THE KINETICS OF ALBUMIN METABOLISM IN EXPERIMENTAL PROTEIN CALORIE MALNUTRITION.

A Thesis submitted to the University of Cape Town

for

the Degree of Doctor of Medicine

by

Ralph E. Kirsch M.B., Ch.B. (Cape Town).

Cape Town

February, 1968.

F R O N T I S P I E C E.

Weight matched rats pair-fed 5% and 20% protein diets for 60 days. (Page 33)

To

BILL HOFFENBERG

A C K N O W L E D G E M E N T S.

Professor J.F. Brock has been the stimulus and guiding hand throughout the progress of this study. He has provided both the stimulus and the opportunity for me to do research and during the two years I have spent working as Research Bursar in the C.S.I.R. Nutrition Unit under his direction, he has taught me a great deal both by example and by spoken word. More specifically, he first suggested the production of an experimental model of kwashiorkor and its use in studying, amongst other things, albumin synthesis. Despite his extremely crowded programme, he has always set aside time for our meetings and discussions, often sacrificing his lunch hour in the process. For all these things it is a great pleasure to thank him.

Dr. Stuart Saunders voluntarily involved himself in this work at a stage when everything was going wrong. He cheerfully assumed the responsibility of guiding my work and placed his laboratory at my disposal. In addition, during the last months of this project he made his technician

available to assist me. I greatly value both his help and his friendship.

Dr. Bill Hoffenberg, to whom this thesis is dedicated, is an acknowledged expert in the field of protein turnover. He unselfishly imparted a great deal of knowledge to me and many of his ideas are to be found in this thesis. He was always willing to help me where two pairs of hands were needed and without his experience and guidance this study could not have succeeded.

Dr. A.S. McFarlane of the National Institute for Medical Research, Mill Hill, London, whose technique I have used in this study, not only advised me initially on the methodology but answered my many written queries with amazing alacrity.

My technical training was in the hands of Miss. Elizabeth Black, C.S.I.R. Senior Technical Assistant. That I was able to acquire the technical skill involved in the McFarlane technique and the techniques for iodination of protein, bears testimony to her ability not only as a technician but as a teacher and as an authority on radioactive isotopes and their use in research.

This work could not have been completed without the assistance, during the past months, of Mrs. Lesley Frith.

She rapidly involved herself in the project and became more than a technical assistant in that she helped me with many of the modifications of the methods used. Our experiments involved a great many hours of working overtime, which she took as a matter of course.

The many beautiful diagrams used in this thesis are the work of Mr. A.February, while the photography was performed by Mr. S. Hendriks.

I would like to thank all those members of the medical, technical and secretarial staff of the Department of Medicine who have helped me over the past two years. To the librarian and staff of the Medical Library go my sincere thanks.

Finally I would like to thank my wife, not only for her encouragement and help during the progress of this study, but also for typing this thesis.

C O N T E N T S.

PART PAGE

PART PAGE

PART PAGE

PART 1.

INTRODUCTION.

(a). THE IMPORTANCE OF PROTEIN CALORIE MALNUTRITION:

Protein Calorie Malnutrition (PCM) is a term which embraces the broad spectrum of diseases occurring where diets are poor in protein. In most cases the diseases are due to an inadequate intake of utilizable protein. A small percentage of cases are due to excessive protein loss, (e.g. burns, nephrosis, protein loosing enteropathy) or to defects in protein absorption (gastro-enteritis, steatorrhoea) or synthesis (liver disease). Where the caloric intake is relatively normal the disease produced, kwashiorkor, is characterised by retardation of growth and development, muscular wasting, oedema and psychic changes, usually peevishness or apathy. In addition, some cases have skin lesions and the colour and texture of the hair is often abnormal. Diarrhoea is almost constant in the recent history and is usually present on admission to hospital. The disease is most common in children who have recently been weaned.

When the calorie intake is considerably below optimum, the resultant clinical state is one of emaciation and is termed marasmus. This can occur even on a perfectly balanced diet as the protein is used to provide the energy lacking in the diet. The above syndromes are those found at opposite ends of the spectrum and they merge to form atypical cases termed marasmic kwashiorkor.

Kwashiorkor has long been known by the African peasants as an expected and ordinary occurrence. They recognised its pathogenesis i.e. removal from the breast, and named the disease accordingly. The word kwashiorkor means the deposed child in the language of the Ga tribe in Accra.

The first published description of the disease was that by Czerny and Keller in Germany in 1906 under the name Mehlnährschaden.(1) This was followed by a description of Culebrilla, a disease encountered in Mexico by Correa in 1908.(2) In 1913, Guillon,(3) working in Indo-China, described "Bouffissure d'Annam" (Annam's swelling) and McConnel's (4) report on a"new oedema disease" (1918) was the first from Africa. These reports clearly established the fact that kwashiorkor occurs in all parts of the world but because of lack of

communication, the isolation of the above workers, and because of the obscure journals in which their descriptions were published, each continued to believe that his disease was peculiar to the area in which he worked and their descriptions failed to attract any attention.

The disease was first described under the name "kwashiorkor" by Cicely Williams (5) who, while working in Accra in the Gold Coast Colony, reported to the Medical Department in 1931 on a "well marked syndrome, not uncommon among the children here which I cannot find described in any of the ordinary text books".

The disease occurred in children from 1 to 3 years and was characterised by peevishness, diarrhoea, skin lesions, oedema and hepatomegaly. At post-mortem an enlarged fatty liver was the only definite finding. She reported curing one child by continuing with breast feeding and adding condensed milk to the diet. The name "kwashiorkor" however, only appeared in 1935 when Dr. Williams (6) published a description of sixty cases of the disease seen in three years in the children's hospital in Accra. The etiology of the disease remained unrecognised, however, due to a red herring! It was the skin lesions of the children suffering from the disease which most impressed observers and these certainly resembled those of pellagra.

In addition, Williams, when discussing these, stated that "the fact that this disease is found in people living almost exclusively on a maize diet, makes it at any rate comparable to pellagra. The differences are obvious - but there are also points of similarity".

Meanwhile Trowell [7] who arrived in Kenya in 1929, had become interested in"a nutritional oedema of unknown cause". A few years later, Gillon,[8] working at Tumutumu Hospital and quite unaware of the work in West Africa, wrote the first full clinical description of kwashiorkor in East Africa (1934-35). He minimised the skin lesions and no references were made to the possibility of pellagra. However, he was impressed by the loose "pale, greasy or soapy looking" stools which were typical of his cases and he discussed the possibility of a specific infective agent in the small intestine.

Stannus in 1934 [9] launched the first of many attacks on Cicely Williams. He wrote with the backing of much experience in Nyasaland that this condition was pellagra. In addition, Trowell had sent him a draft of a paper with photographs and Stannus had allowed a note to be added to the paper that it had "further confirmed in his opinion that the skin condition was certainly typical of pellagra in native children".

This argument and its confirmation by Loewenthal [10] and Sequira [11] among others, were so convincing, that Brock in 1949, at the first Food and Agricultural Organisation/World Health Organisation Joint Expert Committee on nutrition, had to use the subterfuge of raising the subject of kwashiorkor under the misnomer "infantile pellagra" in order to get it into the agenda.

Brock's communication was the beginning of a new era, for at their next meeting in 1951, the Joint Committee had before it a report by Brock and Autret [12] which firmly established the name "kwashiorkor" and its relationship to a low protein diet. This survey concluded that even if the term were restricted to severe cases with arrested growth and development, oedema, dyspigmentation and fatty liver at the post weaning stage, it was still an important syndrome. In discussing milder cases, Trowell is quoted as saying that the condition was "so common that many doctors would regard it as almost normal that an African child in the second year of life should grow but little, have brown soft hair, a pale skin and a low serum albumin. If this is a true appraisal of the situation then in certain parts of Africa it is probable that the majority of children in the second and third year of life suffer from kwashiorkor".

Further surveys established the occurrence of the disease in Latin America, while from the rest of the world reports established its world wide prevalence and by 1959, Jelliffe in his review "Protein Calorie Malnutrition in Tropical Preschool Children" was able to state that the disease was the main contributor to the very high morbidity and mortality in children in the sub-tropics and tropics.

(b) THE IMPORTANCE OF THE SERUM ALBUMIN IN PCM:

When it became apparent that kwashiorkor was a protein deficiency disease, an effort was made to define the existence and degree of protein depletion in biochemical terms and attention was focused on the serum proteins. The reason for this was twofold; the serum proteins were the most accessible proteins in the human body, requiring for their estimation venipuncture or heel prick only, and in addition they were easy to estimate.

It was soon found that the total serum proteins were diminished in cases of kwashiorkor. However, when the individual proteins were assayed, the low serum proteins were found to be due to hypoalbuminaemia, the total globulins being normal.

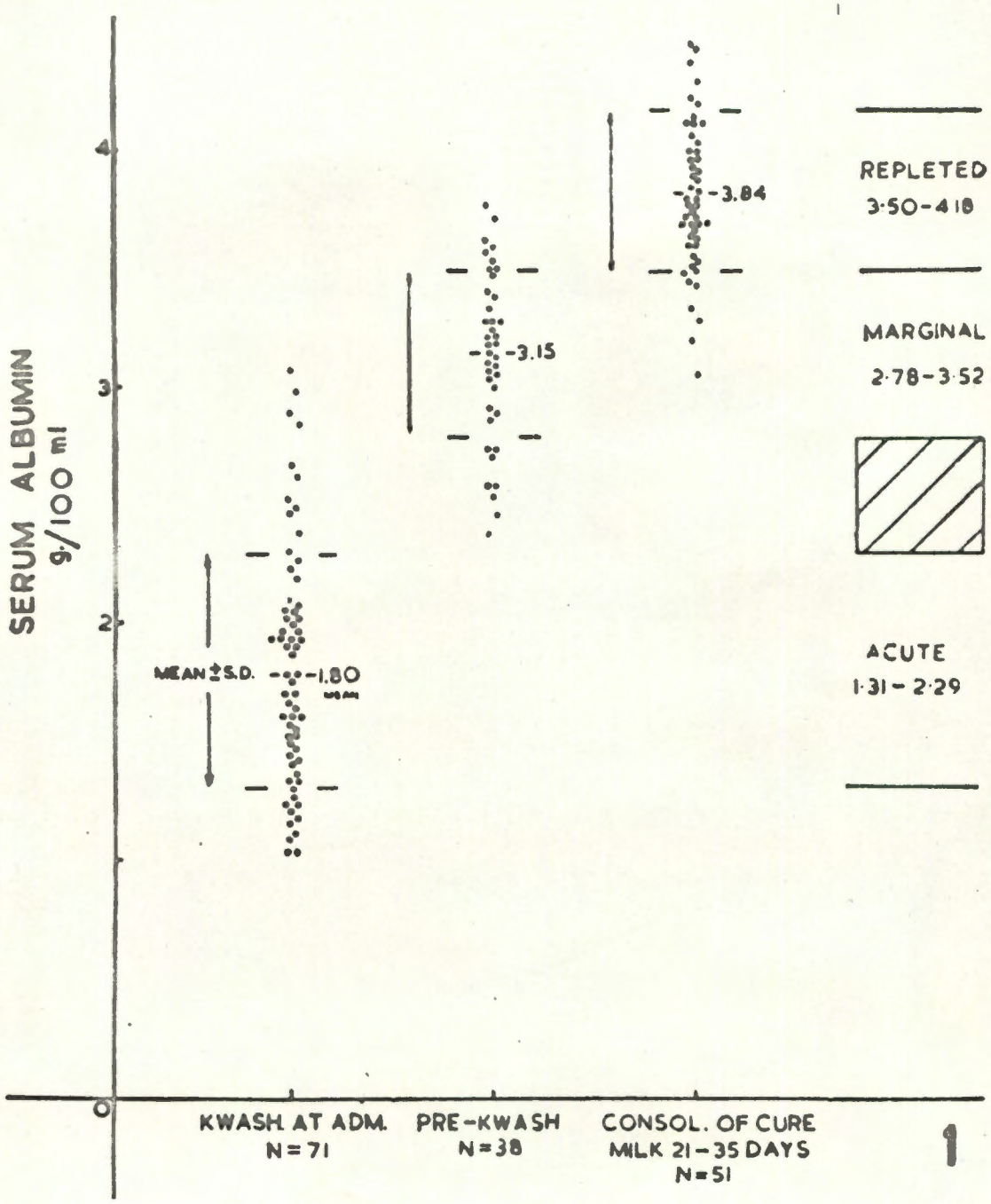


Fig. 1.Marginal hypoalbuminemia. The range and mean ± S.D. on the three groups of children. The groups of 71 and 51 children represent, respectively, untreated kwashiorkor and fully treated kwashiorkor. The group of 38 children had been maintained for 11 weeks on a low-protein (wheat) diet (32).

In their book "Kwashiorkor", Trowell, Davies and Dean [14] summarised work done up to 1953 and the results quoted proved beyond doubt that in kwashiorkor there is a significant hypoalbuminaemia.

The next step was to correlate the severity of the clinical state with the degree of hypoalbuminaemia and this was done by Schendel, Hansen and Brock [15] (Fig. 1.)

While the serum albumin level is always lowered in severe PCM, it is accepted that considerable depletion can exist before this reduction occurs. [16] For this reason the plasma albumin level is a poor index of threatening or marginal protein depletion. However, in order to maintain normal plasma albumin levels, in the face of mild or impending protein depletion, various adaptative processes must be occurring. In the light of our present knowledge it has been postulated that the following sequence of events may occur. The immediate effect of reduced protein intake is reduced albumin synthesis, which would tend to diminish the intravascular pool of albumin. This tendency could be met by a lowering of catabolic rate, and, at the same time, increased transfer of albumin from the extravascular pool - measures that would protect the intravascular pool. [17]

This sequence of events has never been proved because of the lack of a reliable technique for the direct measurement of albumin synthesis rates. Recently such a technique has been developed [18] and accordingly it was decided to use it in order to test the above hypothesis as its confirmation could lead to a test for early or marginal PCM and would greatly increase our understanding of the sequence of events leading to the hypoalbuminaemia characteristic of this disease. For ethical and technical reasons it was decided to limit this study to laboratory animals and we thus set out to produce an experimental model of PCM. Before embarking upon this project therefore, the literature on experimental PCM was reviewed and this review is found in Part 11.

PART 11.

PCM IN ANIMALS.

(a) INTRODUCTION:

In 1920, fourteen years after Czerny and Keller's article on Mehlnährschaden,[1] Emma A. Kohman [19] published an article entitled "The experimental production of oedema as related to protein deficiency". Her rats, on a diet of carrots and cornstarch, developed oedema which failed to respond to fat, or to fat soluble and water soluble vitamins. However, the oedema could be prevented or cured by the substitution of protein for the cornstarch in the diets. Since then, particularly after the recognition of protein malnutrition as the cause of kwashiorkor in the late 1940's, many workers have used animal models in an attempt to increase their understanding of the complex deficiency state. Although the fundamental role of protein deficiency in the etiology of kwashiorkor is now established, this human syndrome with its multiple deficiencies, intercurrent infections and infestations, is a poor model for the study of the precise role of protein deficiency per se.

TABLE I.

DESCRIPTIONS OF PRINCIPAL DIETS AND REGIMENS USED TO PRODUCE PROTEIN CALORIE DEFICIENCY IN PIGS AND DOGS.

Abbreviation Used	Animal	Protein Value (NDpCal%)	Brief Description of Diet and/or Regimen
LP (3.5)	Pig	3.5	All vegetable "Gambia type".
LP + CH (2.5)	Pig	2.5	LP (3.5) diet + 100gm carbohydrate given between meals.
5 CLP (6.6)	Pig	6.6	LP (3.5) diet with 5gm casein replacing 5gm starch in every 100gm diet; two meals per day.
20 CLP (8.3)	Pig	8.3	LP (3.5) with 20gm casein replacing 20gm starch in every 100gm diet; two meals per day.
NI (12.1)	Pig	12.1	Commercial "weaner meal" fed ad lib.
UK (8.6)	Dog	8.6	United Kingdom type diet: fed ad lib.
UK (6.8)	Dog	6.8	Modified UK (8.6) diet
B (12.0)	Dog	12.0	A complete diet, fed ad lib.
B (6.3)	Dog	6.3	B (12.0) diet in which 16gm casein in every 100gm diet is replaced by dextrinized maize starch.
Stock	Dog	9.4	Stock diet + commercial dog food and skimmed milk powder

From Mammalian Protein Metabolism. ed. Munro, H.N. and Allison J.B. (20)

Many species have been used, the commonest being the rat, dog, pig and monkey and the models produced were designed to represent all types and grades of protein calorie malnutrition.

