iron deficiency anaemia, where availability of iron becomes the rate-limiting factor which causes the interval between differentiation and attainment of the critical CHC value to be extended. The morphological characteristics of erythrocytes predicted by this method (Figure 4) to be found during the treatment of severe iron deficiency anaemia by administration of varying doses of iron are in good agreement with the results of such experiments on animals and on human beings (Stohlman, 1960; Brecher and Stohlman, 1961; Stohlman et al., 1963; Leventhal and Stohlman, 1966). Erythropoietin production was high and iron was the rate-limiting factor in haemoglobin synthesis before treatment, and, when iron was given in varying doses, restoration of haemoglobin to normal values was achieved at a rate dependent on the dose of iron. With administration of high doses of iron, sufficient to no longer limit the rate of haemoglobin synthesis, a macrocytic response was observed. When low doses were given, microcytes continued to be produced, while intermediate doses resulted in the production of normocytes.

Many attempts have been made to pinpoint the biochemical mechanisms involved in the action of erythro-

poietin on bone marrow and on erythroid cells. In vivo studies have indicated very rapid incorporation of specific labelled compounds into DNA and RNA of the haematopoietic tissues upon administration of erythropoietin (Hodgson, 1967; Rudolph and Peretta, 1967; Hodgson and Nakuche, 1968; Orlic et al., 1968). Hodgson (1967) demonstrated incorporation of ^{3}H-labelled uridine into RNA of polycythaemic mouse spleen within 2 hours after erythropoietin administration and uptake of ^{3}H-thymidine into DNA beginning 12 hour after erythropoietin, with both these effects observed before ^{59}Fe incorporation into haemoglobin could be detected. Associated with these changes in DNA and RNA, there are increases in the enzymes required for nucleic acid synthesis, in DNA polymerase, RNA polymerase and thymidilate kinase, and additionally an increase in the enzyme ALA synthetase which is rate-limiting for haem synthesis (Bottomley and Smithes, 1968; 1969; Nakao et al., 1968). In vitro effects of erythropoietin demonstrable on bone marrow cultures include stimulation of haem synthesis (Erslev, 1964; Dukes and Goldwasser, 1965b; Fukioka, 1966; Erslev and Silver, 1967; Powsner and Burman, 1967; Krantz and Fried, 1968; Miura et al., 1968; Peretta and Tipapegiu, 1968; Hrinda and Goldwasser, 1969) and incorporation of amino acids (glucosamine) both into a stroma-like fraction and into the lipid fractions of the cells (Dukes and Goldwasser, 1965a; Dukes, 1968). The latter effect is particularly

interesting in view of the observation that the blood group antigen characteristics of the mature red blood cell stroma are evident in the earliest forms of erythroblast, i.e. in proerythroblasts (Yunis and Yunis, 1963).

The differentiation caused by erythropoietin is a complex process, requiring induction not only of the apparatus for haemoglobin synthesis but, in addition, of that for the production of stromal and of other specific characteristics of the red cell. Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, has been observed to inhibit the responses to erythropoietin stimulation both in vivo and in vitro (Reissmann and Ito, 1966). Stimulation by erythropoietin of the incorporation of labelled uridine into marrow cell RNA was also found to be blocked by actinomycin D, but not if there was a delay between administration of erythropoietin and of inhibitor. Many types of RNA are synthesised, with sedimentation constants from 4S-150S, some types short lived, some stable and some containing methyl groups and probably representing messenger or transfer RNA. It is not known what role the the large rapidly-labelled RNA molecules of 150S play in the differentiation of the erythropoietic cell, but it was observed to have a relatively short life and to be unique to erythropoietin-sensitive systems (Gross and Goldwasser, 1969). Protein synthesis occurs following the formation of these RNA molecules. The sequence of the

biochemical changes observed in bone marrow following erythropoietin stimulation has been summarised by Goldwasser and Gross (1969), and their view of these effects is indicated in Figure 5.

The biochemical mechanism of action of erythropoietin has not yet been completely established, particularly in regard to the time sequence of the various changes, but the above findings appear to be consistent with the following mechanism. Erythropoietin acts at the level of genetic transcription by reducing the repression of DNA-dependent RNA synthesis, possibly by virtue of its ability to bind with DNA of certain tissues (Pinto, 1968). The RNA produced so rapidly in response to erythropoietin is likely to represent reasonably stable messenger RNA, which then supplies the templates necessary for synthesis of the various enzymes that are required both for cell division and for the form of differentiation characteristic of erythroid cells, including particularly haemoglobin production.

5. INFLUENCE OF PROTEIN DEFICIENCY ON ERYTHROPOIETIN AND ON ERYTHROCYTE PRODUCTION

Erythropoietin has an erythropoietic stimulation effect and controls red blood cell production at several stages, influencing both the rate at which the marrow stem cells differentiate into definitive erythroblasts

FIGURE 5

(After Goldwasser and Gross, 1969).

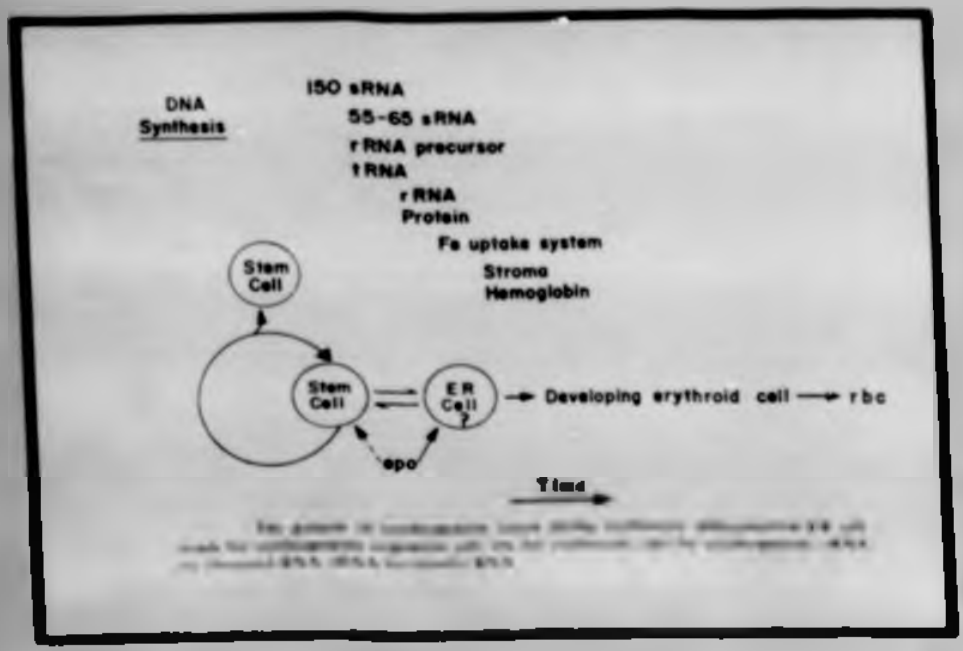
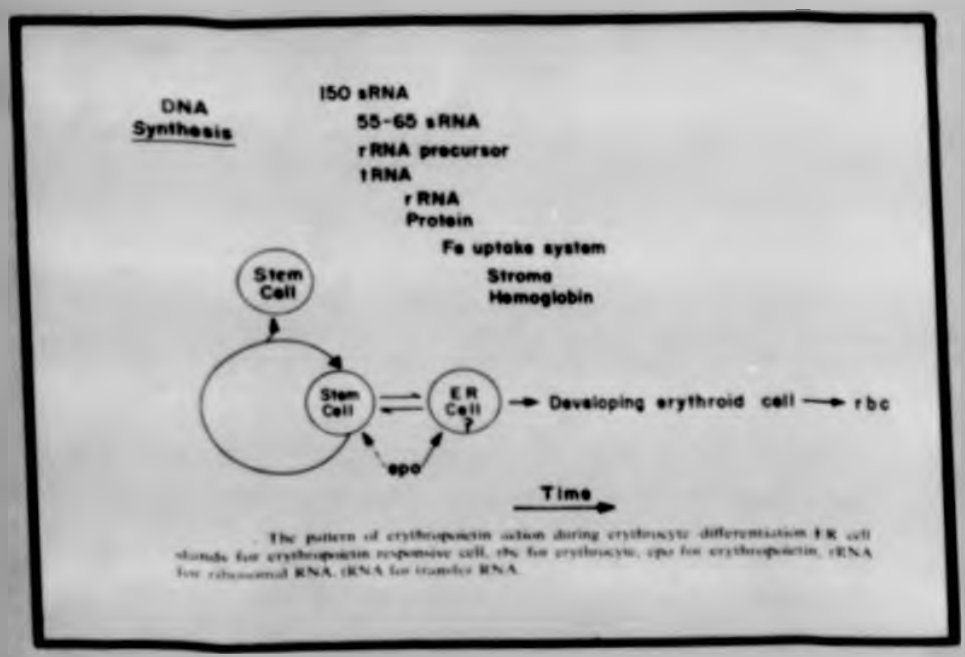


Figure 5

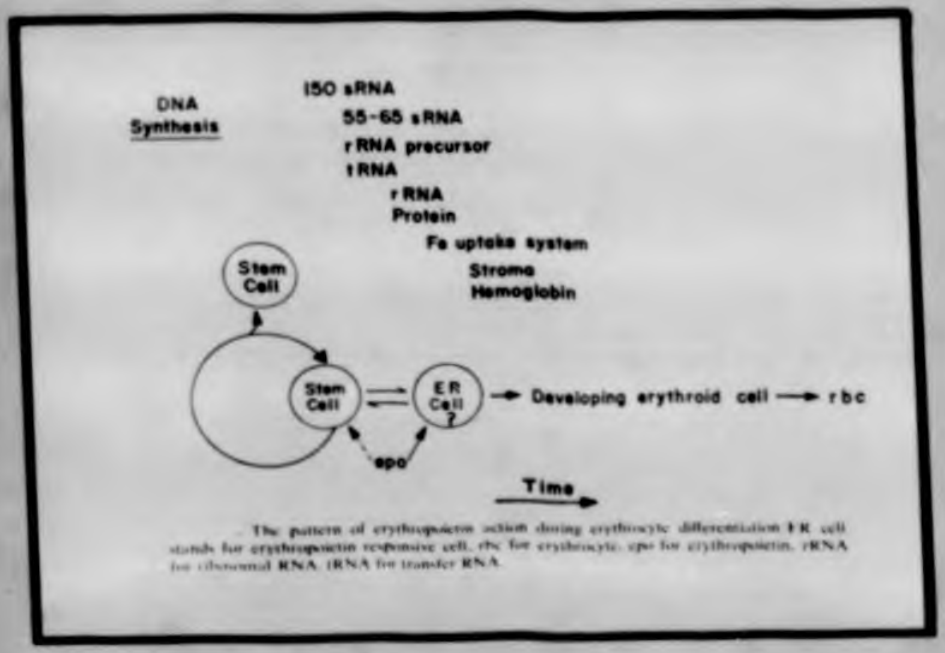
(After Goldwasser and Gross, 1969).



. The pattern of erythropoietin action during erythrocyte differentiation. ER cell stands for erythropoietin responsive cell, rbc for erythrocyte, epo for erythropoietin, rRNA for ribosomal RNA, tRNA for transfer RNA.

FIGURE 5

(After Goldwasser and Gross, 1969).



The pattern of erythropoietin action during erythrocytic differentiation ER cell stands for erythropoietin responsive cell, rbc for erythrocyte, epo for erythropoietin, rRNA for ribosomal RNA, tRNA for transfer RNA.

and the rates of maturation, haemoglobin synthesis and release of the cells from the marrow into the circulation. Thus, changes in erythropoietin level due to protein deficiency might affect red blood cell production in two possible ways, modifying either the quantity or the quality of red blood cells produced in bone marrow.

There are no reports of direct measurement of plasma erythropoietin levels in experimental animals maintained on low protein diet, but direct assessments have been made in human subjects with kwashiorkor. El Hidi et al. (1963) measured serum erythropoietin concentration in anaemic infants with kwashiorkor or marasmus, as well as in anaemic adult patients with ancylostomiasis, by using the uptake of ^{59}Fe by erythrocytes of starved rats as a measure of erythropoietin activity. These authors reported that the anaemic infants with kwashiorkor and marasmus did not exhibit as high titres of erythropoietin in their blood as might have been expected from the severity of their anaemia. They suggested that hypoproteinaemia may cause some depression of erythropoietin production. Murthy (1965) also investigated the erythropoietin levels in children suffering from kwashiorkor with anaemia, using the reticulocyte response in starved rats for assay of erythropoietin. He found a higher than normal reticulocyte response in the starved rats on injection of plasma from these children. He concluded that serum erythropoietin

was elevated in these children with kwashiorkor and anaemia, and he also observed elevation of erythropoietin to a similar extent in these kwashiorkor children as in subjects with a comparable degree of anaemia due to other causes. Moreover, he reported that there was no consistent or significant rise in erythropoietin during a 4 week realimentation period on high protein diet, after which serum albumin was elevated while haemoglobin was rather reduced. Murthy's (1965) studies thus suggested that there was no basic defect in the production of erythropoietin in kwashiorkor and he indicated that the failure of serum erythropoietin to rise significantly during repletion was not consistent with the assumption (El Ridi et al., 1963) that hypoproteinaemia depressed erythropoietin synthesis. It was also commented that the effects of other factors, such as circulatory changes and the present of infection, in reducing the erythropoietin level should perhaps be taken into account.

Further investigations into the changes in erythropoietin level in the anaemia of kwashiorkor were performed by McKenzie et al. (1967), who determined serum erythropoietin titres by injection of the anaemic serum into polycythaemic mice and measurement of the resulting erythrocyte ^{59}Fe uptake. These workers found that the serum erythropoietin levels were initially normal or raised in these kwashiorkor cases and that the levels

continued to rise after protein refeeding when the haemoglobin concentration was further reduced, despite increased erythropoiesis. This continued fall in haemoglobin probably provided the stimulus for the increased synthesis of erythropoietin, by increasing the degree of hypoxia, and there was thus no reason to postulate that protein feeding had itself enabled the increase in erythropoietin to occur. These authors found, in addition, that anaemia in kwashiorkor was frequently complicated by iron and folate deficiency in the cases studied in Cape Town, while the anaemia was not cured unless haematinics were administered in addition to a protein diet. The aetiology of this anaemia was thus considered to be complex and probably was not solely attributable to protein deprivation.

Protein deficiency in man is often accompanied by vitamin and mineral deficiency, so the characteristics of the resulting anaemia may not be comparable to the pure protein deficiency anaemia that can be induced in experimental animals. Although this means that the interpretation of results obtained in human studies of protein deficiency may be open to some doubt in regard to the effects of protein deprivation per se on the anaemia in this condition, these studies in man do have the important advantage that direct measurements of erythropoietin titre were possible whereas indirect methods only

could be used in animal studies. Evidence from these indirect methods suggests that erythropoietin may be reduced in animals fed on a protein-free diet.

Aschkenasy (1960; 1963) examined the effect of a protein-free diet on erythropoiesis in rats and found that the effect was acute with the reticulocyte count falling from 2-5% to less than 1% after only one day. Erythroblastic mitosis and the uptake of ^{59}Fe in the bone marrow also diminished. It was observed that these changes could be prevented by injection of plasma from rats made anaemic by phenylhydrazine. Therefore, during protein deprivation the animal still was able to draw on the protein of other tissues for the formation of new red cells in response to unusual stimuli, such as bleeding (Whipple, 1947) or administration of cobalt (Orten, 1935) or anaemic plasma (Aschkenasy, 1963).

The relationship between protein metabolism and erythropoiesis in protein-deprived rats was investigated in more detail by Heisenmann (1964a; 1964b) who found a rapid suppression of erythropoiesis in protein deficiency, as judged by decreasing iron incorporation into red cells, but observed that this change was reversed by realimentation. Red cell mass declined in a linear fashion in the protein-deprived animals, indicating a removal of senescent red cells without any significant replacement, with anaemia of increasing severity arising as a cumulative result of

these changes. Injection of exogenous erythropoietin into these protein-deprived rats was found to prevent this reduction in the red cell mass, implying that these animals were still capable of red cell formation in response to stimuli. This behaviour could be explained either by a reduced erythropoietin level causing the anaemia during protein deprivation or by the administration of exogenous erythropoietin greatly elevating the erythropoietic stimulus and overcoming another form of limitation to erythrocyte production. In an attempt to distinguish between these alterative, further investigations were carried out on protein-starved and control rats subjected to hypoxic conditions, when it was observed that the plasma erythropoietin titre in the protein-starved rats was significantly lower than that in the group on a normal diet in these hypoxic conditions (Reissmann, 1964b). Moreover, realimentation resulted in a significant and rapid increase in erythropoietin level in the hypoxic environment. From these results, it was concluded that protein deprivation did not directly affect cytoplasmic protein synthesis in erythroid precursors and the depression of erythropoiesis was attributed to a diminished formation of erythropoietin.

Ito et al. (1964) reported a rapid and almost complete disappearance of erythroid marrow elements in rats with protein malnutrition. A single injection of erythropoietin

would then generate a wave of erythroid proliferation commencing with an increase in proerythroblasts, progressing in an orderly sequence through the erythron and terminating in the release of reticulocytes. The percentage of erythroblasts present in the bone marrow at an interval after erythropoietin injection was found to be related approximately linearly to the logarithm of the dose of erythropoietin. Ito and Reissmann (1966) were later able to show that daily injection of 1.8 units of rabbit erythropoietin induced a steady state erythropoiesis which, on the basis of the parameters studied, could not be distinguished from that found in normal rats.

With these differences between the results of studies in man and those from animal investigations it remains uncertain what effect protein deficiency has on the serum erythropoietin concentration. There is little doubt, however, that protein deficiency results in a severe reduction in erythropoiesis, by one mechanism or another. Bethard et al.(1958)reported that subjection of rats to acute protein deficiency resulted firstly in haemoconcentration, then in a drastic reduction in erythropoiesis. These changes were reversed on addition of protein to the diet and these authors suggested that protein intake is more essential for the maintenance of normal erythropoiesis than is total caloric intake. Similarly, Ghitis and his co-workers (1963a; 1963b) postulated that the anaemia in

kwashiorkor and in experimental animals (monkeys) subject to severe protein deficiency was primarily due to the protein deficiency resulting in a decreased production of red cell precursors. Furthermore, Woodruff et al. (1970) observed a very significant lowering of haemoglobin in protein malnourished dogs compared with normal animals. Further investigations, involving measurement of iron clearance from plasma and of iron utilisation, by means of ^{59}Fe, as well as assessment of red cell life span by ^{51}Cr, indicated that the main aetiological factor in the anaemia was a reduction in the ability of malnourished dogs to produce new erythrocytes, the anaemia being truly dyshaemopoietic. This limitation of erythrocyte production in protein-energy malnutrition may reflect a decrease either in substrates (amino acids) required for the synthetic processes of the stem cell or in the hormone erythropoietin (Wintrobe, 1967).