(b) METHODS USED IN THE PRODUCTION OF EXPERIMENTAL PCM:

Many types of low protein diets have been used in the production of experimental models of PCM. The diets used varied from sophisticated diets in which the net dietary-protein calories percent was known,[20]

$$\left(\text{NDP cal \%} = \frac{\text{retained protein (Kcal)}}{\text{total metabolizable energy in food consumed}}\right)$$

(see Table I) to diets made up of indigenous cereals in areas where PCM is rife.[21]

In all cases one common factor soon emerged, i.e. animals put on to low protein diet lost their appetites. This loss of appetite has been the most important limiting factor in producing an animal model of PCM and workers have had to choose between tube-feeding an adequate amount of calories of a very low protein diet,[22] or using a diet with a high enough protein concentration to ensure a reasonably normal caloric intake.

TABLE II.

THE MAINTENANCE LEVELS OF DIETARY PROTEINS.

TYPE OF PROTEIN	MAINTENANCE PROTEIN LEVEL
Egg Protein and herring meal	2%
Casein	2 - 3%
Soya beans and linseed meal	3%
Wheat gluten	3.5%
Pea Meal	5%
Zein and lysine and tryptophan	More than 10%

When animals have been force-fed the experiments are usually of short term duration.

Rats require a daily intake of 1.3G of ideal diet/10G body weight. (Platt)[23] or 200 Kcal/Kg^{0173} [24] for maintaining their weight. The corresponding protein intake (for an ideal protein) is about 190mg N/$Kg^{0.73}$. The minimal percentage of protein in the diet required to maintain the rats weight varies from protein to protein. (Table II).

As can be seen from this table, it is impossible to compare any two diets in which the protein contents are expressed as a percentage without knowing the exact composition of these diets. The various diets were fed for different periods of time. In general the animal force-fed a no protein diet developed PCM more rapidly than those animals fed a low protein diet ad lib. Halder and Platt force-fed rats a no protein diet for 3 days[25] Bhuyan et al[26] and Best et al[27] used a 3% casein diet and Widdowson and McCance 6% protein for longer periods[28] usually for at least 3 weeks. Rose et al [29] and Sidransky and Farber[30] used diets deficient in various amino acids while Shils[31] fed protein of plant origin.

Animal experiments are usually designed to produce either undernutrition or protein calorie malnutrition

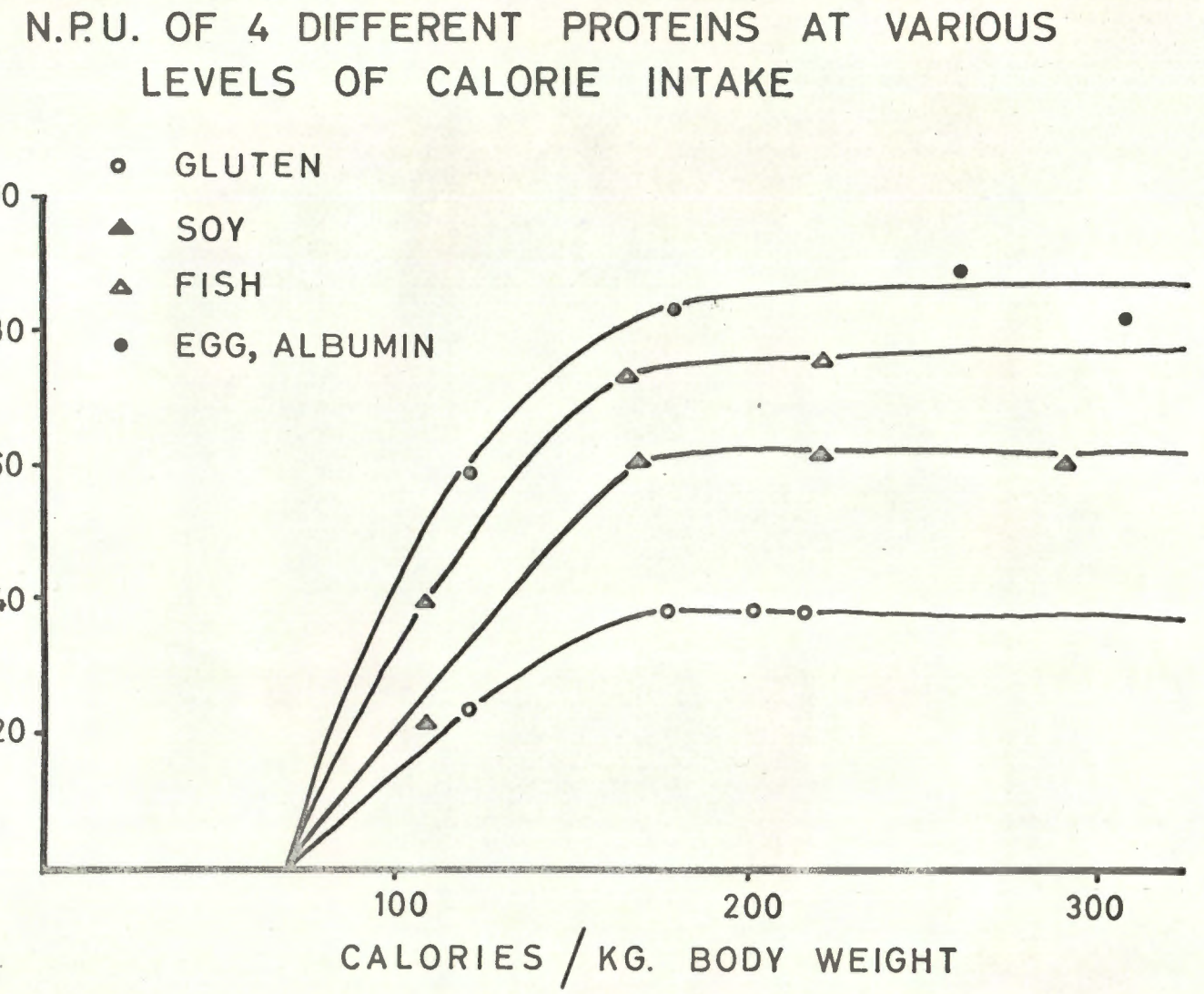

N.P.U. OF 4 DIFFERENT PROTEINS AT VARIOUS LEVELS OF CALORIE INTAKE
GLUTEN
SOY
FISH
EGG, ALBUMIN
0
30
60
40
20
100
200
300
CALORIES / KG. BODY WEIGHT

Fig. 2.

with a low protein but adequate calorie intake. In order to prove that the effects produced are due to protein deficiency the animals are either force-fed or else pair-fed, i.e. they are divided into pairs and one member of each pair receives a low protein diet while the other receives an isocaloric amount of a high protein diet. If the animal on the high protein diet fails to gain or maintain its weight, the effects in both are due to undernutrition. As protein malnutrition is frequently associated with inadequate caloric intakes, Miller et al [32] using rats, assessed the protein values of diets with differing caloric values. They found that as the caloric value dropped, more and more protein was being burnt for energy purposes. They concluded that the protein values of diets when fed in restricted amounts are a function of the caloric intake only. In Fig. 2 the net protein utilisation (NPU) values for four different proteins at various levels of caloric intake are given.[33]

(c) CLINICAL FEATURES OF EXPERIMENTAL PCM:

In rats force-fed a diet devoid of an essential amino acid,[30] the hair of the experimental animals became rough and shaggy within a few days and they developed alopecia of their heads and hind legs.

TABLE III.

COMPARISON OF CHEMICAL AND MORPHOLOGICAL CHANGES PRODUCED IN IMMATURE RATS BY FORCE-FEEDING AND BY AD LIBITUM FEEDING OF THREONINE-DEVOID DIETS.[30]

	Force-Feeding	Ad Lib. Feeding
Change in body weight	Slight decrease	Considerable decrease
Liver.		
Weight	Increase	Decrease
Protein	No change	Decrease
Lipid	Increase (peri portal)	Questionable decrease
Glycogen	Increase	Questionable decrease
Pancreas.		
Weight	Increase	Increase
Protein	No change	Decrease
Amylase	Decrease	Decrease
Morphologic changes	Acinar atrophy and oedema	No change
Skeletal Muscle (gastrocnemius)		
Weight	Decrease	Decrease
Protein	Decrease	Decrease
Salivary Glands.		
Parotid	Atrophy	No change
Submaxillary	Atrophy	No change
Stomach.		
Glandular portion	Atrophy and loss of mucin	No change
Adrenal.		
Zona glomerulosa	Atrophy	No change

In rhesus monkeys there were no appreciable differences between the animals on low and high protein diets for the first few weeks. However, as the deficiency progressed, by the tenth week, considerable weight loss had occurred in the experimental group and some of them had oedema of the lower eyelids. Ascites was found in three of the four animals sacrificed after 15 weeks on the low protein diet. (34) Dogs given a low protein diet intended to produce mild PCM, were smaller than control animals. (20) They all had diarrhoea but were not obviously ill. As the animals grew older the discrepancy in size became less for although the malnourished pups grew more slowly, their growth period was extended. The differences between animals on low protein adequate caloric diets and those on low protein low caloric diets are well illustrated in Table III. Sidransky and Farber (30) force-fed one group of rats with adequate amounts of a diet devoid of threonine and allowed another group of rats to eat the same diet ad lib. In the latter case the rats lost their appetites and thus their caloric intake was low. These two groups of animals are compared in Table III and show striking resemblances to children suffering from kwashiorkor and marasmus respectively.

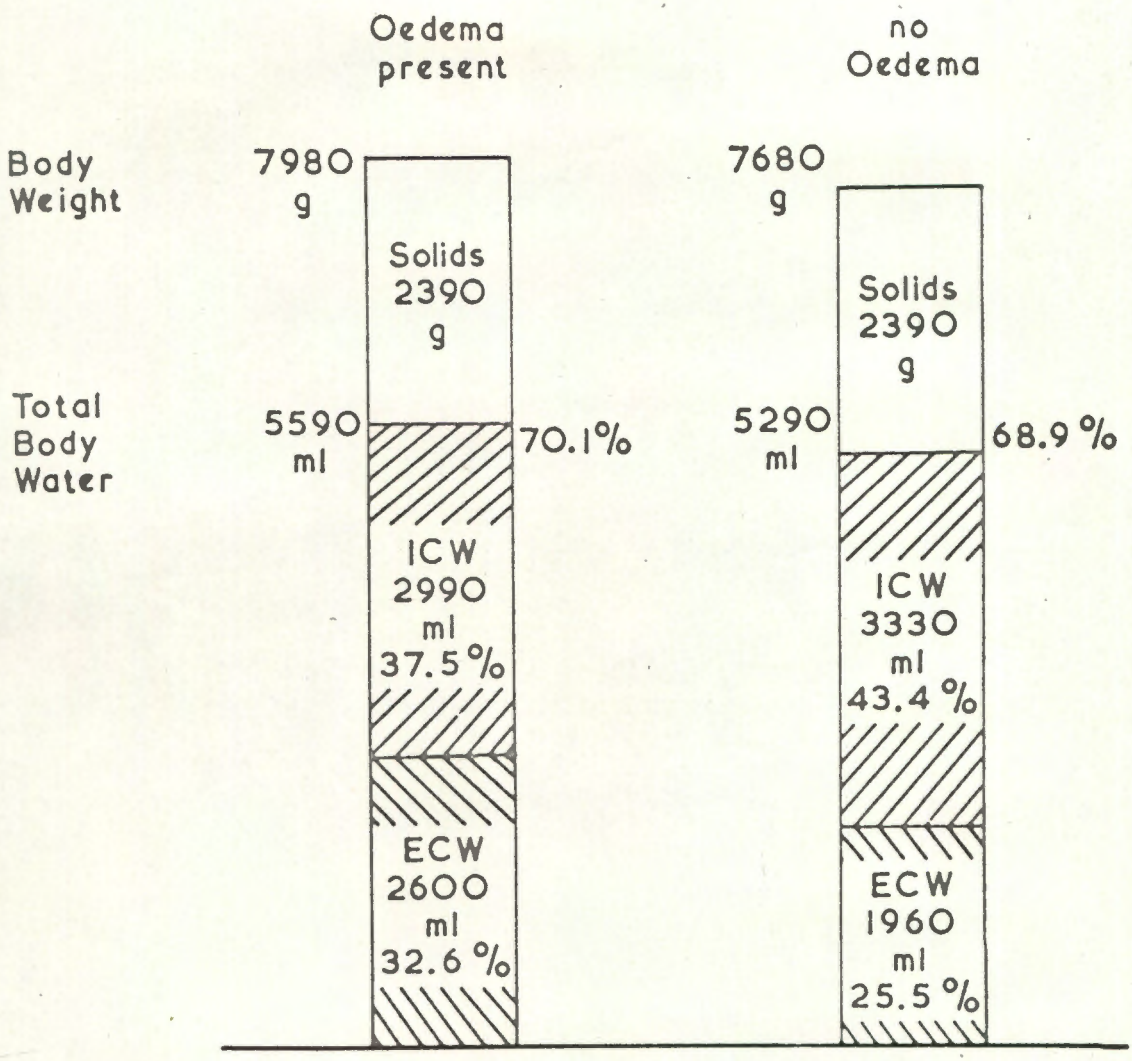


FIG. 3.Body water during recovery from kwashiorkor. Average of fourteen cases.

TABLE IV.

ORGAN WEIGHTS EXPRESSED IN TERMS OF BODY WEIGHT FOR PIGS FED ON VARIOUS DIETS.[20]

	Age Control	Deficient animals		Weight Control
Diet	N1 (12.1)	5CLP(6.6)	LP(3.5)	N1 (12.1)
Pig No.	90[a]	88[a]	92[a]	142
Age at death (days)	65	66	63	15
Weight (kg)	30.0	6.85	4.05	4.05
Spleen (gm/100 gm)	0.17	0.23	0.16	0.20
Liver (gm/100 gm)	2.47	2.76	4.05	3.56
Kidneys (gm/100 gm)	0.52	0.48	0.63	0.65
Brain (gm/100 gm)	0.24	0.92	1.48	1.09
Adrenals (mg/100 gm)	9.70	19.20	33.20	16.50

a. Littermates.

In addition to the failure of growth observed in experimental PCM, the organ weights as a percentage of body weight are usually comparable to those of a much younger animal. (Table IV).

Widdowson et al [35] state that "the outstanding changes that take place in the chemical structure of the body of an undernourished man or animal are a loss of fat and protein and a gain in the proportion of water.

(d) BIOCHEMICAL FEATURES OF EXPERIMENTAL PCM:

i) BODY WATER:

The measurement of body water in experimental PCM is difficult as some doubt has been cast on the "thiocyanate space" as a reflection of the extracellular volume. In children suffering from kwashiorkor, the total body water as a percentage of body weight, was found to be slightly increased due to a disproportionate increase of the extracellular water. In contrast, there was a reduction in the volume of the intracellular water.[36] (Fig. 3.). These changes, which have been reproduced in experimental animals on low protein diets, were found to be aggravated by the feeding of extra calories in the form of carbohydrate. [20]

ii) ANAEMIA AND PROTEIN MALNUTRITION:

In 1947 Trowell [37] stated that when cases of macrocytic anaemia and dimorphic anaemia in African men and non-pregnant women were investigated in Uganda, a large number were found to show one or more of the features of the kwashiorkor syndrome. When one considers that 96% of the haemoglobin molecule is formed by the protein globin, it is not surprising that workers [38] [39] have found that their animals on low protein diets have low haemoglobins which respond to protein alone. Al Rabii [40] in 1962, treated one of two severely anaemic protein deficient pigs with iron and the other with protein and noted an improvement only in the latter. In addition, serum iron levels of anaemic dogs with PCM were normal or above normal. [20]