The period of maturation and division during erythrocyte production is associated with the synthesis of nucleoproteins, haemoglobin, enzymes, carbohydrates and lipoproteins. Deoxyribonucleic acid (DNA) synthesis appears to be necessary for the erythropoietin-induced stimulation of haemoglobin synthesis in erythroid precursors, and DNA represents the genetic material by means of which characteristics are transmitted to, and through, subsequent generations. This DNA has been assumed to be

responsible for the synthesis of globin, so, if a structural gene becomes altered during replication or transcription, this in turn will affect both the type of globin formed and its rate of formation. Since the mRNA-ribosome complex is the main site of globin synthesis in the cytoplasm of the cell, changes in structure or activity either of mRNA or of ribosome could also strongly affect the control of globin synthesis. There is evidence that both DNA replication and protein synthesis are affected in children suffering from protein-energy malnutrition (Gitlin et al., 1958; Waterlow et al., 1960; Metcoff, 1967; Villee, 1967; Cheek, 1968; Waterlow, 1968; Cheek et al., 1970). Development of chromosome abnormalities has been reported in children with advanced protein-energy malnutrition (Armendares et al., 1971). This evidence suggests that in the erythrocyte precursors of these children a reduction in composition of DNA occurs, which, if it is to a severe extent, could give rise to a mutation within the genetic material and thereby result in a structural abnormality of globin.

In addition to inducing the differentiation of stem cells into erythrocytes, erythropoietin is associated with increasing the rates of haem and globin synthesis, normally promoting comparable increases in the rates of synthesis of the α and β polypeptide chains of globin (Mizoguchi and Levere, 1971; 1972). An imbalance in synthesis between

there α and β polypeptide chains of globin can result in the formation of abnormal haemoglobin. A relative excess in α-chain production leads to unstable haemoglobin Leiden (Heider and James, 1974), whereas there is a decrease in synthesis of the α-chain relative to that of the β-chain in iron deficiency anaemia (Ben-Bassat et al., 1974). It is conceivable that abnormalities in erythropoietin production in protein-energy malnutrition might result in abnormal control of the rates of synthesis of haem and of the polypeptide chains of globin and could thus possibly lead to the production of haemoglobin with an abnormal structure. This behaviour should not occur with small changes in erythropoietin production rate, such as those that arise during the normal day to day regulation of erythropoiesis or in response to mild anaemia resulting from physiological bleeding, but abnormal behaviour could occur in the event of a severe reduction or a drastic increase in erythropoietin level during protein-energy malnutrition, particularly if such changes in erythropoietin were accompanied by constraints on erythrocyte production imposed by a shortage of protein substrates. There have been no reports of effects of abnormalities in erythropoietin level on the red cell membrane structure, but it is also possible that erythropoietin might influence the construction of this membrane since it appears to control many different aspects of erythrocyte production. It is conceivable that

changes in the lipid composition of the erythrocyte membrane in kwashiorkor (Coward, 1971) might be related in some way to irregularities in erythropoietin production.

6. [redacted]

ANAEMIAS

General knowledge of erythropoietin has been expanding rapidly in both physiological and pathological fields. Erythropoietin may be used as a fundamental indicator in the classification of anaemias into two distinct types, those associated with increased erythropoietin levels and those associated with low erythropoietin levels.

(i). Anaemias Associated with Increased Erythropoietin levels

Anaemias of this type can arise in two ways :-

a. As a result of a loss of red cells by haemorrhage or by haemolysis. These anaemias are accompanied by elevated levels of blood and urinary erythropoietin and by increased red cell production (Jones and Klingberg, 1960; Penington, 1961; Van Dyke et al., 1961; Nakao et al., 1963).

b. As a result of decreased red cell production in the bone marrow. These anaemias are also associated with increased blood levels of the hormone, which are apparently

ineffective, however, in the reversal of the lowered red cell count owing to alteration or damage to the marrow. Increased amounts of erythropoietin have been demonstrated in the plasma of patients with aplastic anaemias (Penington, 1961; Naets and Heuse, 1962; Nakao et al., 1963; Hammond et al., 1968), iron deficiency anaemias (Penington, 1961; Van Dyke et al., 1961; Naets and Heuse, 1962; Novarnaghi et al., 1967; Gutnisky et al., 1968; Hammond et al., 1968) and megaloblastic anaemias (Penington, 1961; Zalusky, 1967). These are the most common types of underproduction anaemia, which to be due to a primary failure of the bone marrow, either from injury or as a result of a lack of a necessary nutrient, and where the increase in erythropoietin secretion occurs in response to the anaemia. Blood erythropoietin levels have also been reported to be high in cases of leukaemia (Naets and Heuse, 1962; Thorling, 1965), and it is considered that the cause of anaemia in the leukaemias is a primary marrow failure to respond to the hormone (Thorling, 1965).

(ii). Anaemias Associated with Low Erythropoietin Levels

Most anaemias of this type are underproduction anaemias associated with low hormone levels. Examples include various forms of endocrine deficiency, where a decrease in metabolic rate and in oxygen consumption leads to a reduction in erythropoietin production. Such changes have been observed in hypothyroidism (Heinecke and Crafts,

1964) and following hypophysectomy (Heineke and Crafts, 1959) as well as in starvation (Morgulis, 1923). Presumably the juxtaglomerular apparatus has a role in this anaemia, finding itself at first provided with surplus oxygen, owing to reduced oxygen consumption, and responding with a decrease in its R&F output, with a reduction in erythropoiesis occurring as a consequence.

Many investigators have studied the anaemias associated with uraemia and with chronic renal disease in man and their experience has been that, in spite of the presence of severe anaemia, the majority of these patients did not have raised serum or urinary erythropoietin levels (Lange and Gallagher, 1962; Naets and Heuse, 1962; Brown, 1965; Denny et al., 1966; Finne, 1968). There was evidence to suggest a toxic depression of marrow function in these conditions, as well as a decrease in erythropoietin, with toxic factors possibly inhibiting or neutralising erythropoietin (Naets and Heuse, 1964; Mann et al., 1965; Bozzini et al., 1966; Brown, 1965; Shaw, 1967; Fisher et al., 1968). In addition, anaemia is often associated with chronic infection and with malignancy, and decreased erythropoietin production appears to be the major cause in many such cases (Ward et al., 1971).

The appropriate position in this classification scheme for the anaemia associated with protein-energy malnutrition remains uncertain since it has not yet been established

unambiguously in which way the erythropoietin level changes in such anaemias. Studies of the anaemia accompanying kwashiorkor in man appear generally to point to an elevation of the plasma erythropoietin titre in this condition (Murthy, 1965; McKenzie et al., 1967) although the extent of this elevation may not be as great as in other types of anaemia (El Ridi et al., 1963). Investigations into protein deficiency in animals, in contrast, appear to point to the opposite conclusion (Reissmann, 1964b; Ito and Reissmann, 1966).

CHAPTER 2 MATERIALS AND METHODS

MATERIALS AND METHODS

I. STANDARD ERYTHROPOIETIN

To ensure comparability of results, a standard preparation of erythropoietin should be brought into universal use once a stable preparation has become available and its biological effect has been carefully analysed. A stable standard material is an essential requirement for valid biological assay, since otherwise its activity will decrease with time, making the labelled potency misleading and resulting in overstimulation of specific activity of sample preparation. Bangham (1962) has recommended that a standard for bioassay should have the following characteristics :- (a) possession of specific biological activity and a demonstrable dose/response relationship, (b) similarity of type of preparation to that to be assayed, (c) stability, and (d) a fixed unit of potency. The WHO International Reference Preparation (IRP) of erythropoietin (Cotes and Bangham, 1966) meets many of these requirements, providing a satisfactory dose/response relationship in a wide variety of assay systems. The slopes of regression lines of log-dose against response obtained with this preparation are effectively parallel to those provided by a wide range of other preparations of erythropoietin, from various species and sources and with differing degrees of purification.

This preparation has been found to be stable in accelerated degradation tests (Cotes, 1968).

Erythropoietin Standard B has now been established as the International Reference Preparation of Erythropoietin, and the International Unit for erythropoietin is defined as the activity contained in 1.48 mg of this International Reference Preparation. Material for this reference preparation was obtained from the urine of patients with paroxysmal nocturnal haemoglobinuria (The WHO Expert Committee on Biological Standardization, 1964a; 1964b), but has been shown to have similar characteristics in bioassay to plasma extracts from rabbit, sheep and monkey as well as from man (Cotes and Bangham, 1961; 1966). Valid results can therefore be expected despite differences in species and assessments of erythropoietin in plasma from rats in the present study should be realistic.