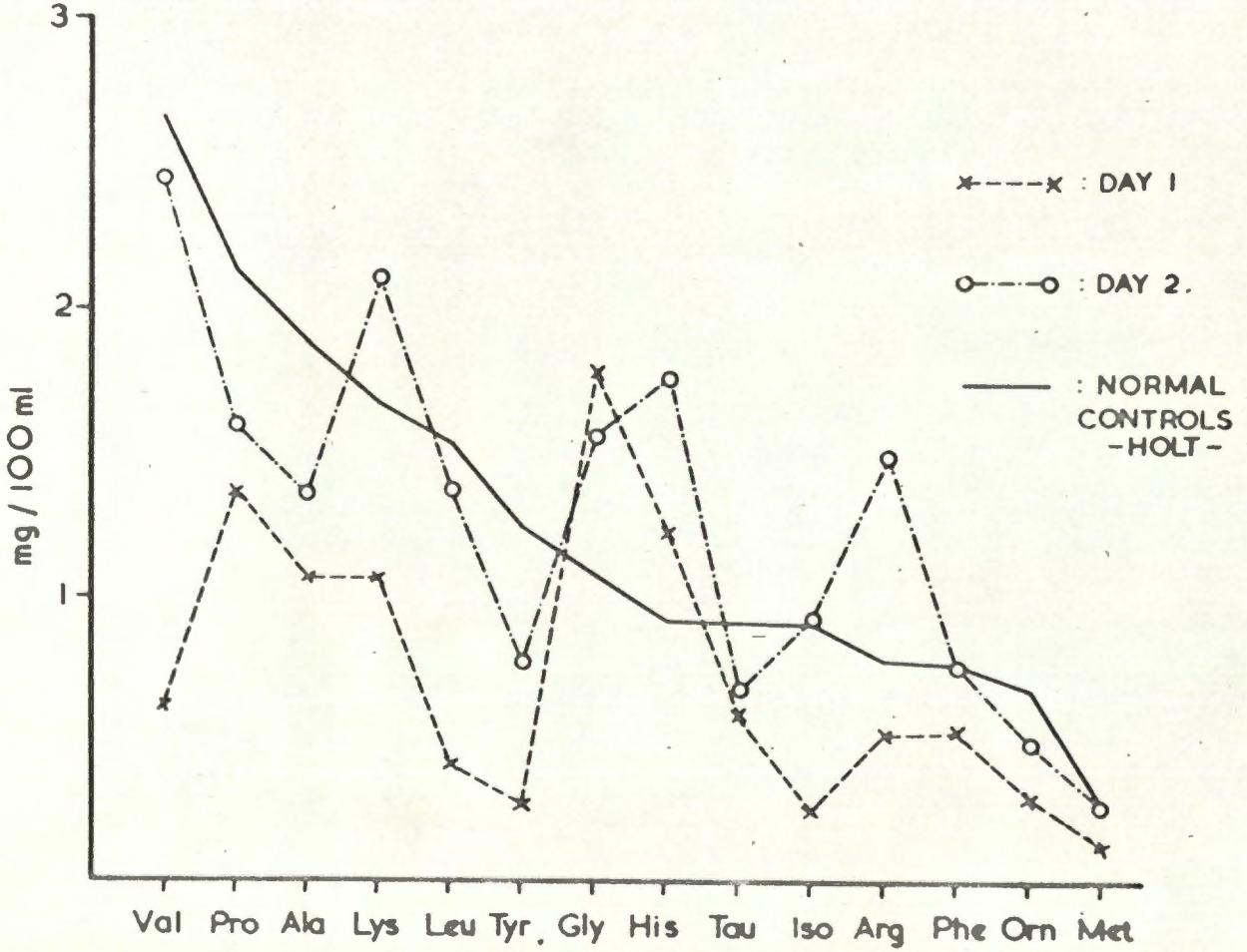
iii) SERUM PROTEINS:

In their book on kwashiorkor (1954) Trowell et al [14] state the simplest biochemical manifestation and the one which has been the most frequently studied, is the level of the total serum proteins. Brock and Autret, [12] quoting Trowell's results, stated: "almost without exception, when the serum albumin has been determined in oedematous cases of kwashiorkor it has

TABLE V.[42]

EFFECTS OF PROTEIN DEPLETION ON THE PARTITION OF SERUM PROTEIN IN THE ADULT RAT.

Group	Total Serum Protein (gm%)	Albumin (gm%)	Globulins			
			Alpha 1. (gm%)	Alpha 2. (gm%)	Beta (gm%)	Gamma (gm%)
A. Normal	5.8 ± 0.04	2.0 ± 0.03	1.5 ± 0.03	0.7 ± 0.03	1.1 ± 0.03	0.5 ± 0.02
B. Inanition	5.1 ± 0.02	1.7 ± 0.05	1.3 ± 0.04	0.5 ± 0.03	0.9 ± 0.03	0.7 ± 0.03
C. Protein-Free	4.8 ± 0.05	1.7 ± 0.02	1.2 ± 0.03	0.5 ± 0.02	0.8 ± 0.02	0.6 ± 0.03
D. 2% Protein	4.9 ± 0.07	1.7 ± 0.04	1.3 ± 0.03	0.4 ± 0.02	0.1 ± 0.05	0.5 ± 0.04



-Mean free plasma aminoacid levels of patients with kwashiorkor on days 1 and 2 and in normal American controls (Holt et al. 1963).

been found to be reduced." Thus the serum albumin level remains "the most sensitive biochemical index of mild or impending protein deficiency," [41] and has been one of the first signs looked for by workers in this field. In their chapter in Munro and Allison, Platt et al [20] detail their results in dogs and pigs. Wiemer, [42] Ramalingaswami [43] and McCance [44] have produced hypoalbuminaemia in rats and Follis [45] and Ramalingaswami [46] have produced hypoalbuminaemia in monkeys. Friend et al [47] have shown a definite relationship between diet and serum albumin in pigs by changing their diet from high to low protein. There was, in their animals, no change in the alpha beta or gamma globulins. Wiemer, [42] using albino rats, observed a decrease in the serum albumin, the alpha globulins and the beta globulins. The gamma globulin level was significantly increased. (Table V.)

iv) THE PLASMA AMINO ACIDS:

The aminogram of the plasma amino acids in kwashiorkor has a profile which is peculiar to this form of PCM no matter where it occurs. [48,49,50,51,52] Holt, [49] who was the first major author in this field, attempted but failed to find a correlation between the severity of the disease and the plasma amino acid levels.

Fig. 4 is a combination of Holt's normals and Cape Town cases of kwashiorkor.(53) As can be seen, the branch chain amino acids i.e. leucine, isoleucine and valine are low while the glycine and histidine levels are above normal. After 1 day of protein re-feeding, there is an immediate increase of the plasma amino acids to above normal and their levels then slowly decline to normal. (53) It is possible that the rise in the plasma amino acids seen after only one day of re-feeding, is due to a temporary impairment of their utilisation for protein synthesis. This rapid reversal in children still suffering from severe malnutrition, suggests that the plasma aminogram has no place as a biochemical measure of protein nutritional status, as a glass of milk given to the patient at an outlying clinic would result in a normal aminogram on his arrival at hospital. The aminogram therefore only reflects the immediately preceding diet.

(d) THE PATHOLOGY OF THE GASTRO-INTESTINAL TRACT IN EXPERIMENTAL PCM:

i) THE MOUTH:

Epithelial cells scraped from the buccal mucosa of pigs and dogs with PCM when compared with those from normal animals, were distorted, often fragmented

and stained poorly. (54)

ii) SALIVARY GLANDS:

Deo et al (34) in their studies on PCM in the rhesus monkey, found microscopical changes in the salivary glands similar to those found in the pancreas of their animals. There was shrinkage in size of the acini and loss of cytoplasmic material. The parotid gland was most severely affected. Severe damage was noted in the salivary glands of rats force-fed a low protein diet, (20) while Sidransky and Farber (30) found a striking loss of cytoplasm in the cells of the parotid gland of rats force-fed a diet devoid of an essential amino acid.

iii) STOMACH:

In the rhesus monkey all parts of the stomach were affected. (34) There was a flattening of mucosal folds and gross atrophy of the muscularis externa whilst histologically the most marked change was an atrophy of the mucosa with reduction of all three secreting types of cells. Due to uncooked haricot beans in their diet, some of Platt's (20) pigs were found to have gastric ulcers. The latter were however, in his opinion, greatly aggravated by the deficiency of dietary protein.

The pigs when killed demonstrated the delayed emptying time of the stomach, found in children with PCM, as their stomachs often contained a considerable bulk of undigested food 12 to 18 hours after their last meal. The stomachs of rats with PCM were atrophic and almost devoid of mucin which was abundant in controls.(30)

iv) SMALL INTESTINE:

The "tissue-paper-like" small intestine of kwashiorkor has been reproduced in animals on a low protein diet by Deo et al. (34) In general the small intestines of animals with PCM are smaller in diameter and their walls are thinner. Histologically, the villi are flattened and their tips often bare of epithelial cells. As the lining cells of the small intestinal mucose are renewed every two or three days, this high turnover rate makes them particularly susceptible to PCM. Deo (55) studied the kinetics of these cells using tritated thymidine. His results indicated a disturbance of cellular proliferation of protein deficient monkeys as compared with controls. The transit time of jejunal epithelial cells was markedly lower in the protein deficient group. Whereas a cell from the jejunum of an animal with an optimal protein intake would cover a distance of one mile in 10,700 years, a cell from an

MIGRATION OF H^3- THYMIDINE LABELED CELLS IN PROTEIN DEFICIENT & CONTROL MONKEYS

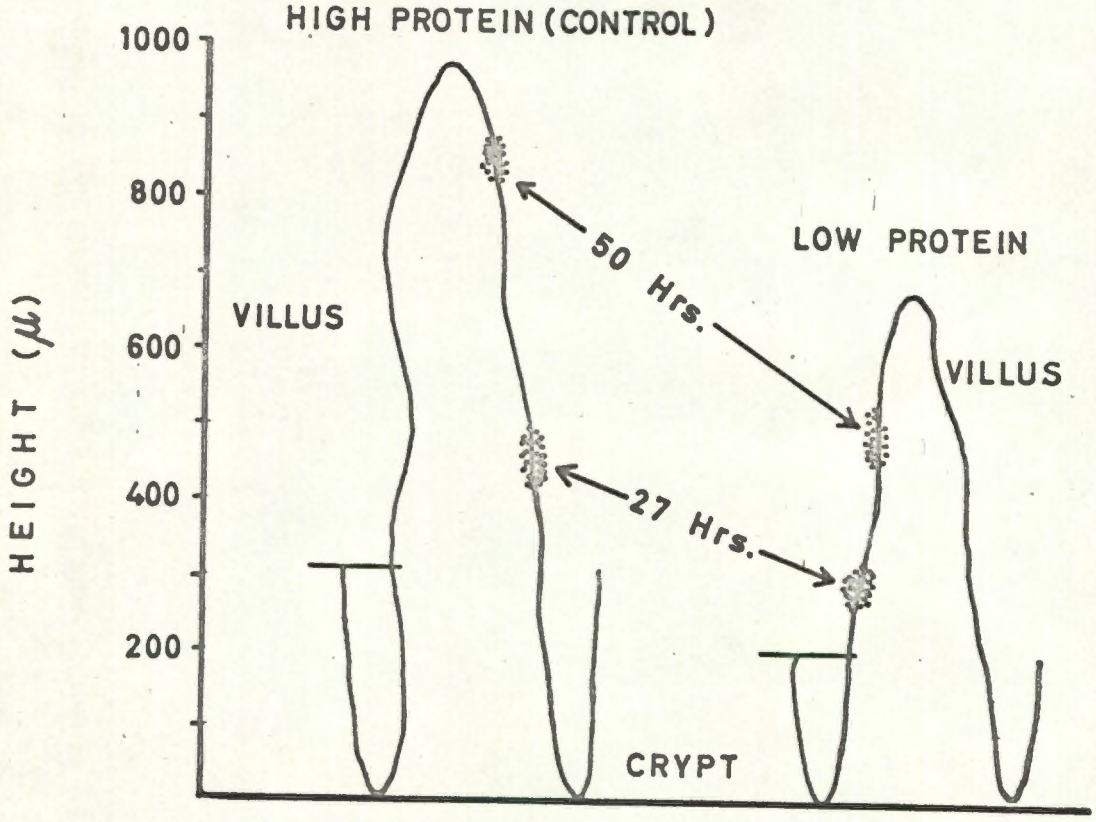


Fig. 5.

animal on a low protein diet would take 23,000 years. (Fig. 5.). Sirsat et al [56] have examined the epithelial lining of the small intestine of the rhesus monkey under the electron microscope and describe the brush border with its microvilli as being blunted, loosely orientated and having lost its terminal web, They conclude that as this is the site of active absorption of sugars and proteins, it could be expected that disorders of their absorption would result. Using cobalt 58 cyanocobalamine in their protein-malnourished monkeys they have demonstrated defective absorption of this vitamin.

v) THE EXOCRINE PANCREAS:

While this organ is severely affected by a low protein diet per se the lesion is more grave, both in extent and nature, if carbohydrate and fat are present in adequate amounts. If the latter is the case then the rapid loss of cytoplasm, which is reversible, progresses to destruction starting with dissociation of the glandular tissue and ending with necrosis.

Veghelyi [57] et al fed rats a diet deficient in methionine (10% of requirement) and tryptophan (50% of requirement). This "released a chain of events consisting of disappearance of the zymogen granules from

the supranuclear zone, acidophilia and then vaculolation of the cytoplasm, cyst formation and finally diffuse scarring strangulating the acini!' These changes were prevented by the addition of methionine to the diets but the addition of tryptophan had no effect on the lesions.

Similar changes have been reported in rats by Friedman and Friedman, [58] Platt et al [20] and Sidransky and Farber [30] by Deo et al [34] in the rhesus monkey and by Platt et al in pigs. [20]

Using an electrophoretic method of separating the pancreatic enzymes, Veghelyi [57] found the following changes in enzyme production in rats on a diet devoid of methionine. In two weeks trypsin had disappeared while lipase esterase and chymotrypsin were reduced in amount. Eventually amylase was the only remaining enzyme. When he treated his rats with carbon tetrachloride in addition to the diet, the changes, both functional and histological, were more rapid and severe. By using crossed circulation between a protein malnourished dog and the pancreas of a well-nourished dog, the authors proved that the functional changes were inherent in the organ itself by observing no deviation from the expected pattern of secretion of the pancreata in control and malnourished groups.

In their paper Friedman and Friedman [58] discuss the part played by the pancreas in the etiology of the fatty liver. They found that changes in the pancreas preceded those in the liver and compared this sequence of events with that occurring in pancreatectomised animals which rapidly develop fatty livers. Montgomery et al [59] claimed that pancreatic juice contained a factor which prevented fatty livers. However, it appears more likely that the etiology in both cases i.e. pancreatic and hepatic involvement, is the same and that the pancreas, by virtue of its higher protein turnover rate, is affected before the liver is.

(e) THE PATHOLOGY OF THE LIVER IN EXPERIMENTAL PCM:

The early recognition of the fatty liver of kwashiorkor and its prominence at post mortem in this disease, has led to considerable research in experimental animals.

Many authors have produced fatty livers in animals by decreasing the amount of protein in the diet while maintaining the caloric intake. In fact, the amount of fat in the liver bears a direct relationship to and is proportional to the amount of calories fed to animals on low protein diets. [60] [61]

Closely comparable to this are the normal or increased values for liver glycogen obtained in rats [30] and pigs.[20] (Table III).

Macroscopically the livers are often a yellow-brown or even a bright yellow colour. The fatty changes due to PCM occur first in the periportal regions and while they spread throughout the lobe, the most marked changes are in the former. These changes closely resemble those found in children with kwashiorkor and are unlike experimental choline deficiency where the involvement is centrilobular. [27] The earliest light microscopical changes are the angular, vacuolated appearance of the cells; the cytoplasm, broken up by the accumulation of fat and glycogen, loses much of its basophilia. The cells appear bloated and enlarged and the sinusoids are relatively narrow. In severe cases fatty vacuoles fill the entire lobule. These changes have been produced by force-feeding low protein diets for 6 days [20] [62] but occur later (12-26 days) when diets are fed ad lib.[63] Most studies last from 6 days to 3 months or longer. The electronmicroscopical changes have been described by Svoboda et al. [64] They found a decrease in the mitochondria with cavitation of their matrices and dissolution

of membranes by 9 weeks. Many mitochondria contained a new type of inclusion consisting of stratified fibrils with a beaded or helical configuration. The endoplasmic reticulum dilated to form large cytoplasmic lakes which appeared to communicate with the widely separated nuclear membranes. Nucleoli were enlarged and showed separation of the pars amorphia from the nucleolonema and widening of the interchromatinic space.

Much interest has been shown in the relationship between PCM and the high incidence of cirrhosis of the liver in areas in which the former is particularly rife. Their possible relationship has been intensively investigated but has to date not been established. Various workers e.g. Bhuyan et al [65] and Shoental et al [66] have used hepatotoxins in animals on low protein diets. The former used carbon tetrachloride and the latter senecio alkaloids; in both cases there was less fibrosis in the livers of the animals on low protein diets. This may not be entirely unexpected as the laying down of fibrous tissue requires protein which in these animals was not available.