At present, the best standard for daily use in bioassays is a commercially produced, freeze-dried preparation of erythropoietin from the plasma of anaemic sheep. Its preparation involves the irradiation of specific pathogen-free sheep, making them anaemic by subcutaneous injections of phenylhydrazine and removing their blood by exsanguination when the haematocrit has fallen below 10%. For this study, a standard erythropoietin preparation purified to step III and freeze-dried (Connaught Medical Research Laboratories, Ontario, Canada) was used : this

had been assayed by the manufacturers against the WHO International Standard B Unit and its activity was quoted as 3.50 units/mg. It was also confirmed that there was a close relationship between the manufacturer's unit and the WHO Standard B Unit in the bioassay method adopted.

II. PREPARATION OF POLYCYTHAEMIC MICE FOR THE BIOASSAY OF ERYTHROPOIETIN.

Gordon (1959) found that erythropoietin could promote an increase in the total red cell mass of an intact normal animal, but this property is not used in the estimation of erythropoietic activity in biological fluids from human subjects or animals since a greater amount of active material would be required than is usually available. Common methods of assay instead make use of test animals whose red cell production rate has been suppressed to provide a lower base-line for the measurement of stimulation of erythropoiesis. Test animals frequently used for bioassay purposes have included starved rats (Fried et al., 1957; Hodgson et al., 1958), hypertransfused polycythaemic rats and mice (Gurney and Pan, 1959-Jacobson et al., 1959; DeGowin et al., 1962) and post-hypoxic polycythaemic mice (Cotes and Bangham, 1961). Zivny et al. (1970) made a comparison of erythropoietin bioassays in mice rendered polycythaemic by three methods : (a) by hypertransfusion, (b) by hypoxic hypoxia, and (c) by hypoxic hypoxia and

transfusion. They found that the mice in which polycythaemia had been induced by hypoxia provided the most sensitive erythropoietin assay. For this reason, mice were used in the present study and the hypoxic method was chosen for the induction of polycythaemia.

1. Animal Selection

It has been shown to be very important that the test animals should have uniform characteristics. This was ensured by the selection of a pure-bred strain of mouse and the use of animals of the same sex, age and weight. The importance of consistent weight and age characteristics to the maintenance of uniformity in this type of assay has been stressed by Cread (1969).

Female CBA/Ca mice were chosen for the assay for the following reasons:-

(a). They are an inbred line that has been proved satisfactory in the erythropoietin standard assay made by Cotes and Bangham (1966).

(b). In the investigations by Bernstein et al. (1968), into the effects of mouse strain differences on erythropoietin assay response, it was found that strains of small black mice showed a greater response to erythropoietin than did other types. The CBA strain falls in this group with optimal erythropoietin assay performance and CBA/Ca mice are a pure inbred strain, brown/black in colour, which

proven to be the best for assay purposes.

(c). Female mice are always used as the male has an androgen which has an effect on erythropoietin (Fried and Gurney, 1965; 1966).

2. Diet

The iron requirement increases in test animals during the development of polycythaemia as a result of the increased red cell production rate. Clearly, if iron deficiency occurred in the test mice when used in the bioassay, then the amount of radioactive iron (^{59}Fe) utilisation would vary as a result of the iron deficiency and would thus provide an invalid measurement of erythropoietin activity. This difficulty has been illustrated by the report of a higher haematocrit seen in hypoxic polycythaemic mice when given extra iron (DeGowin et al., 1962).

The following diet was chosen to avoid the development of iron deficiency as a result of the marked increase in haemoglobin synthesis during the hypoxia.

(a). The mice were fed exclusively on PBU diet (obtained from E. Dixon and Sons, Ware, Herts.), a standard rodent food with a fairly high iron content.

(b). The mice received iron supplemented drinking water, in the form of ferrous sulphate at 30 mg/l in 5% dextrose solution, during the hypoxic period. Fresh

drinking water was frequently prepared by 1 in 100 dilution in sterile 5% dextrose of a stock solution containing 3 g/l ferrous sulphate in sterile 30% dextrose, as Wrightly and Brown (1966) have shown the lowest concentration of dextrose able to maintain the iron in its reduced state for 2-3 weeks to be a 30% solution. Oxidation of the iron would soon occur if the solution were not sterile. This concentration of ferrous sulphate has been used previously by Fogh (1966). The ferrous sulphate-dextrose solution was preferred for iron supplementation since it was shown to result in a trend towards weight gain from the beginning of the hypoxic period and a reduced variation in weight between the individual animals. Its ease of preparation and storage was also an advantage.

3. Hypoxia Chamber (Low Pressure Tank)

(a). Tank Construction

The tank (Figure 1) was made of steel with the dimensions shown in Figure 2, i.e. 92 cm high and 61 cm diameter, measured internally, with a wall thickness of 5 mm. Safety valves which operate in case of excess negative pressure and a spring loaded valve which opens on failure of the pump were incorporated. The animal cages were made of galvanised iron and had perspex lids. Water for the mice in each cage was contained in a

FIGURE 1

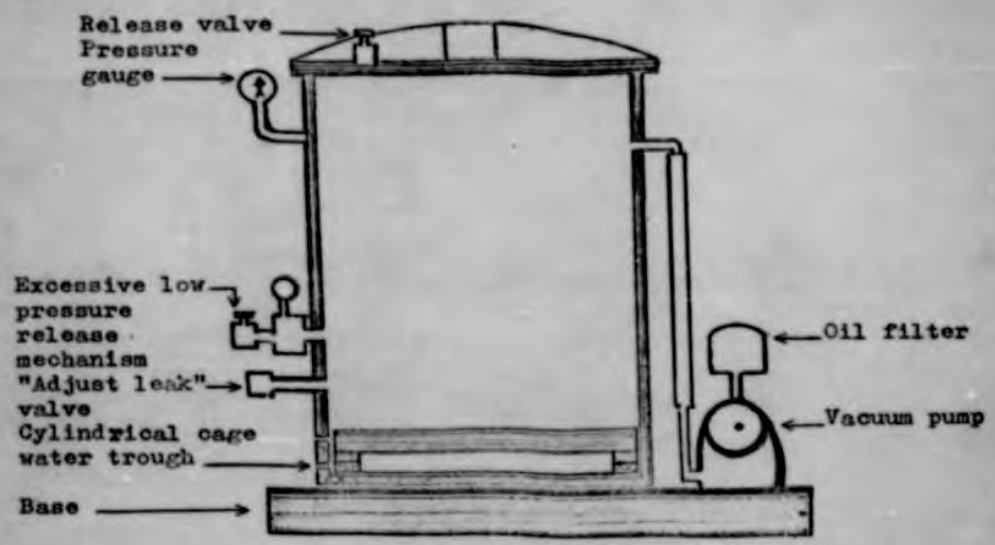
Hypoxic chamber (low pressure tank).

FIGURE 1

Hypoxic chamber (low pressure tank).

FIGURE 2

Plan of the low pressure tank.



semicircular trough, which acted as a reservoir to the drinking areas (Figure 3). Holes in the lid of the trough allowed equalisation of air pressure during evacuation and repressurisation.

(b). Pressure

Cotes and Bangham (1961; 1966) reported that the most suitable pressure for inducing polycythaemia in mice by the hypoxic method was 0.5 atmosphere and this was the pressure used in their assay for the standardisation of erythropoietin. Weintraub et al. (1963), however, have indicated that a lower pressure of only 0.4 atmosphere provided a faster development of an adequately polycythaemic state. The pressure used in this study was 0.4-0.5 atmosphere (40-50 kNm^{-2}).

4. Choice of the Most Suitable Time for Assay

Before the actual assay can be performed, an essential requirement is that polycythaemia should be demonstrable in the test animals. In addition, an assessment must be made of the optimal day after completion of the exposure to hypoxia, for commencement of the assay.

Cotes and Bangham (1961) found an increased red cell mass, an elevated haematocrit and a depression of ^{59}Fe incorporation into red cells when TO mice had spent 14 days in air at half atmospheric pressure and then 4-6 days at normal pressure before making these measurements. They

FIGURE 3

Animal cage in the low pressure tank, showing the semicircular trough.

FIGURE 3

Animal cage in the low pressure tank, showing the semicircular trough.

thus advised that a suitable time for routine assay of erythropoietin was 4 days after ending the hypoxic exposure. This method was modified in 1966 when CBA mice were used, the duration of the hypoxic period was extended to 14-21 days and it was found that 3 days after hypoxia was the most suitable time for the assay (Cotes and Bangham, 1966).

Other workers have used slightly different durations of hypoxic exposure and have recommended slightly different intervals after exposure as the optimum for erythropoietin bioassay. Such differences in technique, however, have only been small and were probably related to differences in choice of pressure for the hypoxic exposure and to the use of different strains of mice. Thus, DeGowin et al. (1962) found that an appropriate degree of plethora was most efficiently obtained by placing CF No. 1. female mice in a hypoxic chamber at half atmospheric pressure for 3 weeks and then the assay was started 5 days after their return to normal pressure. Meanwhile, Weintraub et al. (1963) exposed mice to a pressure of 0.4 atmosphere for 2 weeks and found they were ready for assaying by 3 days after return to ambient pressure.