The pathogenesis of the fatty liver is probably a combination of two defects. The first is the tremendous free fatty acid (FFA) flux from the depot tissues

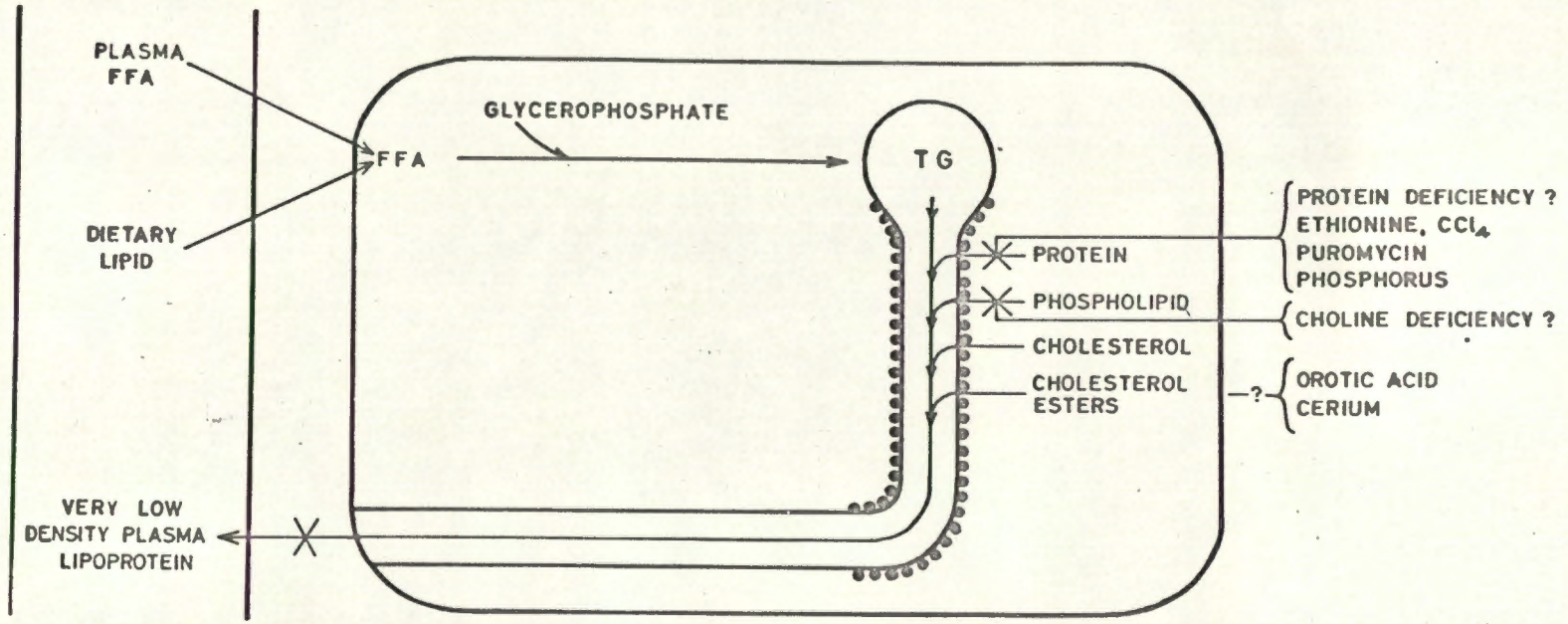


Fig. 6. The Pathogenesis of the Fatty Liver. (68) (69)

TABLE VI.

THE PERCENTAGE OF ORIGINAL PROTEIN LOST FROM DIFFERENT ORGANS IN FASTING RATS. (a)

Organ	7 Day Fast	20 Day Fast
Liver	40	20
Kidney	20	4
Heart	18	4
Other organs	10	4

(a) Data from Addis et al (1936) [70]

to the liver.[67] The second defect is the lack of protein in the liver to act as carriers for the triglycerides and other fats formed from the FFA. While the first defect is proven the second is inferred from the high fat level of the liver and the low plasma lipoprotein levels. The pathogenesis of the fatty liver is summarised diagrammatically in Fig. 6 which is taken from reviews by Lombardi [68] and Farber.[69] Puromycin, which inhibits protein synthesis, is probably similar to, though more severe in its action than, the effects of protein calorie malnutrition.

While the fatty liver has received the attention of many workers other changes have been demonstrated which are less well known. The protein loss from the liver is rapid and is large in amount.[70] (Table VI)

In addition there are changes in the activities of liver enzymes.[71] As these are highly specialised findings and are beyond the scope of this review, they are referred to and not elaborated upon.

(f) THE PATHOLOGY OF THE ENDOCRINE GLANDS IN PCM:

i) THE ANTERIOR PITUITARY GLAND:

In protein-calorie deficient pigs there is cellular vacuolation, loss of cytoplasm and crowding

together of cells in the anterior pituitary. (20) These changes have caused researchers to postulate hypo-function of this gland. In recent years Srebnick and Nelson (72) found diminished follicle-stimulating hormone, interstitial cell-stimulating hormone, growth hormone and thyroid-stimulating hormone in the pituitaries of protein depleted rats. It must be remembered however, that the hormonal content of a gland need not reflect its activity, e.g. the thyroid when active lacks colloid. However, recent work on children (73) has shown conclusively that the plasma growth hormone levels in kwashiorkor are higher than normal. This is not surprising as Platt et al (20) in their discussion on PCM in dogs state "The disparity in size and weight between pairs of littermates (on different protein diets) tended to lessen with age since, in the deficient animals, although the rate of growth was reduced, the period of growth was extended!" It would seem from this that growth hormone, despite the lack of building blocks, eventually succeeds in promoting growth by remaining elevated until such time as the animal has achieved a nearly normal adult size.

ii) THE PANCREAS: (ENDOCRINE)

During starvation there is a reduction of the insulin content of the pancreas.(74) Pigs with PCM

show crowding, loss of size and loss of aldehyde-fuchsin positive granules in their beta-islet cells.[20] In humans it appears that there is an insulin response similar to that shown in cases of beta-cell atrophy in children with kwashiorkor.[75]

iii) THE ADRENAL GLANDS:

In PCM these glands, while small when compared with animals of a similar age, are large when expressed as percentage body weights.[76] [20] There is at present conflicting evidence as regards histological and functional changes.[20]

iv) THE THYROID GLAND:

The follicular cells in PCM in pigs are flat and there are almost no colloid droplets in their cytoplasm. When dietary cure is initiated, there is, on the other hand, intense activity as evidenced by cubical follicular cells with many colloid-like droplets in their cytoplasm.[20] The ^{131}I uptake is reduced in rats on a 3.5% protein diet.[78] There is some debate whether this is a primary thyroid defect or whether it is secondary to pituitary failure.

v) THE GONADS:

McCance [44] and Halder [62] respectively have described large cystic follicles in pig ovaries, and a reduction in interstitial cells in rat testes, in their animals with PCM.

PART 3.

THE PRODUCTION OF AN EXPERIMENTAL MODEL OF PROTEIN CALORIE MALNUTRITION.

(a) INTRODUCTION:

The reproduction of human disease under controlled conditions is a major pursuit of medical science, and whenever this has been achieved, understanding has improved. (46) In order to study the kinetics of albumin metabolism in protein calorie malnutrition, it was considered essential to produce an experimental model of kwashiorkor. This protein deficiency disease, occurring in young children soon after weaning, is characterised clinically by retardation of growth and development, muscular wasting, oedema and psychic changes.

In addition some cases have skin lesions and the colour and texture of the hair are often abnormal. Biochemically hypoalbuminaemia is an invariable finding in developed cases while the lack of protein in the immediately preceding diet is reflected in the characteristic profile of the plasma aminogram. Histologically the outstanding feature is periportal fatty infiltration

of the liver. These features have been discussed in great detail in Parts 1 and 2.

Animals fed low protein diets rapidly lose their appetites and thus in order to produce a replica of kwashiorkor, animals have, in most cases, been tube-fed in order to ensure relatively normal caloric intakes. As tube-feeding is both time consuming and unphysiological it was decided to produce an experimental model allowing ad libitum feeding of a marginally low protein diet.

(b) CHOICE OF ANIMAL:

As can be seen from the preceding review, many types of animals have been used as a model of PCM. The best model to date has been the tube-fed monkey as used by Follis [45] (Cereopithicus Aethiops) and Ramalingaswami et al [46] (Rhesus Monkey). Their animals had clinical, biochemical and histological signs comparable to kwashiorkor. However, monkeys are relatively large, difficult to handle, expensive and require tube-feeding in order to produce PCM. They were thus rejected as the experimental model in our studies as were pigs and dogs for the same reasons. On the other hand, rats are small, tough and large numbers are easily handled and housed. Their short life span is an asset as they double their

TABLE VII.

COMPOSITION OF RAT CUBES.

	Weight (kg)	Protein	Weight of Protein (Kg)
Yellow straight ran mealie meal	960	9.6%	92.2
Germ meal	150	10%	15.2
Bran	275	13½%	35.8
Ground nut cake	150	47%	70.5
Fish meal	50	60%	30.0
Carcose meal	250	60%	165.0
Lucerne meal	200	16%	32.0
Whey powder	60	12%	7.2
Bone meal	30	28%	8.4
Limestone powder	40	0%	0.0
Fine salt (NaCl)	10	0%	0.0
Ethol concentrate	60	7%	4.2
Vitamin premix	2		
Mineral premix	2		
1 gallon raw linseed oil	-		
	2239	20.6%	460

weight in under three weeks and thus are ideal for growth rate studies. Against the use of rats are the facts that PCM as such, has not been easy to produce in this animal because, as in other species, low protein diets lead to fall-off in appetite and because tube-feeding such small animals for any length of time is extremely difficult. Despite this, it was felt that the rat should be used as the experimental animal for this study.

(c) MATERIALS AND METHODS:

i) EXPERIMENTAL ANIMALS AND DIETS:

Young male albino rats of the Wistar strain, weighing approximately 100 grams, were placed in individual cages or in cages containing four rats, in an air conditioned room. (Temp. 22°C, Humidity 50%). The animals had been weaned onto a diet of ground rat cubes obtained in bulk from Vereeniging Milling Company. Table VII shows the composition of the rat cubes. The protein content of these cubes was found to be constant when measured by Kjeldahl. The experimental diets, which were isocaloric to the control diet, were made up by diluting the ground cubes with cornstarch. A full vitamin and mineral mix was added to the experimental diets in

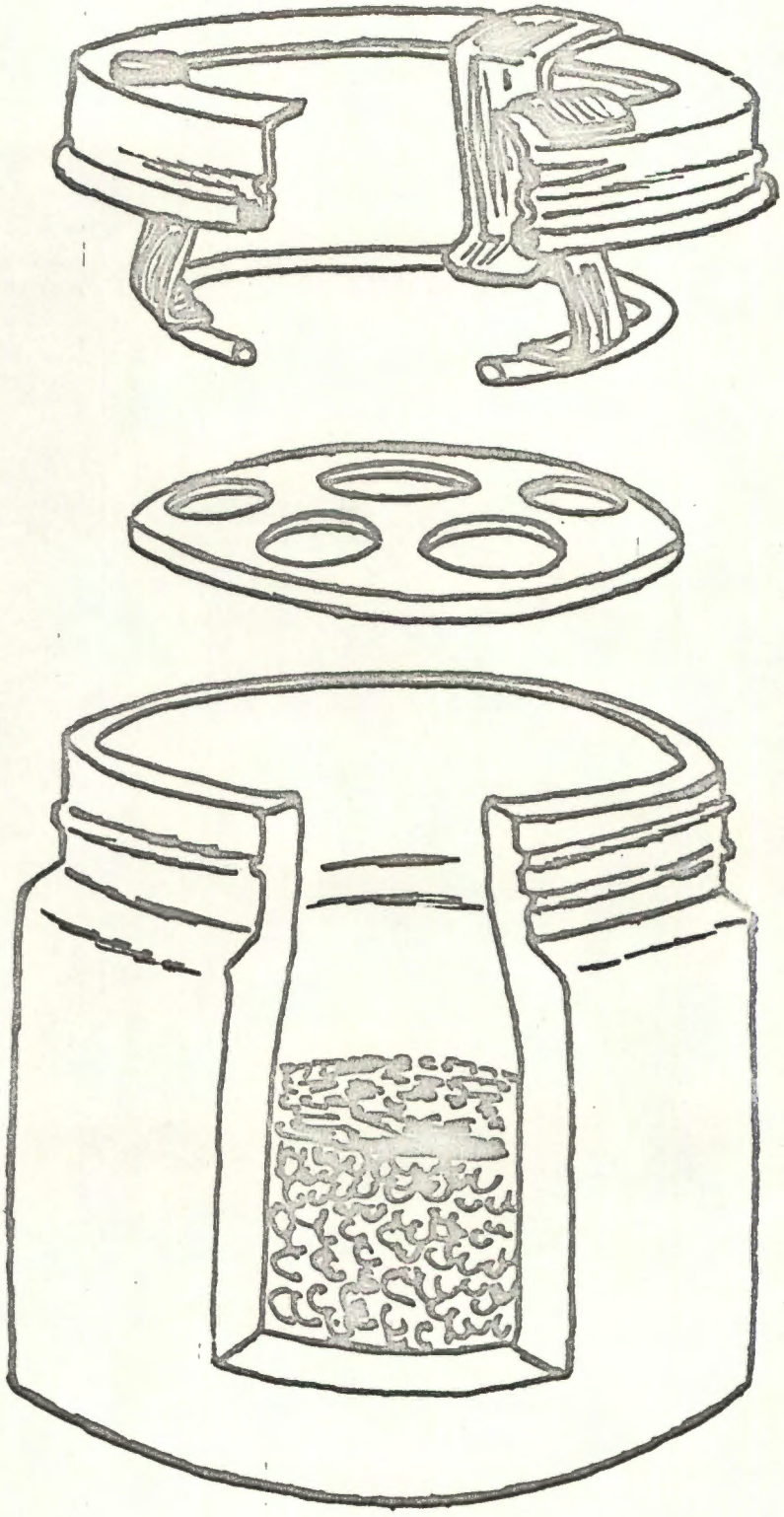


Fig. 7. Food dishes used in "pair-feeding" experiments.

sufficient quantity to compensate for the dilution, thus avoiding the production of multiple deficiency states. 5 kg. amounts of the diets were mixed in a commercial cake mixer for five minutes by which time it was impossible to separate the ground cubes from the cornstarch. The diets were fed dry in a special dish with a perforated disc which rested on top of the food in order to prevent spillage. Fig. 7. The dishes were filled, weighed and placed in the cages for 24 hours, after which they were weighed again, and the amount consumed calculated. All animals were weighed daily. Two types of controls were used: one group of animals was allowed to consume the control diet ad lib. Another group of weight matched animals was pair fed the control diet. The intake of the latter animals was isocaloric to that consumed by the rats on low protein diets. Five diets were fed containing 20, 12, 8, 5 and 0 percent protein respectively. The protein free diet was made up of cornstarch with added vitamins and minerals.

ii) PLASMA PROTEINS:

After a period of 60 days on their respective diets the rats were anaesthetised with ether and exsanguinated by cardiac puncture into a heparinised syringe.

Plate 1. Weight matched rats pair-fed 5% and 20% protein diets for 60 days.

Plasma samples were stored frozen until analysed. Total proteins were measured with the biuret reagent of Fernandez et al.[78] The albumin concentration was derived from this reading by electrophoresis [79] on cellulose acetate strips or by the method of Fernandez et al.[78] (Appendix 1.)

iii) PLASMA AMINO ACIDS:

After protein precipitation with salicyl sulphonic acid the protein-free filtrate was analysed on a "Technicon" amino acid analyser after the methods of Hamilton [80] and Efron.[81] (Appendix 2.)

iv) HISTOLOGY:

After an overnight fast the animals were killed by a sharp blow on the head. The abdomens were immediately opened and the small intestine and liver removed. The specimens were fixed in 10% formol saline. Sections of liver were stained with haematoxylin and eosin (H & E) and Sudan 4 for fat. Transverse and longitudinal sections of the jejunum were stained with H and E.

(d) RESULTS:

i) CLINICAL FEATURES:

The daily food consumption and weight gains

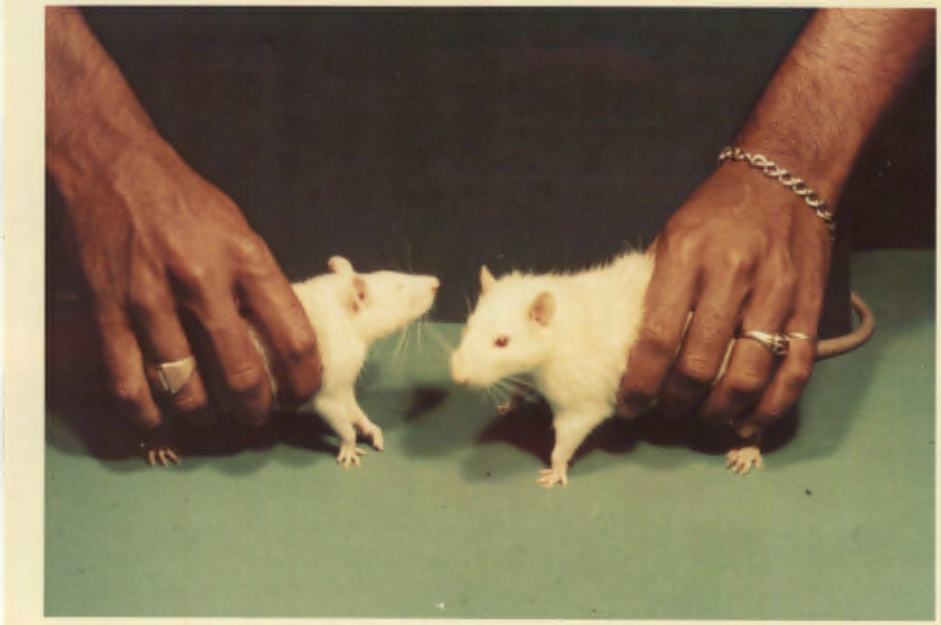


Plate 2. Oedema, as evidenced by puffiness around the eyes, is seen in the rat on the 5% protein diet (left) and is absent in the pair-fed partner (right).