Polycythaemic mice with haematocrit values of about 70% were shown by Jacobson et al. (1960) to be suitable for erythropoietin determination as erythropoiesis had practically ceased in these animals. Cotes and Bangham

(1961) advised that for routine assays, the haematocrit of polycythaemia mice should be over 50% and that any with a lower haematocrit should be discarded. Similarly, Weintraub et al. (1963) discarded any experimental animals with a haematocrit below 53% in their work, and Fogh (1966) found that endogenous stimulation of erythropoiesis had virtually ceased when the haematocrit had reached 55% and any test animals with a haematocrit below 55% were discarded.

In the present study, the fourth day after return to ambient pressure was chosen for commencing the assay procedure and any test animals with a haematocrit below 55% were discarded from the analysis.

III. ERYTHROPOIETIN ASSAY

When polycythaemic mice are ready for the assay, erythropoietin standard or test sample is administered followed by ^{59}Fe after a suitable time interval, dependent on the method adopted.

(a). Administration of Standard Erythropoietin or Test Material

Gurney et al. (1961) observed a smaller erythropoietic response with a single submaximal dose of erythropoietin than with the same amount of erythropoietin administered in divided doses. In accordance with this

finding, the divided dose method was used in this study.

(b). Routes of Administration of Erythropoietin and of Radioactive Iron (^{59}Fe)

Sivny et al. (1970) found, in their erythropoietin bioassay in polycythaemic mice (H strain), that the standard deviation of the ^{59}Fe incorporation response to a standard dose of erythropoietin was ministered when the erythropoietin was administered by intraperitoneal (ip) and the ^{59}Fe by intravenous (iv) injection. Peleppa (1972) also investigated the routing of ^{59}Fe administration in relation to its effect on the sensitivity of an erythropoietin bioassay. He found that incorporation of ^{59}Fe into the blood was depressed when a crude preparation of erythropoietic stimulation factor (ESF) and ^{59}Fe were both administered ip. The problem with ip administration of ^{59}Fe appeared to be one of absorption and was overcome by the injection of test material and ^{59}Fe by different routes, the best results being achieved with administration of erythropoietin standard or test material ip and ^{59}Fe iv.

On the basis of these observations, erythropoietin standard or test material was administered ip (Figure 4) while ^{59}Fe was injected iv (Figure 5) in the present investigation.

FIGURE 4

Intraperitoneal injection of standard or test plasma.

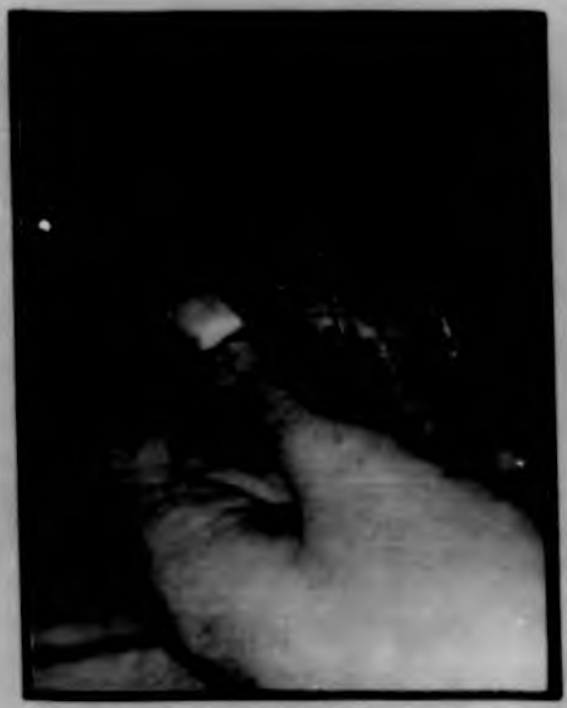


FIGURE 4

Intraperitoneal injection of standard or test plasma.

FIGURE 5

Intravenous injection of ^{59}Fe.

FIGURE 5

Intravenous injection of ^{59}Fe.

PROCEDURE FOR ERYTHROPOIETIN ASSAY USING POST-HYPOXIC POLYCYTHAEMIC MICE

Bearing in mind the aforementioned experiences of other workers, the following plan for the assay of erythropoietin was evolved :-

Test Animals : Female CBA/Ca mice of weight 20 ± 2 g were used.

Preparation of Polycythaemic Mice :

For at least one week before being placed in the hypoxic chamber and for the entire hypoxic and post-hypoxic periods, the animals were fed on PRM diet. Throughout the hypoxic period, the mice received an iron supplement in their drinking water in the form of 30 mg/l ferrous sulphate in 5% dextrose. The animals were kept in the hypobaric chamber at a pressure of 0.4-0.5 atmosphere (40-50 kNm^{-2}) for 20 hours each day for a total of 20 days. Temperature and humidity within the chamber were regularly monitored and maintained within safe limits. The carbon dioxide level has been measured previously with a group of 120 mice (the maximum possible) in the chamber and found to be satisfactory.

Erythropoietin Assay :

The post-hypoxic regime was as follows :-

Day 0: Animals were removed from chamber.

Day 1-3:	Animals were maintained at normal atmospheric pressure.
Day 4:	1 ml (half of total dose) of test (or standard) erythropoietin was given ip.
Day 5:	1 ml (remaining half) of erythropoietin dose was given ip.
Day 7:	^{59}Fe was injected iv-approx. 0.2 µCi in 0.2 ml isotonic saline.
Day 8:	20 hours after iron injection the animals were killed and bled into EDTA. PCV was determined and 0.5 ml whole blood was counted in a gamma-ray counter for 1000 seconds (to give a counting error of less than 2%).
Notes:	Each test sample was assayed at two dose levels, and standards at two dose levels (0.1 and 1.0 units/ml) and diluent (saline) controls were included in each batch. Normally 7 animals (always a minimum of 4) were used for each variant. The results of each assay were analysed by a standard method based on analysis of variance (Finney, 1964).
Reference:	This method was based on that of Wrigley (1970).

VI. SELECTED GROUPS OF RATS [illegible] FOR ERYTHROPOIETIN ASSAY

It was decided to assay the erythropoietin concentration in plasma samples taken from four groups of male weaned hooded rats, each group containing 7 animals. These groups received the following diets and treatment :-

Group 1. Rats were fed on control diet (10 NDoCal%) for 8 weeks.

Group 2. Rats were fed on control diet (10 NDpCal%) for 5 weeks and then rendered anaemic by bleeding 0.5 ml daily, while still being maintained on control diet, until their haemoglobin and haematocrit levels were similar to those found in the protein deficient rats of group 4.

Group 3. Rats were fed on control diet (10 NDpCal%) for 6 weeks and then rendered anaemic by bleeding 1 ml daily, while still being maintained on control diet, until their haemoglobin and haematocrit levels were similar to those found in the protein deficient rats of group 4.

Group 4. Rats were fed on the 2 NDpCal% low protein diet for 8 weeks.

The rats of group 1 and 4 were killed after 8 weeks on the appropriate diet; while those of groups 2 and 3

were killed when their haemoglobin and haematocrit levels had fallen to the required levels, also after about 8 weeks on the diet. A blood sample from each rat was taken by heart puncture and put in a heparinsed tube. Haemoglobin and haematocrit were measured for each sample, then the blood from each group of rats was pooled. The plasma was separated and stored at -20°C until the erythropoietin assay could be performed.

CHAPTER 3 RESULTS

A comparison was made of plasma erythropoietin level between a group of rats fed on a protein sufficient diet (Group 1) and a group of protein deficient rats (Group 4). The protein deficient animals, fed on 2 NDpCal% diet, developed anaemia, with their haemoglobin level falling to a mean value of 10.5 g/100 ml and their haematocrit to a mean of 29.8%. Two further groups of rats fed on control diet (Group 2 and 3) were bled daily (by 0.5 ml for Group 2 and by 1.0 ml for Group 3) until they developed anaemia of similar severity to that of the protein deficient animals, as judged by their haemoglobin levels and haematocrit. The plasma erythropoietin levels in these anaemic groups were compared with that found for the protein deficient rats. Table 1 shows the results of the erythropoietin assays for these four groups of animals, and the corresponding haemoglobin levels and haematocrit to indicate their degree of anaemia.

It was found that the erythropoietin level in normal control rats (without anaemia) was too low to be measured, and a similar finding was made in the case of control rats rendered anaemic by bleeding to a haemoglobin level of about 10 g/100 ml. The lower limit of erythropoietin concentration for quantification by this assay has been estimated as about 0.05 International 'B' units/ml, and

TABLE 1

Plasma erythropoietin levels of rats with anaemia resulting from feeding on low protein diet (group 4), of control rats brought to the same degree of anaemia by bleeding(groups 2 and 3) and of control rats that are not anaemic (group1).

Group of rats providing the plasma	Hb (g%)	PCV	Mean EP level (International 'B' units / ml)
1. Control rats Non-anaemic	15.3	45.8	No detectable erythropoietin
2. Control rats Anaemic (Bled 0.5 ml daily)	10.3	29.7	No detectable erythropoietin
3. Control rats Anaemic (Bled 1.0 ml daily)	10.3	29.6	No detectable erythropoietin
4. Protein deficient rats Anaemic	10.5	29.8	0.86 (95% confidence limits. 0.33-2.2 units /ml)

the erythropoietin levels in these groups must have been below this limit. On the other hand, the erythropoietin level in rats with anaemia resulting from protein deficiency (at a haemoglobin level of about 10 g/100 ml) could be measured, although these animals were if anything marginally less anaemic, and the estimated mean value for these rats was 0.86 International 'B' units/ml.

Measurements were made on two groups of rats bled at different rates, but to the same degree of anaemia, in case the resulting erythropoietin level were dependent on the rate of development of the anaemia as well as on its final severity. Any dependency on the rate of development of anaemia might affect the interpretation of any differences that were found in erythropoietin level between the protein deficient and the bled rats. In practice, however, although dependency on the rate of development of anaemia could not be assessed since the erythropoietin levels were unmeasurable with both rates of bleeding, it was clear that the plasma erythropoietin was more elevated as a result of the anaemia of protein deficiency (Group 4) than from the anaemia due to bleeding at either rate (Group 2 and 3).

CHAPTER 4 DISCUSSION AND CONCLUSION

DISCUSSION

The mechanism by which anaemia occurs under conditions of low dietary protein appears to be complex, in that anaemia persists in spite of apparent hyperplasia in the bone marrow. The present observations on the levels of various metabolic components and other factors relevant to the anaemic condition should help to elucidate various aspects of this mechanism.

The greatest effect of the low protein diets, specifically the 2 %DpCal% diet, on each of the measured variables was observed to occur at or near week 8. For this reason, the primary purpose of this discussion will be to establish an explanation of the mechanism of anaemia which is consistent with all the experimental observations after that period of diet.

The following observations were made at week 8:

(1). The erythropoietin level in plasma was high.

(2). The bone marrow exhibited hyperplasia, as judged by marrow smears.

(3). The animal was in an anaemic condition.

(4). All measured plasma protein components (albumin, total globulin, β-globulin) were present in lower than normal concentrations.

(5). The mean red blood cell life-span was shorter than normal.

These observations above were not sufficient, however, to derive a complete explanation of the mechanism of anaemia under low protein conditions and it is necessary, in addition, to make some assumptions about various stages in the mechanism. It did not prove possible to measure directly α-globulin concentration in the plasma, but it is assumed that α-globulin should behave generally similarly to total globulin concentration in rats on a low protein diet. Thus a drop in α-globulin concentration in plasma would be expected at week 8 on the 2 NDpCal% diet, compared with the control diet, but the extent of this reduction is anticipated to be relatively small (comparable with the 10 - 3% reduction in total globulin at that stage). Since the globulins appear to have more specific biochemical roles in the body than does albumin, it might be expected that plasma globulin concentrations should fall less severely than albumin concentration under conditions of dietary protein shortage (Clamp, 1967; Sandstead et al., 1965).

The normal mechanism for control of anaemia is a feed-back mechanism principally operating between the kidneys and the bone marrow. A reduction in the oxygen supply to the kidneys, resulting in hypoxia, due to a low level of haemoglobin in blood, promotes the synthesis of a renal erythropoietic factor (REF) in the kidneys. REF is regarded as an enzyme which then acts on its protein substrate (α-globulin) in plasma to produce an erythro-

poietic stimulating factor(HSF), or erythropoietin (EP) (Gordon, 1966), whose function is to stimulate various stages of erythrocyte production in bone marrow. Erythropoietin primarily affects the rate at which marrow stem cells give rise to pronormoblasts, but is thought also to affect the rates of maturation, haemoglobin synthesis and release of red blood cells from the marrow into the circulation. The level of erythropoietin can thus control both the quantity and the quality of red blood cell production in bone marrow. An increase in erythropoietin level following the onset of anaemia under physiological conditions leads to an acceleration in the rates of all stages of red blood cell production in the bone marrow and in the rate of release from the marrow (Fisher et al., 1964; 1965). The mechanism by which erythropoietin is broken down and the relative extent to which it is excreted are not clearly established, but there is some evidence that erythropoietin is destroyed when it exerts its effect on the bone marrow (Stohlman and Brecher, 1959; Hammond and Ishikawa, 1962; Carmena et al., 1967; LoBue et al., 1968).

The response of such a feed-back control system under conditions of shortage will depend on which factors limit the various stages in the control mechanism. In the anaemia feed-back control system there are two stages at which two different components have to combine to

produce the required response :

(1). The reaction of REF with α-globulin to form EP.

(2). The effect of EP in stimulating the bone marrow to utilise its protein and other substrates to form mature red blood cells.

The response at each stage depends to a great extent on which of the two reactant components normally represents the limiting factor, in the first case on whether REF or α-globulin is normally present in excess and in the second case on whether EP or the required substrates are present in excess.

It is useful to consider the normal response to physiological anaemia after bleeding in order to elucidate which of these pairs of factors normally represent the limiting and controlling factors, then the response to protein deficiency can be more readily predicted. The response to physiological anaemia after bleeding is known to be a large elevation of the plasma EP level, and this response clearly suggests that REF is normally the factor limiting EP production in plasma while α-globulin should be present in an excess. The second stage in the response to this type of anaemia is an acceleration of the rates of production in and release from the bone marrow of red blood cells, and this response requires that EP is the limiting factor controlling red blood cell production

while the various substrates required by the bone marrow are normally present in excess. Thus the REF and EP concentrations, respectively, should normally represent the limiting factors at these two stages of the feed-back mechanism. It is interesting to observe at this stage that the difference in the response of the feed-back mechanism in the case of iron-deficiency anaemia results from the difference in the limiting factor at the second of these stages: a shortage of the iron substrate in the bone marrow limits the production of mature red blood cells in this condition.

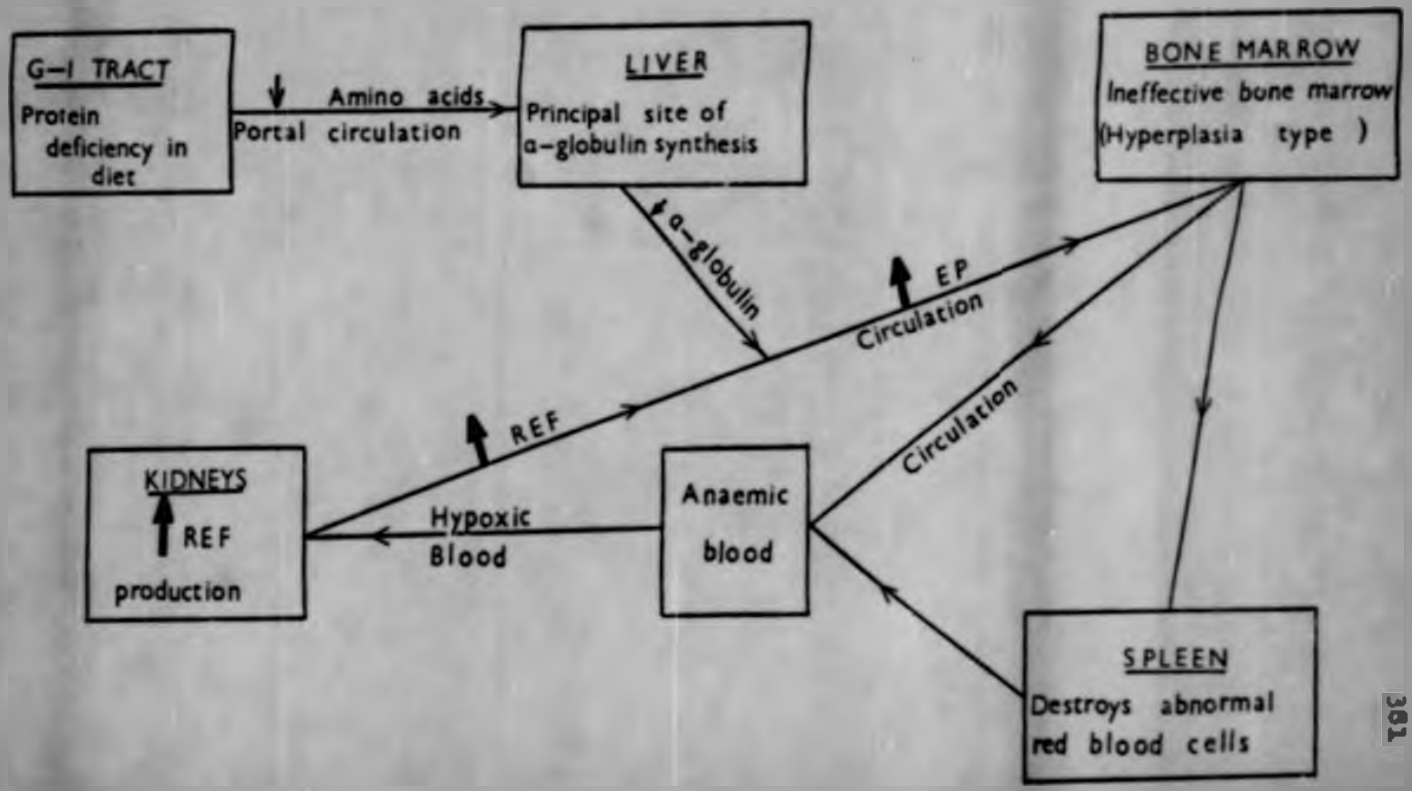
Now it is possible to consider the probable response to a reduction in the dietary protein content and a consequent shortage of protein at various stages. The concentration of REF should remain as the limiting factor controlling the production of EP, since the reduction in plasma α-globulin concentration is thought to be small, and EP should thus respond in the normal way to any changes in REF. The limiting factor determining the rate of production of mature red cells, however, is less readily predicted. Under normal dietary conditions EP appears to be the limiting factor at this stage but it is clear that the protein supply to the bone marrow must have a greater influence on red blood cell production as the extent of protein deprivation increases in severity. Bone marrow normally exhibits a rapid turn-over of protein