TABLE VIII.

GROWTH RATES & FOOD CONSUMPTION OF RATS ON LOW PROTEIN DIETS

ROTEIN DIET	GROWTH RATES (gm. Day)			FOOD CONSUMPTION (gm./Day)	
	Groups of 4 Rats	Individual Rats	Pair fed Partners	Group of 4 Rats	Individual Rats
20	16·1	5·0(E)	–	60·6	14·3(A)
12	11·7	3·7	4·0	59·6	17·2(C)
8	8·75	2·7	3·7	58·4	21·8(D)
5	2·2	0·7(F)	2·8(G)	32·3	13·7(B)

A vs. B - $p > 0{\cdot}1$

A vs. C - $p < 0{\cdot}02$

A vs. D - $p < 0{\cdot}002$

E vs. F - $p < 0{\cdot}001$

F vs. G - $0{\cdot}002 > p > 0{\cdot}001$

of the rats on the various diets are given in Table VIII. The grouped animals appeared to eat less and to gain less weight than those in individual cages. These differences were not statistically significant. There was an obvious gradual step-wise reduction in weight gain directly proportional to the protein content of the diet.

The amount consumed varied from diet to diet. The individual rats on the 12% and 8% protein diets consumed significantly more food than those on the control diet. There was no significant difference in food consumption between those animals on the 5% protein diet and those on the control (20% protein) diet.

There was no real difference in weight gain in the pair-fed partners of those rats fed the 12% and 8% protein diets. There was however a significant decrease in weight gain in the rats fed the control diet in an amount isocaloric to that consumed by the animals on the 5% protein diet. The rats on a protein-free diet rapidly lost weight (3g./day), their daily food consumption dropped (8g./day) and they became emaciated.

There were no external signs of abnormality in those rats on the 8% and 12% protein diets. The rats on the 5% protein diet were obviously stunted and retained

TABLE IX.

DIETARY PROTEIN INTAKE & PLASMA PROTEINS IN RATS ON LOW PROTEIN DIETS

DIET	% PROTEIN	No. OF RATS	MEAN TOTAL PROTEINS (gm./100ml.)	MEAN ALBUMIN (gm./100ml.)
Control	20·6	10	5·76	2·82
B	8	5	5·65	2·45
D	5	10	4·0	1·60
E	0	6	—	1·33

TOTAL PROTEIN C vs. B $p > 0{\cdot}1$
C vs. D $p < 0{\cdot}001$

PLASMA ALBUMIN C vs. B $p > 0{\cdot}1$
C vs. D, C vs. E $p < 0{\cdot}001$

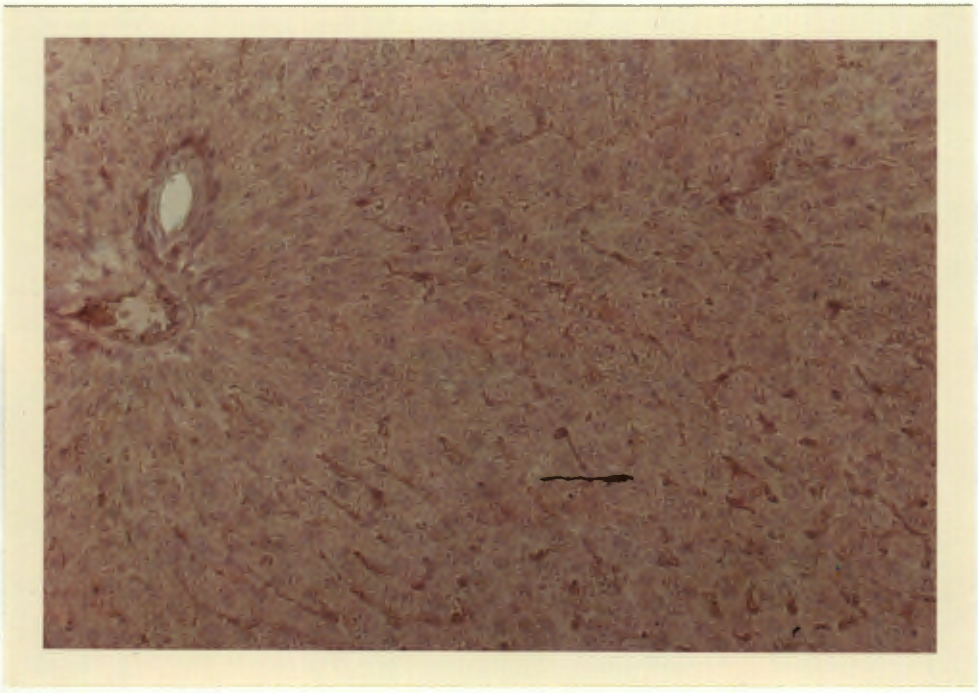


Plate 3. Sudan 4 stain of liver of a rat on control diet (20% protein) showing absence of fat.

DIETARY PROTEIN INTAKE & PLASMA ALBUMIN
IN RATS ON LOW PROTEIN DIETS

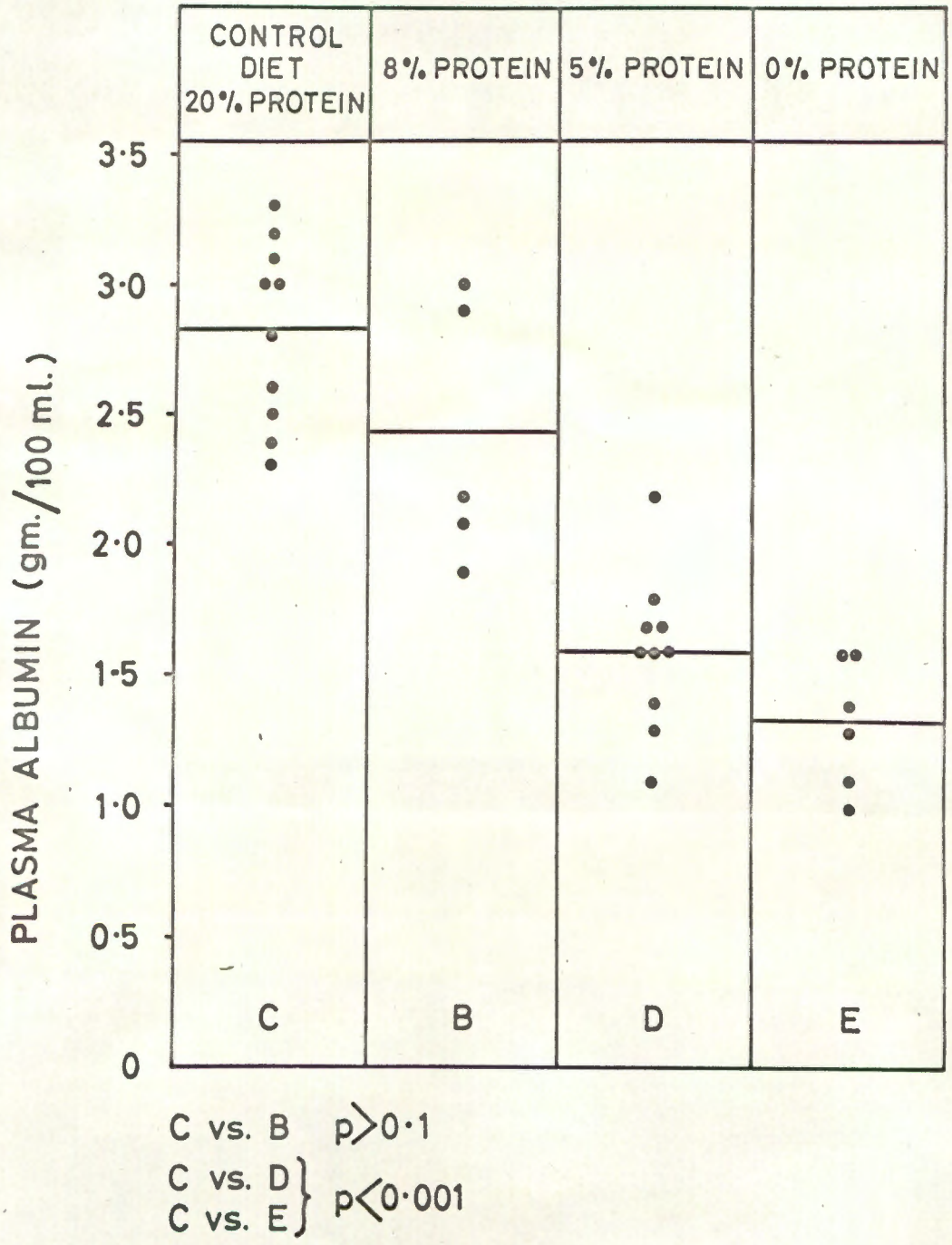


Fig. 8.

the fine hair of the young rat long after their peers had grown their coarser adult coats. (Plate 1.) In addition there were signs of oedema, as evidenced by puffy eyes and bulging cheeks, amongst the rats on the 5% protein diet. (Plate 2.) These rats were irritable and resisted handling while those rats on control diets rapidly became accustomed to being handled.

ii) PLASMA PROTEINS:

The results are shown in Table IX and Fig. 8. The rats on the 12% protein diets had normal plasma proteins. While the total plasma protein and plasma albumin levels in rats on the 8% protein diets were lower than in control animals, these differences were not statistically significant. In contrast the total protein and plasma albumin levels in those rats on a 5% protein diet were significantly lower than in control rats. The decrease in total plasma protein levels was almost entirely due to the drop in the albumin concentration. The mean total globulin level was 2.4G/100 ml. in the 5% protein group as opposed to 2.9G/100 ml. in the control group ($P > 0.1$).

iii) PLASMA AMINO ACIDS:

The plasma levels of 18 amino acids of rats

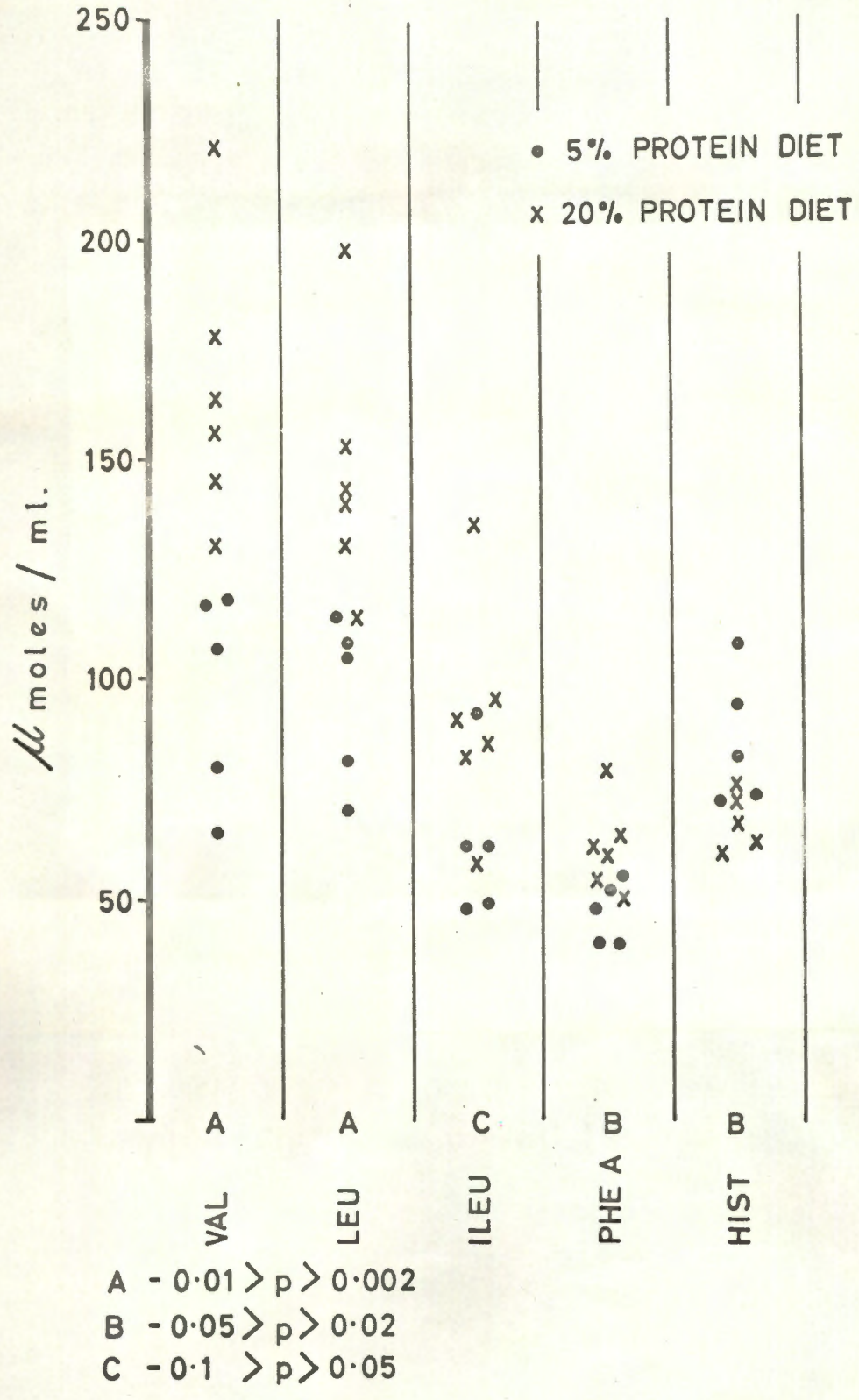


Fig. 9. Dietary protein intake and plasma free amino acids.

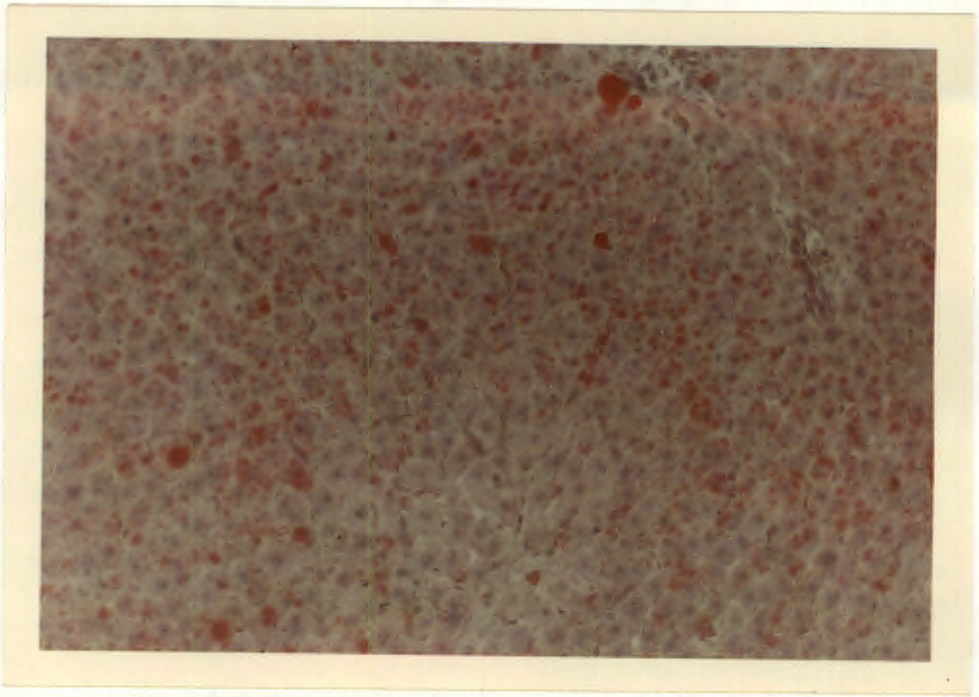


Plate 4. Sudan 4 stain of liver of a rat on 5% protein diet showing periportal fatty infiltration.

fed the 5% protein diet were compared to those of rats on the control diet. The significant differences are shown in Fig. 9. The levels of the branch chain amino acids - leucine, valine and to a lesser extent isoleucine - were significantly lower in the experimental group. This change was even more marked when their combined values were compared. Of the other amino acids only phenyl - alanine was significantly lower in the experimental group while levels of histidine and glutamic acid were higher in these animals. These changes closely resembled those found in children suffering from PCM.

iv) HISTOLOGY:

Liver: Fat stains revealed no fat in the livers of five control animals or in the livers of the rats on the 12% and 8% protein diets. (Plate 3.) All five animals on the 5% protein diet showed marked periportal fatty infiltration. (Plate 4.) This sometimes extended to involve the central zone as well. The fat appeared as small globules in the cytoplasm and occasionally formed large globules distending the cell.