and thus requires a large and continuous supply of the protein substrates, while it is thought that this tissue and other tissues showing a similar rapid turn-over of protein may be affected to the greatest extent by protein deprivation (Dao and Ramalingaswami, 1960; Dao et al., 1965; Ramalingaswami et al., 1961; Sood et al., 1965). The protein substrate content of the bone marrow could thus be dramatically reduced on the low protein diet (2 NDpCal%) and this factor could then become the limiting factor for red blood cell production instead of the EP level. Such a change in the limiting factor at this stage in the feed-back mechanism would then explain the inadequacy of the response and its inability to prevent the occurrence of anaemia. The observations of Ghitis et al. (1963a; 1963b) have provided evidence that depletion in protein supply to the bone marrow may be a direct cause of the anaemia of severe protein deprivation both in humans and in monkeys. They found that protein re-feeding led directly to an improvement in the blood haemoglobin level and this pattern of behaviour would be consistent with protein substrate rather than EP level being the factor limiting red blood cell production. Unfortunately, however, these workers did not measure EP levels. The observation of bone marrow hypoplasia during the early stages of protein deficiency (at week 2 on 2%DpCal% diet - see Part 3, Chapter 2) in the present investigations may be indicative that

shortage of protein substrate was starting to limit bone marrow activity at that stage. The results of the present study themselves thus provide some evidence that protein substrate supply at the bone marrow becomes the limiting factor controlling erythrocyte production in protein deficiency, but there is some evidence against this view which will be discussed later.

Most of the stages in the cycle of feed-back control of anaemia at week 8 on the 2 MDpCal% diet can now be readily explained (as shown in Figure 1). The anaemic condition of the blood directly causes an elevated REF production rate in the kidneys and consequently a high plasma EP level. The reduced plasma globulin levels, presumably including a reduction in α-globulin, have little influence on the EP level as REF is the limiting factor. Owing to the reduced level of plasma substrate in the bone marrow, this is unable to respond to an adequate extent to the EP stimulus and does not produce sufficient red blood cells to eliminate the anaemia. This pattern, however, might be more consistent with hypoplasia rather than hyperplasia of the bone marrow and Ghitis (1963a; 1963b) did observe bone marrow hypoplasia both in monkeys fed on a protein-free diet and in children with severe protein-energy malnutrition (kwashiorkor and marasmus). A complete absence or an extremely low level of protein in the diet must inevitably lead to bone marrow hypoplasia

FIGURE 1. Illustration of a plausible mechanism of anaemia induced by abnormal production of erythropoietin resulting from protein deficiency.



when the bone marrow has insufficient protein to produce its normal complement of red blood cells, but this need not necessarily be the response to a less severe form of protein deprivation. Bone marrow hyperplasia was observed on the 2 HDpCal% diet in the present study, but this might be explained by the shortage of protein affecting the maturation of red blood cells to a greater extent than the conversion of marrow stem cells into proerythroblasts. The observation of hyperplasia could result from a greater number of cells in the early stages of red blood cell production, while the shortage of protein could prevent these cells from maturing at the normal rate and in the normal way into erythrocytes and could thus explain the inability of this erythropoiesis to eliminate the anaemia. Observations of the relative numbers of cells in the three stages of normoblast maturation in the bone marrow preparations suggested that there might be a larger proportion of cells in the earlier and middle stages on the low protein diets than on the control diet although these differences were not statistically significant, possibly owing to the difficulty of maintaining a consistent standard to judge the different stages of normoblast maturation. These observations thus tended to support the view that protein deprivation might predominantly affect the maturation of erythroid cells and thus make erythropoiesis ineffective. Protein shortage during the maturation of the red blood

cells would also tend to result in a greater proportion of malformed and damaged cells which would tend to aggravate the anaemia as these cells are broken down either in the bone marrow itself or rapidly in the spleen after release from the marrow. The shorter than normal mean life-span of the red blood cells is indicative of a greater proportion of malformed or damaged cells.

The plasma EP level in the rats on the low protein diet (2 NDpCal%) was even found to be higher than that observed on physiological bleeding to the same degree of anaemia (EP undetectable in that condition). This difference would appear to reflect either the slower rate of usage and breakdown of EP by the bone marrow in the low protein condition or a slower excretion of EP owing to impairment of renal function, or a combination of both these effects. The lower α-globulin level on the low protein diet should hardly affect this comparison as long as α-globulin is still present in an excess compared with REF.

In this manner it is possible to construct an explanation for the mechanism of anaemia after an extended period (8 weeks) of protein deficiency. Murthy (1965) similarly observed that plasma erythropoietin was elevated in protein deficiency, in kwashiorkor children with anaemia, but only elevated in this case to a similar level to that found with other anaemias of comparable degree. He thus

considered that there is no fundamental defect in EP production in protein deficiency, although he did not attempt to construct a complete mechanism to account for the observed anaemia. Other workers, however, from animal investigations involving a shorter period or more severe protein deficiency than used in the present study, have postulated different mechanisms to account for the subsequent anaemia. For example, Reissmann (1964a) found that a daily injection of EP into a group of rats on a protein-free diet prevented the onset of anaemia over a period of up to 5 weeks. In studies (Reissmann, 1964b), in which rats were maintained in a hypoxic condition by subjecting them to various degrees of reduction in atmospheric pressure, he also found lower plasma EP levels in the protein-starved rats after 10 days diet than in normal rats. He thus suggested by extrapolation that the plasma EP level at normal atmospheric pressure should be lower in the protein-starved rats than in the normal animals, but such extrapolation is not necessarily valid especially as the difference in EP level between the protein-starved and normal rats appeared to be greater at the lowest pressures than at the less low pressures and could therefore be non-existent or even reversed at normal atmospheric pressure. Such a pattern would be consistent with a reduction in the capability of the kidney in the protein-starved rats to produce large

amounts of REF in response to hypoxia, and is thus not necessarily indicative of a reduced EP level in the protein-starved rats at normal atmospheric pressure.

The response of the anaemic control mechanism at different stages of the low protein diet may in any case vary. Initially the protein supply to the bone marrow may be adequate, allowing the bone marrow to produce sufficient red blood cells to prevent the onset of anaemia for an appreciable period. At this early stage the REF level could be nearly normal and it is possible that a reduction in α-globulin level could lead to a small decrease in plasma EP level, though any such effect would be expected to be very small with REF as the limiting factor in EP production. During the later stages of protein deficiency, the protein supply to the bone marrow appears to become inadequate and the resulting ineffective erythropoiesis is unable to prevent the onset of anaemia.

Fatty changes in the kidney and liver may also have some influence on the feed-back mechanism, particularly in the later stages of protein deficiency when the fatty changes become more prominent. The ability of the kidney to produce REF may be reduced by fatty changes in the kidney thus tending to lower the plasma EP level, but the renal clearance rate would also reduce and tend to raise the plasma EP level, so the overall effect becomes difficult to predict. Fatty changes in the liver might

reduce the α-globulin synthesis rate but such an effect would tend to be roughly compensated for by the deterioration in renal function.

CONCLUSIONS FROM PART 5

The erythropoietin level in rats during protein deficiency (on 2 NDpCal% diet) was found to be high. The anaemia associated with this condition thus could not be accounted for by a defect in the production of erythropoietin. Anaemia occurring in the presence of an elevated plasma erythropoietin level must reflect an inability of the bone marrow to respond adequately to the erythropoietin stimulus and to produce the requisite number of red blood cells. As hyperplasia was noted, the red blood cells, though numerous, were presumably ineffective either because they were not released into the circulation or because of their shortened half-life (see Haemolysis Part). This failure of erythrocyte production or function is considered to be related to a shortage of protein substrate required for the maturation of red blood cells. The presumed maturation defect of erythrocyte production, in the presence of an elevated erythropoietin level, possibly results from globin and protein membrane disorders or malformation.

Plasma erythropoietin was observed to be higher in the anaemia of protein deficiency than in anaemia of the same degree produced by bleeding. This difference is thought probably to reflect the lesser usage of erythropoietin in the bone marrow in protein deficiency, but

could alternatively be explained by a slower excretion rate.

PART 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

One of the major problems of public health in many parts of the world is protein-energy malnutrition. Anaemia is an unavoidable manifestation in severe forms of this condition and the anaemia is usually normocytic normochromic, but has sometimes been described as macrocytic, or as microcytic and/or hypochromic (Woodruff, 1961; Woodruff et al., 1970). Its aetiology remains unknown, although many possible mechanisms have been suggested, as follows:

1. Haemolysis (Woodruff, 1961).
2. Insufficiency of protein substrate in erythroid precursors of bone marrow for production of erythrocytes (Woodruff et al., 1970).
3. Decreased erythropoietin production (Woodruff et al., 1970).

The purpose of this study was to elucidate the mechanism, or mechanisms, of the anaemia associated with protein deficiency in the rat. The investigations were performed with weaned rats in order to simulate the commonest situation in which protein-energy malnutrition is found in humans. The following aspects of the anaemia were investigated.