Intestine: There was a tendency towards shortening and blunting of the villi in animals on the 5% protein diet.

(e) DISCUSSION:

Rats fed the 5% protein diet developed clinical, biochemical and histological disorders similar to those seen in children suffering from kwashiorkor. Growth was markedly decreased, oedema occurred in most animals and they developed significant hypoalbuminaemia, a plasma amino acid profile similar to that seen in kwashiorkor and histological evidence of periportal fatty infiltration in the liver. These lesions could only have been reproduced if the caloric consumption was relatively adequate.

When the level of dietary protein is slightly reduced, the experimental animal, in an attempt to overcome this deficit, responds by increasing its food consumption, and up to a point this inverse relationship of dietary protein level and food consumption is maintained. This is evident from the food consumption of the rats on the 12% and 8% protein diets. That this compensatory mechanism succeeds in some cases is shown by the small decrease in weight gain in the rats (in individual cages) on these diets and by the normal histology and nearly normal plasma protein levels. When further decreased, the dietary protein value assumes a direct relationship to food consumption. The rats on the protein-free diet rapidly lost their appetites.

This has also been observed in animals fed a low protein diet or a diet deficient in one of the essential amino acids. (82) (30) There is however, a point at which the animals have a normal or nearly normal caloric intake but where the dietary protein is inadequate to meet the demands of the young animal. This is evidenced by the greatly diminished weight gain, in spite of the nearly normal food consumption, of the rats on the 5% protein diet. The decrease in weight gain in the pair-fed partners of the rats on a 5% protein diet is surprising as they were fed amounts virtually equal to those consumed when rats were allowed unlimited amounts of food. This may be partially explained by an inversion of their eating habits. The rat is a nocturnal feeder and in those cages where food was limited, the animals took their food a little earlier each day until the stage was reached where the food would be consumed each morning as soon as it was placed in the cage and the rats became increasingly agitated at night in their search for food. In spite of the subnormal weight gain, these animals gained more than four times as much as those on the 5% protein diet thus eliminating caloric deficiency as the major cause of the poor growth rate of the latter. Further supportive evidence for the relatively normal caloric

intake of the rats on the 5% protein diet was the presence of oedema, one of the signs used in human disease to distinguish kwashiorkor from marasmus.

The fatty liver is perhaps the most striking pathological lesion in children suffering from kwashiorkor. This does not occur in marasmus. (83) Histologically small fat droplets appear in the peri-portal area spreading with increasing severity to involve the entire lobule. In severe cases the droplets coalesce forming large globules displacing the nuclei, often rupturing the cell walls and fusing with each other to form large cysts. (83) These changes are similar to those seen in our animals and are different from those found in animals with choline deficiency where the globules first appear in the cells surrounding the central vein. (27)

Children dying from kwashiorkor have atrophy of the mucous membrane and wall of the proximal small intestine at autopsy. (14) Deo et al (34) found that the mucosal height was markedly reduced in their protein depleted rhesus monkeys. The villi were reduced but there was no blunting or fusion. In our animals there was a tendency to blunting and shortening of the villi but these changes were not marked.

Biochemically hypoalbuminaemia is an invariable finding in developed PCM. (83) (14) Similar results have been obtained experimentally in the rhesus monkey, (34) in dogs, (20) rats (42) and in pigs. (20) Schendel et al (15) have found a good correlation between the clinical severity and the serum albumin level in children with kwashiorkor. (Fig. 1 - Part 1) We have produced three degrees of protein depletion and find a similar correlation. (Fig. 8.)

The concentration of free amino acids in the plasma of untreated cases of kwashiorkor has been shown to have a remarkably constant pattern in a number of different countries. (48-53) In general there appears to be a depression of certain essential amino acids, especially valine, leucine, isoleucine and methionine, as well as the non-essential amino acid, tyrosine. In contrast, there is usually a normal or raised level of glycine and histidine. Saunders et al (53) have recently shown that this distinctive profile is abolished within 24 hours of commencing protein feeding and is probably only indicative of the immediately preceding diet. It is therefore not surprising that the rats on a low protein diet have an amino acid profile resembling that seen in kwashiorkor. In recent years much attention has

been given to the production of an experimental model of kwashiorkor. Most workers however, have discarded the rat as an experimental animal and have turned to monkeys, pigs and dogs. (20) These animals are larger and thus easier to tube-feed for a long time. Furthermore, in the case of the monkey, the results obtained are more likely to apply to man than those obtained from the rat. However, when large samples are required, e.g. for statistical analysis, the use of large animals becomes time-consuming and requires more space than is available in the usual laboratory complex. The production of large numbers of experimental rats requires little space and, using the methods described, 40 rats may be weighed and fed in just over an hour each morning. For these reasons I have chosen to use the rat as a model of experimental "kwashiorkor".

Having produced, in rats on the 5% protein diet, an experimental model of kwashiorkor, it was decided to review the methods for measuring albumin turnover and the work done to date on albumin turnover in protein depletion states. These reviews are to be found in Parts 4 and 5.

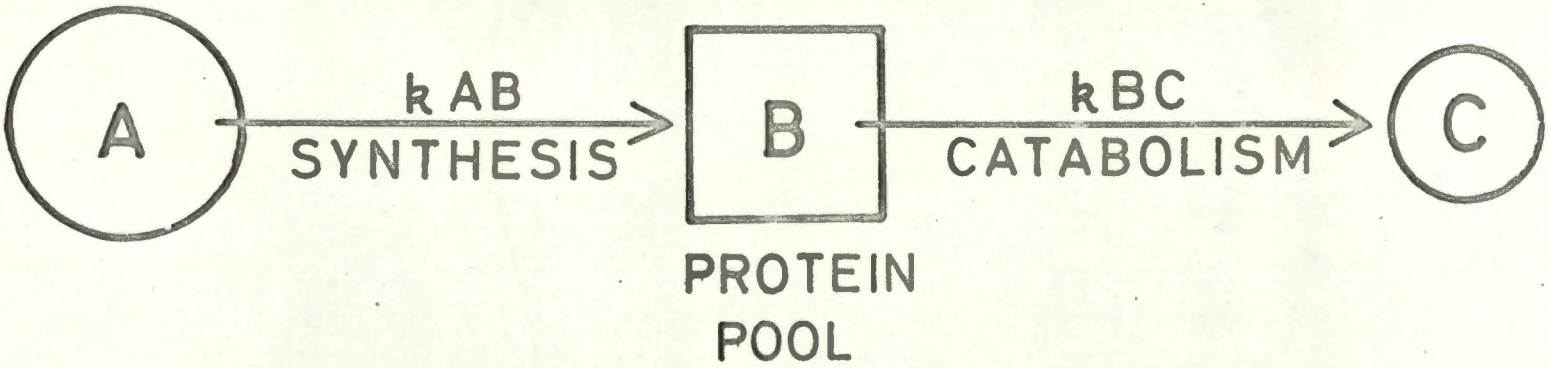


Fig. 10. A simple system demonstrating formation and degradation of protein.

PART 4.

ALBUMIN TURNOVER.

(a) INTRODUCTION:

Turnover may be defined as the replacement of an amount of protein, or any other substance under consideration, by an equal quantity of the same material but newly synthesised from its metabolic precursors or transported into the system from outside.(84)

In order to study turnover isotopically labelled proteins or amino acids, have to be used. Radioactive tracers are readily detected by the researcher, while the body finds them indistinguishable from the naturally occurring substance.

A simple protein system, as illustrated in Fig. 10, consists of a protein pool (B) (The term "pool" is used to denote the mass of albumin present in the body. The total protein pool, or protein pool, is subdivided into the intravascular pool and the extravascular pool) to which newly synthesised protein is continually being added at rate RAB and from which protein is continually

being lost at rate RBC. If RAB = RBC then the protein pool mass will remain constant and conversely if the protein pool mass remains constant the synthesis rate is equal to the catabolic rate.[85]

Until recently synthesis rate (SR) measurements have been derived from degradation studies. This has severely restricted the scope of turnover experiments as synthesis rate could only be derived when the subject was in a "steady state" i.e. the protein pool mass remained constant.

Recently a method has been described for the direct measurement of SR[18] and this method will be reviewed in considerable detail on Page 49. To avoid confusion methods for determining catabolic and synthesis rates will be reviewed separately.

(b) MEASUREMENT OF ALBUMIN CATABOLIC RATE:

Radioactive albumin injected into the body is affected by three processes: dilution, distribution and degradation.

Dilution: This occurs immediately. The albumin injected intravenously mixes with that already present in the plasma and serial samples have revealed that this

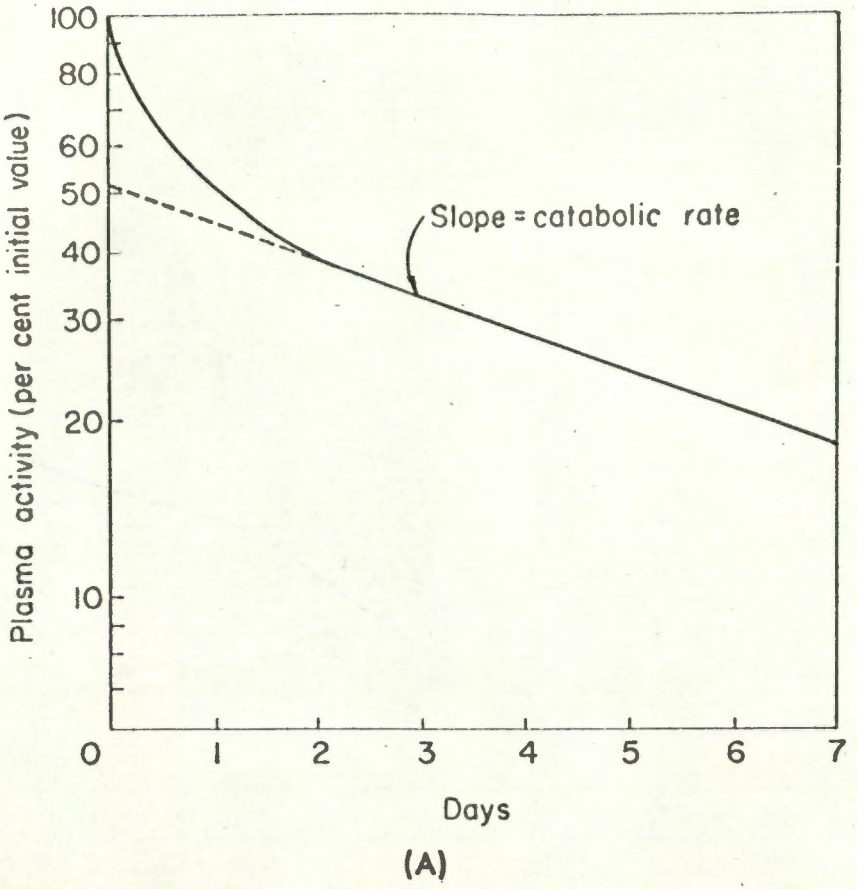


Fig. 11.. The Sterling model. Plasma activity curve following injection of a labeled protein. The dual composition of the curve is evident. According to Sterling (1951), the slope of the later part of the curve represents the catabolic rate expressed as a fraction of the total labeled protein, and the y-axis intercept gives the proportion of the total labeled protein which is present in the plasma.

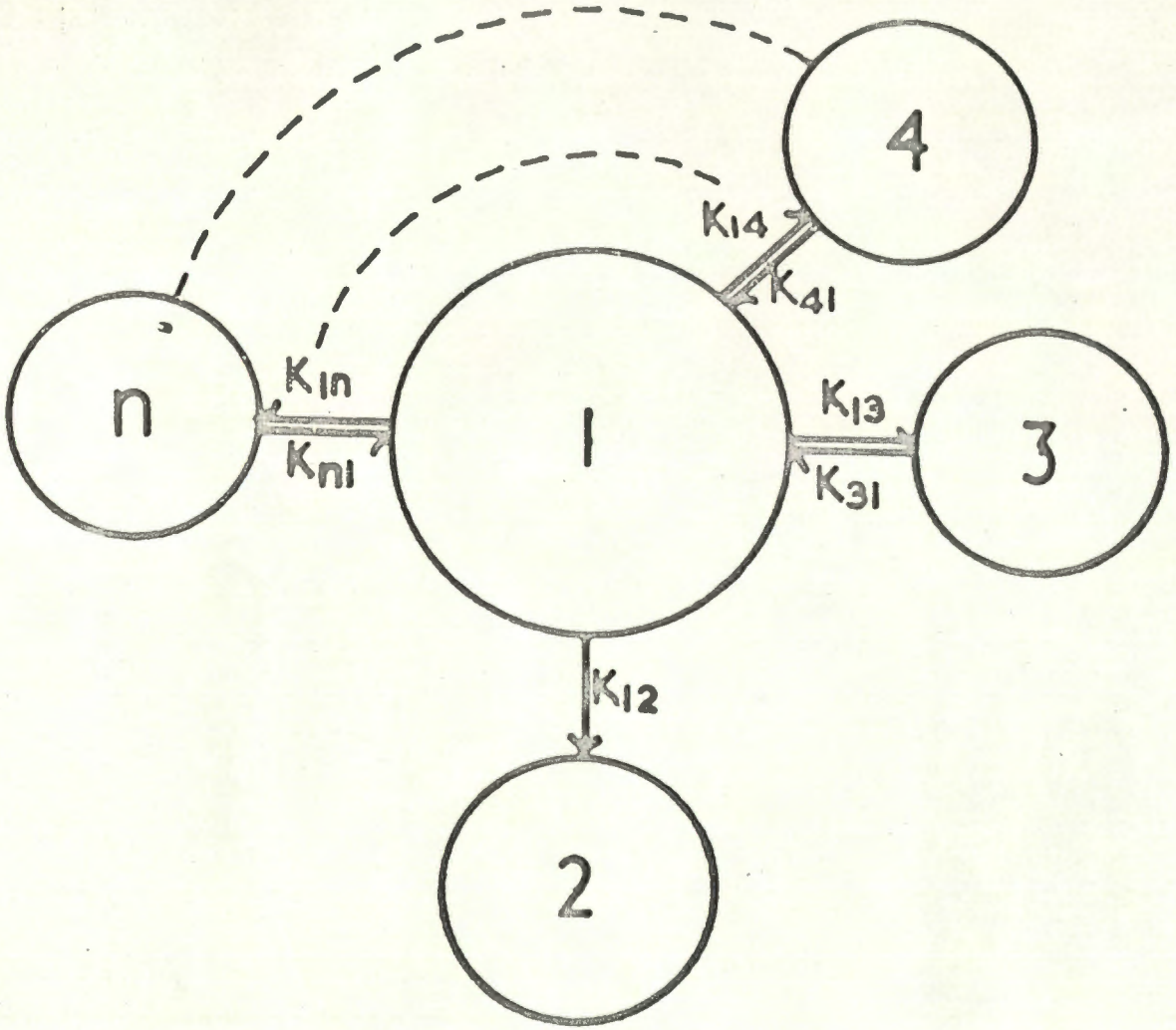


Fig. 12. Diagrammatic representation of an open mammillary system of n compartments reversibly connected to a central compartment: 1, intravascular albumin pool, 2, excretion, 3,4.....n extravascular compartments.