1. THE RELATION BETWEEN THE PROTEIN VALUE AND DURATION OF THE DIET AND THE DEVELOPMENT OF ANAEMIA IN RATS

Anaemia developed in animals maintained on low protein diets, of all the various protein values, while its severity correlated well with the protein concentration ($p < 0.01$) of the diet and also varied with the duration. The diet of lowest protein concentration (2 MDpCal%) resulted in the greatest degree of anaemia. On each diet, anaemia was observed by week 2 and then increased gradually in severity until reaching a maximum at about the 8th week. Beyond this time, the survivors were found to be able to adapt to the diet and their anaemia then gradually improved, to an extent depending on the protein concentration of the diet, its duration and the age of the rat. The higher the protein concentration, the longer the duration and the older the rat, the greater was the improvement.

These changes in the severity of anaemia followed the same pattern as the changes in total serum protein concentration. Reductions in albumin were mainly responsible for the latter changes and the albumin/globulin ratio was markedly lowered in the protein deficient rats, an observation which is a characteristic feature of protein-energy malnutrition.

2. CHARACTERISTICS OF THE ANAEMIA RESULTING FROM PROTEIN DEFICIENCY

The only type of anaemia found in any of the rats fed on the various low protein diets was the normocytic normochromic type. This anaemia was of mild or moderate degree (Hb.~ 10 g%, PCV~28%). The reticulocyte count was in the normal range, but the bone marrow was found to be of hyperplasia type, as indicated by an increased number of normoblasts. These were observed by 4 weeks and the normoblast count gradually increased to a maximum at about 8 weeks. This condition is referred to as " ineffective erythropoiesis ".

The transferrin level was significantly lower ($p < 0.01$) than the control level at weeks 4 and 8 of the low protein diet (2 NDpCal%) and correlated well with the haematological data (i.e. Hb. and PCV), with the lower serum iron level and with the mortality rate. The serum iron level of the rats fed on the low protein diets was significantly lower ($p < 0.02$) than that of the control group, although this reduction appeared to be insufficient to affect either the morphology of the red blood cells (none of hypochromic type were observed) or the mean cell haemoglobin concentration. It is thought that the reduction in serum transferrin was partly responsible for the decrease in serum iron level but that it had little

role in the aetiology of the anaemia. The serum transferrin level did appear, however, to be a useful indicator of the degree of malnutrition and of the pro prognosis of treatment, as previously suggested by McFarlane et al. (1969).

3. HAEMOLYSIS AS A POSSIBLE CAUSE OF THE ANAEMIA INDUCED BY PROTEIN DEFICIENCY

The role of haemolysis in the pathogenesis of anaemia during protein-energy malnutrition was studied and the following observations were made:-

(a). Erythrocyte life span

Using the ^{51}Cr method, it was found that the survival in control receiver rats of erythrocytes from rats fed on low protein diet was significantly shorter ($p < 0.001$) than that of erythrocytes from rats fed on control diet: the survival half-time was 7.5 ± 0.1 days for 2 NDpCal% diet and 9.4 ± 0.1 days for control diet.

(b). Osmotic fragility test

The osmotic fragility of erythrocytes of rats fed on low protein diet was significantly reduced ($p < 0.001$) (more difficult to break). This was considered probably to be due to a higher lecithin content of erythrocyte membranes, as has been reported in kwashiorkor (Coward, 1971).

(c). Electron microscopy

Neither abnormalities in membrane structure nor a tendency for fragmentation were detected by electron microscopic examination of erythrocytes from rats fed on low protein diet.

Thus haemolysis did occur in the rats fed on the protein deficient diet, but this haemolysis is considered to be a secondary cause of the anaemia since it was presumably related to the capture by the spleen of defective erythrocytes released from the bone marrow. Moreover, the observed rate of haemolysis was insufficient to account for the degree of anaemia found in the protein deficient rats.

4. THE ROLE OF ERYTHROPOIETIN IN THE ANAEMIA DUE TO PROTEIN DEFICIENCY

The plasma erythropoietin level was found to be elevated in the rats on low protein diet (2 NDpCal%). This elevation presumably resulted from the normal feed-back control mechanism, with the kidney being stimulated to produce more REF in response to hypoxia of its blood supply arising from the anaemia.

It was observed that the erythropoietin level was higher in the protein deficient rats than in rats on control diet but suffering physiological bleeding to the same degree of anaemia. This difference would appear to

reflect either a lower usage of erythropoietin by the bone marrow or a slower excretion rate for erythropoietin in the protein deficient rats.

CONCLUSIONS

In view of these results, and taking account of the background information on erythropoietin provided by the work of other investigators, the following is proposed as the probable mechanism of the anaemia resulting from protein deficiency:-

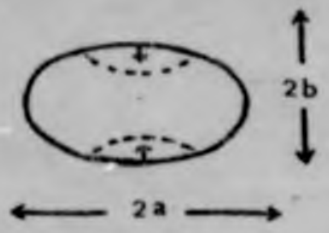
1. The primary cause of the anaemia was an insufficiency of protein supply at the bone marrow. This was the interpretation of the finding that the hyperplasia of the bone marrow, due to the stimulus of an elevated erythropoietin level, was unable to prevent the onset of anaemia.
2. A secondary cause was extravascular haemolysis, with some of the defective erythrocytes being captured by the spleen on their release from the bone marrow. It is thought that a deterioration in quantity of the erythrocytes released to the circulation occurred as a result of the combination of a stimulus from erythropoietin to produce more erythrocytes together with a restriction on this production from the insufficiency in protein supply at the bone marrow.

APPENDIX

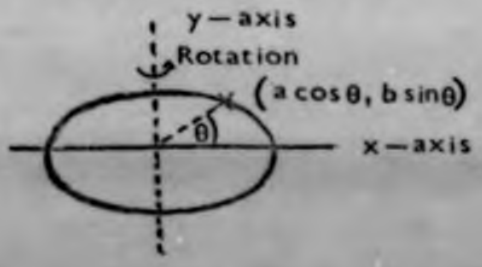
APPENDIX

CALCULATION OF SURFACE AREA OF A RED BLOOD CORPUSCLE

Ponder has indicated that the surface area of a red blood corpuscle can be estimated by comparing the red blood corpuscle to an ellipsoid, since the surface area of an ellipsoid would not be altered by indenting its surface at two opposite poles to produce a discoid shape:



The surface area of the equivalent ellipse can be calculated by the standard methods of calculus. An ellipse of semi-major axis a and semi-minor axis b, with parametric co-ordinates (a cos θ , b sin θ), is rotated about the y-axis to produce the appropriate discoid shape (Rotation about the axis would give an egg-like shape).



The surface area, S, is given by:

$$S = \int_{y=-b}^{y=b} 2\pi x \left[1 + \left(\frac{dx}{dy}\right)^2\right]^{\frac{1}{2}} dx$$

Using the form $x = a\cos\theta$, $y = b\sin\theta$,
we have $dx = -a\sin\theta\, d\theta$, $dy = b\cos\theta\, d\theta$
and thus:

$$S = \int_{-\frac{\pi}{2}}^{+\frac{\pi}{2}} 2\, a\cos\theta \left[a^2\sin^2\theta + b^2\cos^2\theta\right]^{\frac{1}{2}} d\theta$$

$$S = 2\pi a \int_{-\frac{\pi}{2}}^{+\frac{\pi}{2}} \cos\theta\, (b^2 + a^2e^2\sin^2\theta)^{\frac{1}{2}}\, d\theta$$

with $a^2e^2 = a^2 - b^2$

or $= \frac{\sqrt{a^2 - b^2}}{a}$

This can be simplified by the substitution $z = \frac{ae}{b}\sin\theta$,

giving:

$$S = 2\pi a \int_{-\frac{ae}{b}}^{+\frac{ae}{b}} (b^2 + b^2z^2)^{\frac{1}{2}}\, \frac{b}{ae}\, dz$$

$$= \frac{2\pi b^2}{e} \times 2 \int_{0}^{+\frac{ae}{b}} \sqrt{1 + z^2}\, .\, dz$$

$$= \frac{4\pi b^2}{e}\left[\tfrac{1}{2}Z\sqrt{1+Z^2} + \tfrac{1}{2}\sinh^{-1}Z\right]_{Z=0}^{Z=\frac{ae}{b}}$$

$$= \frac{4\pi b^2}{e}\left[\frac{ae}{2b}\left(1+\frac{a^2e^2}{b^2}\right)^{\frac{1}{2}} + \tfrac{1}{2}\sinh^{-1}\left(\frac{ae}{b}\right)\right]$$

$$= 2\pi a^2 + \frac{2\pi b^2}{e}\sinh^{-1}\left(\frac{ae}{b}\right)$$

The surface area of the equivalent ellipse is thus

$$S = 2\pi a^2 + \frac{2\pi b^2}{e}\sinh^{-1}\left(\frac{ae}{b}\right)$$

where a = semi-major axis of ellipse
= radius of red blood corpuscle

b = semi-minor axis of ellipse,
and is related to the thickness of the red blood corpuscle by a factor dependent on the degree of indentation of the corpuscle : Ponder's factor was used here:
b = 0.67 x maximum thickness

$e = \sqrt{\frac{a^2 - b^2}{a}}$ = eccentricity of ellipse

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