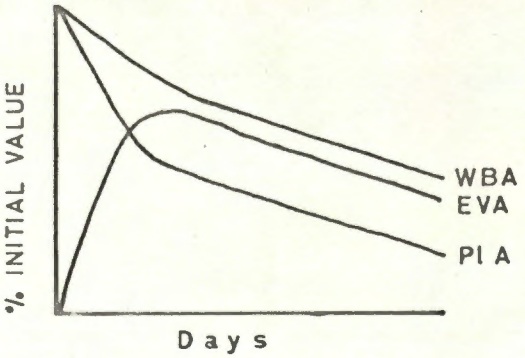
process is completed within 10 minutes. Thus a sample taken 10 minutes after injection can be used to calculate the plasma volume as follows:[86]

$$\frac{\text{Dose injected (counts/min)}}{\text{Plasma Radioactivity (counts/min/ml)}} = \text{Plasma Volume (ml)}$$

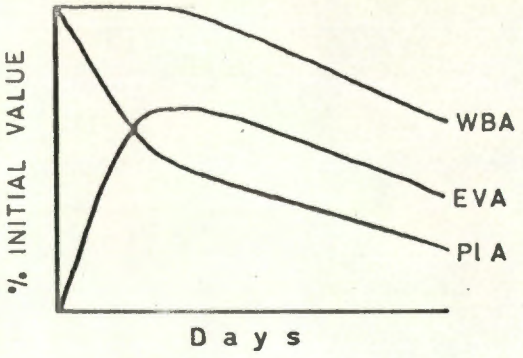
Distribution and Degradation: Repeated plasma radioactivity measurements reveal a rapid decline of activity during the first 48 hours followed by a slower fall off in activity. This gradual decline is an exponential decay as a semilogarithmic plot of the points after the second day approximates a straight line. The rapid fall off of activity during the first 48 hours is due primarily to redistribution of labelled molecules in the plasma and lymph spaces, while the more gradual slope following the attainment of equilibrium reflects the degradation of albumin and the elimination of the label. These changes, represented graphically in Fig. 11, were first described by Sterling in 1951.[87]

The fact that more than one albumin pool exists warrants discussion. Fig. 12 is a diagramatic representation of the system we are dealing with.[88]

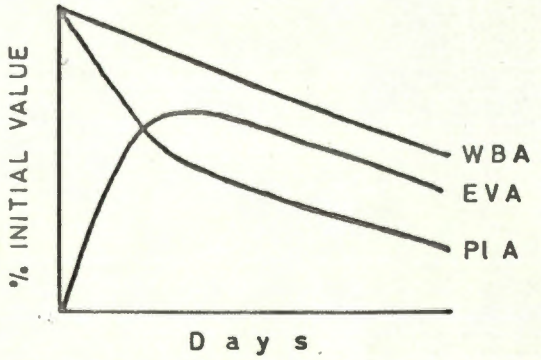
DIAGRAMATIC REPRESENTATION OF HYPOTHETICAL SITES OF CATABOLISM



INTRAVASCULAR CATABOLISM



EXTRAVASCULAR CATABOLISM



INTRA- & EXTRAVASCULAR CATABOLISM

WBA = Whole Body Activity = 100% - cumulative urinary radio-activity excretion

Fig. 13.

The central compartment 1 represents the intravascular (IV) albumin pool while the outer compartments 3,4 represent the protein in the extravascular (EV) spaces. These extravascular spaces are connected reversably with the central compartment. Compartment 2 represents the activity in the urine and is connected with compartment 1 in the direction 1→2 only. The evidence for an intravascular site of catabolism is as follows: when the whole body activity curve, derived from the 24 hour urinary elimination of the ^{131}I label is plotted on semilogarithmic paper, it follows generally the slope of the plasma albumin specific activity (SA), i.e. counts per minute per mg albumin, of the same animal. If the ^{131}I excreted came from degradation in the EV pool its slope would be different from that of the plasma slope during the equilibration of the labelled protein between the IV and EV spaces, while if it came from degradation in both compartments, it would show no sign of equilibration.[89] (Fig. 13) ^{131}I albumin is not, however, broken down when incubated with plasma or blood in vitro and it is postulated that catabolism occurs in cells to which the plasma proteins have rapid access and which themselves contain a negligible amount of protein when compared with the intravascular or extravascular pools of protein (McFarlane).[90] Reeve and Roberts [91]

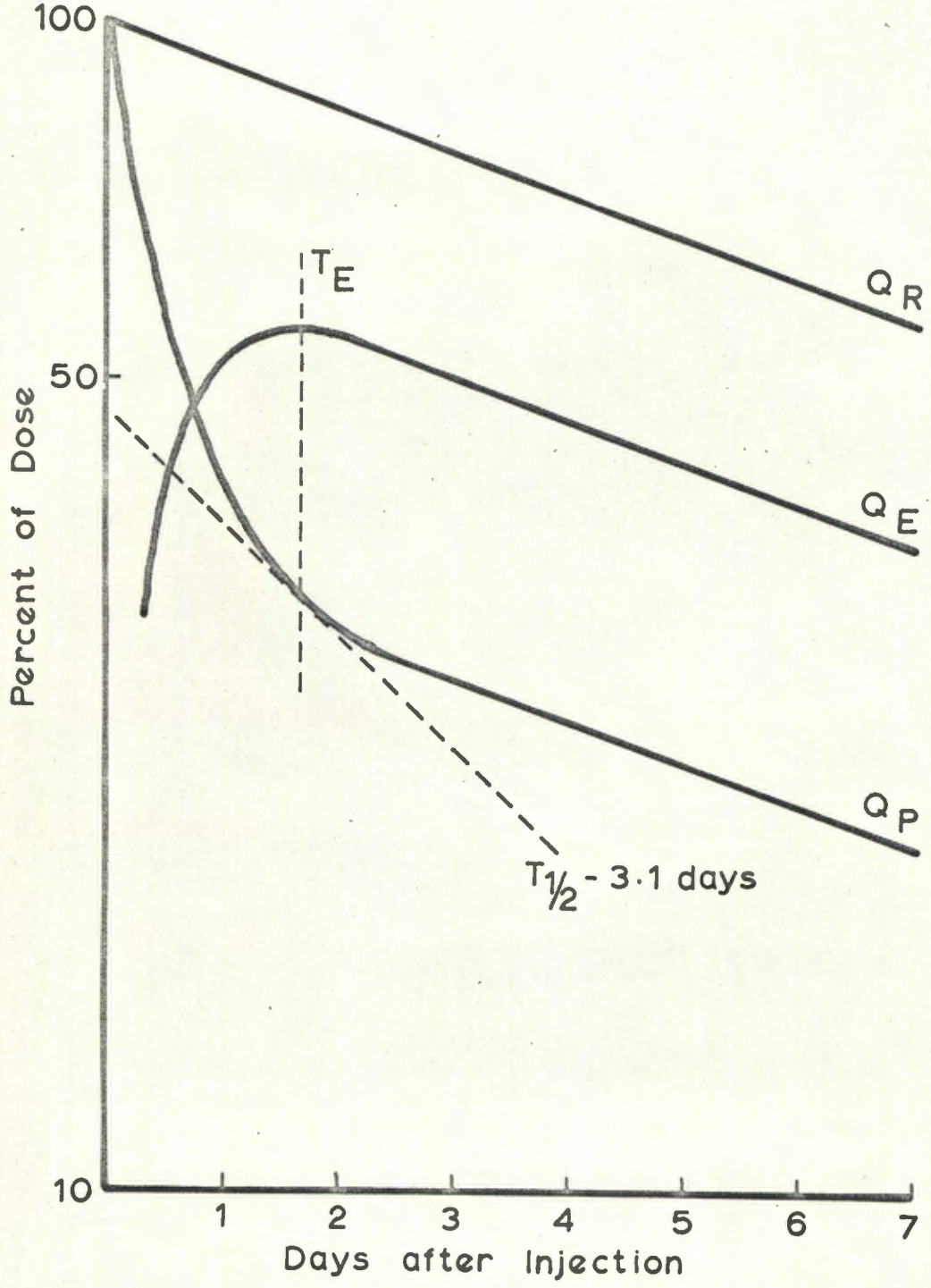


Fig. 15. Equilibrium Time (T_E).
Q_R = Whole Body Activity
Q_E = Extravascular Activity
Q_P = Plasma Activity

postulate the existence of a "breakdown pool" which receives the plasma proteins and delivers their breakdown products to the kidneys where they are excreted. Fig. 14 is a diagram of the model for albumin synthesis and catabolism put forward by these authors.

If serial plasma radioactivity (RA) values are subtracted from the whole body activity (WBA) (WBA = 100% - cumulative excretion of radioactivity in the urine) the values obtained give the percent of activity in the EV pool. The EV activity rises as the plasma activity falls and reaches a maximum after 2 - 3 days. (Fig. 15) The maximum value occurs at the equilibrium time so called because at that time plasma specific activity (SA) equals EV specific activity (and IV activity → EV pool equals EV activity → plasma pool).

Thus we have the formula:

$$\text{At equilibrium time} \qquad \frac{\text{Plasma RA}}{\text{Plasma Alb.}} = \frac{\text{EV.RA}}{\text{EV. alb.}}$$

$$\text{EV albumin} = \frac{\text{EV. RA x Plasma Alb}}{\text{Plasma RA}}$$

Thus the EV albumin pool can be calculated. The total pool is the sum of the plasma and EV albumin pools. Once equilibrium time has been reached the IV and EV activities both decline gradually. These slopes, which

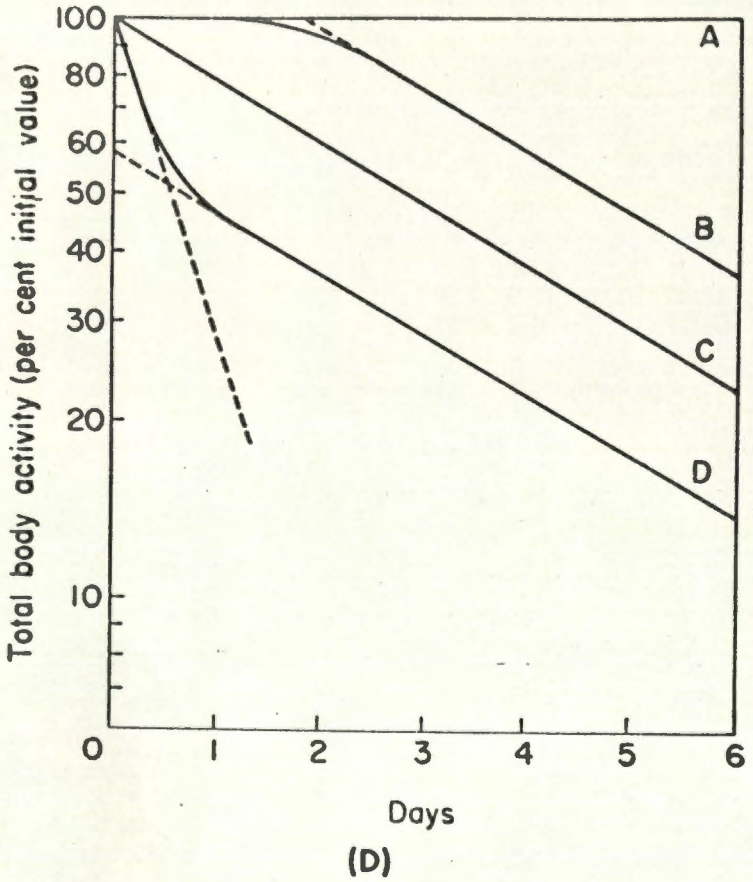


Fig. 16. Hypothetical total body activity curves after injecting a labeled protein intravenously. A, with complete retention of breakdown products; B, with retention of breakdown products in the body water until the renal threshold is exceeded; C, with no retention of breakdown products, or with retention effects exactly counterbalanced by effects due to rapid early catabolism of denatured protein; D, with early catabolism of denatured protein and insignificant retention of breakdown products.

should be parallel, are due to degradation. However, in order to calculate the catabolic rate, WBA measurements are usually required. This may be calculated by subtracting the cumulative urinary excretion from the injected dose or by direct measurement.[90] The latter is far simpler in the case of small animals as they are easily counted and collection of their excreta is not readily accomplished particularly when they have to be removed from their cages at least once every 24 hours in order to be bled. As would be expected, the slope obtained by total body measurements is quite unaffected by the process of equilibration, in fact the slope of the WBA and the plasma activity should be the same (after the period of equilibration in the case of the latter) and both reflect catabolism. It is essential in all cases where ^{131}I turnover studies are undertaken to block the thyroid, as failure to do this would lead to retention of the label. Fig. 16 illustrates the effect this would have on the WBA.[90]

Many methods have been devised for measuring the catabolic rates based on the above method.[86][87][88][89][90] In addition, methods exist for measuring synthesis rate plus transfer from the same data. However, these latter

apply mainly to animals who are in a steady state, and as the animals to be studied were in anything but a steady state, it was decided to limit the ^{131}I albumin study to the measurement of the catabolic rate.

The formula used for deriving the catabolic rate was based on the assumption that catabolism occurs in the IV pools and is as follows: the activity eliminated over a period of 24 hours is divided by the mean plasma albumin SA during the same period.(89) This method is valid even when the catabolic rate and pool mass are varying, since the only assumption involved is that the SA of the albumin catabolised is the same as the plasma albumin, so that in any given period:

$$\frac{\text{Activity in urine}}{\text{Plasma Albumin SA}} = \text{mass of albumin catabolised}$$

The above method was first used in animals who were in an unsteady state by Matthews (1960) (92) who justified its validity.

The label most often used in albumin catabolic rate experiments is ^{131}I. However, ^{14}C labelled albumin has also been used. Full technical details of the method used are found in Part 6 and Appendix 3.

(c) MEASUREMENT OF ALBUMIN SYNTHESIS RATE:

Synthesis rate of albumin may theoretically be measured by using a labelled amino acid and measuring incorporation of that amino acid into the protein. For example, if the specific activity of the intracellular precursor amino acid is P microcuries per gram over an interval t during which new protein containing M gram of precursor and R microcuries are produced, then R = P x M and the synthesis rate, M/t, is equal to R/Pt.[90]

This formula is based on the assumption that the label remains attached to the amino acid injected. Amino acids can be labelled by using isotopes of nitrogen,[90] carbon[90] and in some cases sulphur.[96] Isotopic nitrogen, due to transamination, becomes distributed amongst most of the amino acids present in the body and thus can not be used for measuring the synthesis rate of a particular protein.[94] Carbon and sulphur labels are less labile and may thus be used.

A further assumption made in the theoretical formula is that the intracellular SA of the precursor amino acid remains constant. Isolation of intracellular free amino acids from whole organs (in this case the liver) or from biopsy specimens, is a complicated

procedure which has been carried out on only a few occasions.[95] When the precursor SA has been measured it has been found to be far from constant. Multiple injections or prolonged feeding of the isotope have been used in an attempt to maintain a constant precursor SA, but these have yielded poor results.[90] [96]

As a result of the difficulties experienced in measuring the intracellular precursor SA and the failure to maintain it at a constant level, attention has been focused on the use of extracellular indicators of precursor SA.

Weismann et al (1961) have used urinary hippuric acid as an indicator or intrahepatic glycine specific activities.[97] This method is time-consuming and the alternative method of using a double label, e.g. ^{14}C and ^{13}C, is both expensive and technically difficult.

Recently McFarlane et al [18] and Reeve et al[98] have devised a method which uses urea carbon specific activities as indicators of the intracellular specific activities of the guanidino-carbon of arginine. Deluva and Wilson in 1936, using Na H $^{13}Co_3$, fed orally or injected intraperitoneally, demonstrated the label in the sixth carbon atom of arginine and in urea.[99]

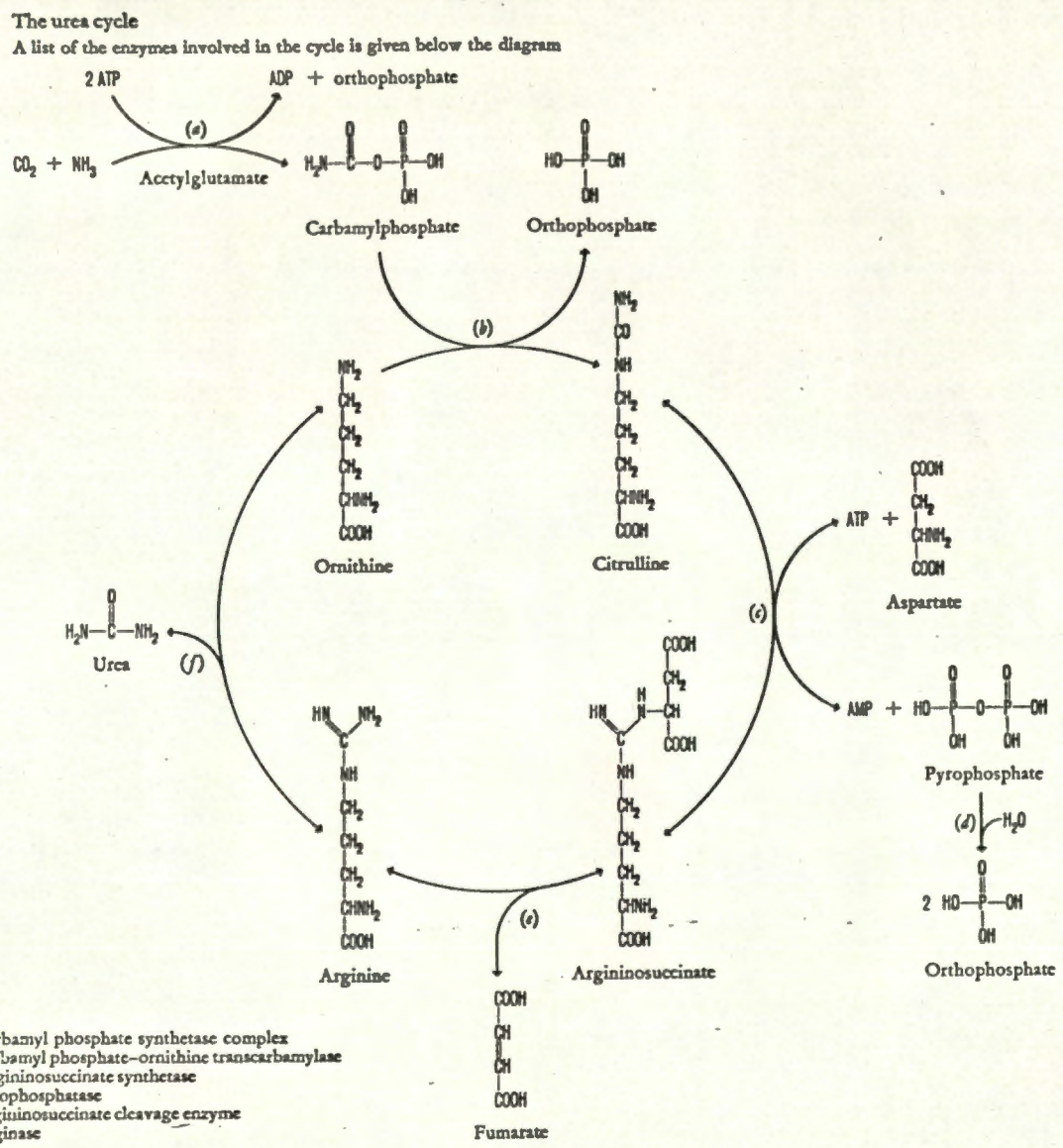


Fig. 17. The Ornithine Cycle.

Swick (1958) confirmed this work and in addition showed that the arginine so labelled was used for the synthesis of plasma proteins.(100)

Fig. 17, the Krebs-Henseleit Cycle, demonstrates the incorporation of Co_2 into the guanidine-carbon of arginine and the subsequent splitting off of that carbon atom to form urea.

The formula used by McFarlane and by Reeve et al was derived as follows: The radioactivity appearing in a protein, and in any other direct product of 6 - ^{14}C - arginine, in a given time, is a function of the integral of the precursor arginine specific activity curve over the interval in question. Thus the radioactivity of the guanidine-carbon appearing in protein and in urea in a given time must bear the same ratio as the respective masses of guanidine-carbon synthesized. In other words, in two products arising from the same precursor the ratio of the activities of the products is equal to the ratio of the rates of incorporation of the precursor into the products. Thus we have the formula:

$$\frac{\text{Synthesis Rate of protein guanidine C}}{\text{Synthesis Rate of urea C}} = \frac{\text{Total activity in protein guanidine C at time t}}{\text{Total activity in urea C at time t}}$$

Dividing both sides of this equation arbitrarily by the sizes of the urea and protein guanidine-carbon pools,[101] then:

$$\frac{\text{Fractional SR of Alb.}}{\text{Fractional SR of Urea(K)}} = \frac{\text{SA in protein guanidine C at t}}{\text{SA in urea carbon at t}}$$

O R

$$\text{Fractional SR of Alb.} = \frac{\text{K x SA Alb. guanidine C at t}}{\text{SA urea C at t}}$$

The above formula can only be applied to the measurement of protein synthesized in the liver as this is the only organ in which both protein and urea synthesis occur simultaneously within the same cells. McFarlane[18] found, that on injecting arginine - 6 - ^{14}C, gamma-globulin SA was many times higher than albumin SA. From this he concluded that albumin was being synthesized within cells possessing a major alternative pathway for this particular carbon atom i.e. urea synthesis. When $Na_2\ ^{14}Co_3$ was injected the SA of albumin was found to be twice that of gammaglobulin. The explanation for this was that $^{14}Co_2$, incorporated into arginine in liver cells only, was used for protein and urea synthesis by this

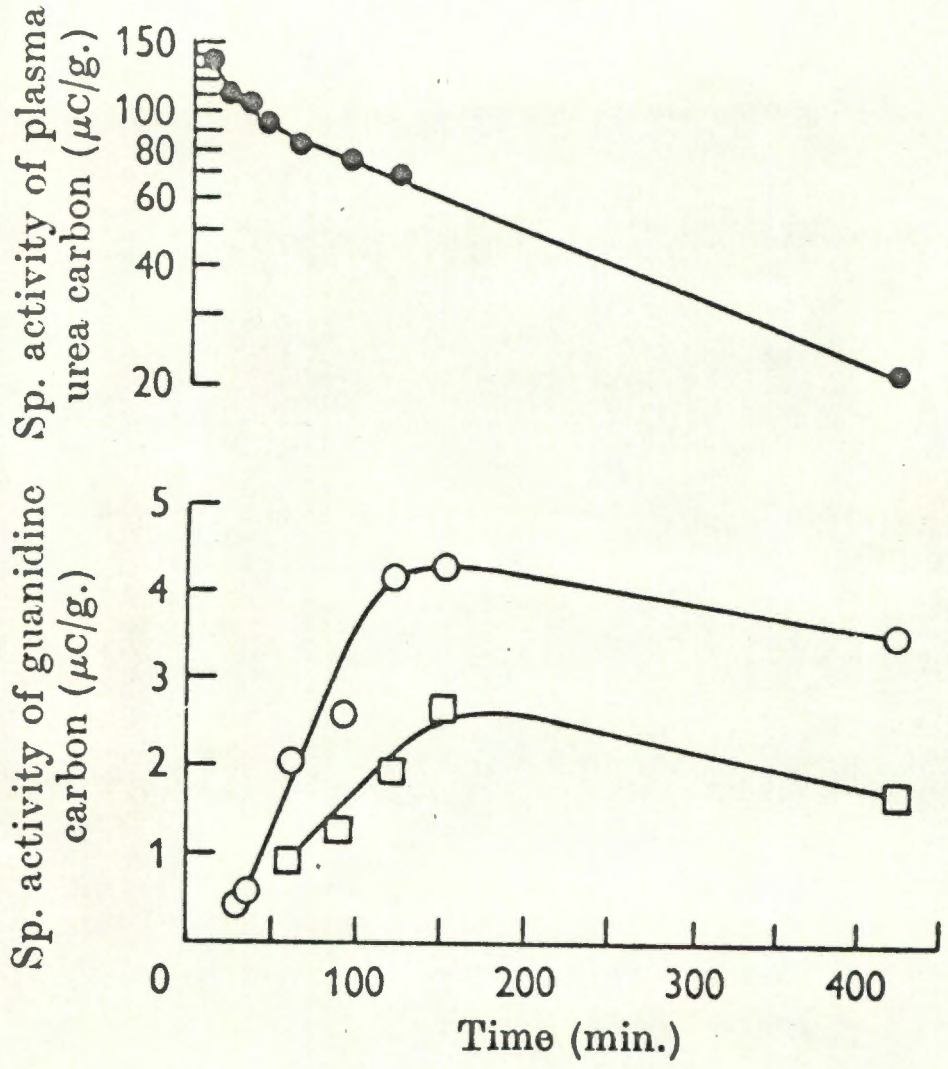


Fig. 18. Specific radioactivities of the guanidine carbon of plasma proteins and of plasma urea after the injection of 500 μc of $NaH^{14}CO_3$ into a rabbit. Experimental details are given in the text. The values for ^{14}C-labelled γ-globulin (□) are about one-half of those for ^{14}C-labelled albumin (○). The values for [^{14}C]urea (●) ($t_{\frac{1}{2}}$ = 3 hr.) are shown in the upper curve.

organ, only a small amount reaching the reticulo-endothelial cells for gammaglobulin synthesis. After the injection of $Na_2{}^{14}Co_3$, plasma urea SA rapidly increases to reach a peak in the first few minutes after which it declines gradually. (Fig. 18)
This decline, which is exponential, is due to dilution of the label by newly synthesized urea and as such reflects urea synthesis. The fractional synthesis rate of urea can thus be calculated from this slope.

Albumin SA, plotted in the same way, rises much more slowly, reaches a maximum in about 3 to 4 hours and then gradually diminishes as an exponential reflecting degradation. When labelled carbonate is injected, the albumin SA slope agrees reasonably well with that of ^{131}I labelled albumin. When labelled arginine is injected, the slopes differ quite markedly. This is due to the fact that the labelled amino acid is incorporated into tissue proteins with high turnover rates and, as these are rapidly degraded, the label becomes available for reutilization and is partly used for albumin synthesis. When the amino acid is labelled in the liver cells, as is the case with carbonate, the specific activities of the tissue proteins are 100 times lower and thus recycling becomes less important.

Both labelled urea and labelled albumin are lost during the period of measurement[18] and for this reason, this period should be kept as short as possible. Within minutes, after the injection of the labelled amino acid, both intracellular urea and albumin are labelled[95][102][103] but whereas the former rapidly diffuses out of the cell, the latter only appears in the plasma after half an hour.[104] For this reason the experimental period is prolonged and is usually 4 to 6 hours by which time the albumin SA has reached its maximum. As the loss of protein due to transfer and catabolism may be considerable during the 6 hour period, ^{131}I labelled albumin is usually injected at the same time as the $Na_2\,{}^{14}CO_3$ and this provides a correction factor for albumin lost. The urea losses are made good by extrapolating the curve of the plasma urea specific activities to zero time. Using this method for measurement of synthesis rate, McFarlane et al,[18][101] Reeve et al [98] and Hoffenberg et al [105] have obtained excellent agreement with ^{131}I albumin catabolic rate studies in rabbits and humans in steady state conditions.

The technical details of this method will be described in Part 6 and in Appendix 4.

PART 5.

THE DEVELOPMENT OF THE CONCEPTS OF PROTEIN TURNOVER AND PROTEIN RESERVES, AND A REVIEW OF ALBUMIN TURNOVER IN PROTEIN DEPLETION STATES.

Before discussing the effects of protein depletion on turnover of plasma albumin it is desirable to summarize work done on the effects of protein depletion on the organism as a whole. This work, based largely on nitrogen balance studies and incorporation of labelled amino acids into tissue proteins, has led to the development of the concepts of turnover and of protein reserves.

(a) THE DEVELOPMENT OF THE CONCEPT OF TURNOVER AND OF PROTEIN RESERVES:

That dietary protein is an essential nutrient which is readily converted into bodily protein was known long before the discovery of nitrogen (N). Indeed Haller, in 1754, two decades before the discovery of nitrogen wrote "....The flesh of animals

appears a necessary part of our nourishment....For it appears that the flesh of animals only contains the gelatinous lymph, ready prepared for the recruit both of our fluids and solids, which, being extracted from broken vessels and fibres, is readily converted into abundance of blood."..... (106) The concept of protein reserves was developed almost 100 years later by Voit, who perfected the technique of nitrogen balance. This technique he applied to dogs fed on different levels of dietary protein and from this work two important facts emerged which led him to suggest the existence of "circulating protein", which we to-day call "labile protein". The main experiments which led to his hypothesis were as follows:

Dogs were fed a normal protein diet over a long period of time and N intake was found to equal N loss in the urine and stools. If these animals were suddenly deprived of dietary protein, their N loss diminished rapidly for the first few days and only after a variable period of time was a new steady state obtained where the loss of N was minimal. During the lag phase of readjustment, the amount of protein lost exceeded that consumed and bore a direct relationship to the dietary

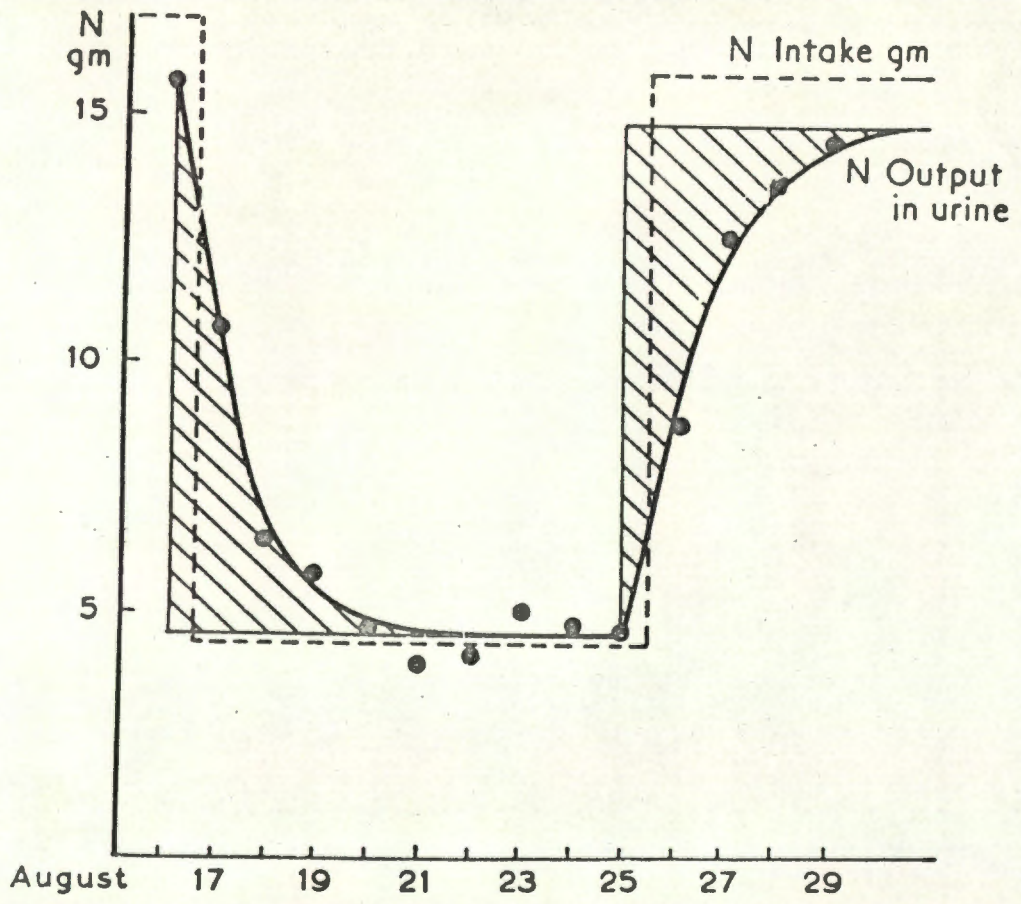


Fig. 19. Nitrogen excretion of a human subject at two different levels of protein intake. (From Martin and Robison, 1922.) Shaded area on the left represents stored N gradually removed on dropping the intake from 17 gm to 4.4 gm; right-hand area represents the amount stored on resuming a diet containing 16 gm N. Curves are reciprocal; the two shaded areas correspond approximately in area.

protein level immediately prior to deprivation.[107] Martin and Robinson (1922) pointed out that the amount of N lost during the first few days of protein deprivation decreased in an orderly and regular manner and the log of the differences between N excretion on any two subsequent days gave a straight line when plotted against time. They concluded that the amount of storage N which is removed from the body on any given day is proportional to the amount still present.[108]

While Martin and Robinson confirmed Voit's work in man, it was confirmed in animals by Campbell and Kosterlitz,[109] Swanson[110] McCollum and Steinbock[111] It has also been shown (Martin and Robinson) to be true if the process was reversed, i.e. if the protein-free diet was changed to a normal diet, nitrogen retained equalled that lost during the deprivation and once again there was a lag phase before N excretion returned to normal.[108] (Fig. 19)

From balance studies, we can thus draw the following conclusion: during periods of high protein diet the body accummulates a small amount of "labile" protein. This is rapidly lost during deprivation and regained on refeeding.

Once the concept of labile protein reserves had become established much work was done on factors influencing its deposition or removal. The labile body protein was increased by increasing the dietary protein level and was further increased by a high carbohydrate content of the diet (protein sparing effect of carbohydrates).[112] Anabolic hormones e.g. growth hormone and testosterone were shown to promote N retention.[113][114] Decreasing the dietary protein, as stated previously, caused a loss of the labile protein as did injury, proteinuria and plasmapheresis. It soon became apparent that to obtain further information an attempt had to be made to ascertain the site or sites of storage of the labile protein. Once again Voit supplied the lead by observing that in starving cats, the livers were extremely reduced in size or weight while the weight of the brains and heart remained virtually unchanges. Addis et al (1936) performed extensive studies on rats.[70] They showed (Table VI, Part 2) that on fasting these animals the liver lost 40% of its initial protein content in seven days while the carcass (muscle, skin and skeleton) only lost 8%. They followed this up by obtaining similar results on feeding rats a protein-free diet. Subsequent

studies by other workers confirmed these findings and showed that the pancreas, gastro-intestinal tract and the plasma proteins also lost protein rapidly. However, because of the dramatic loss of protein from these organs, it is often overlooked that Addis et al found that during the 7 day fast the bulk of the N lost in the urine was provided by the muscle, skin and skeleton (62%) while the liver, although losing 40% of its N content, only accounted for 16% of the urinary loss. Allison and Wannemacher (1965) clarified the concept of protein reserves.(115) They placed dogs and rats on a protein-free diet and found that the daily urinary excretion of nitrogen decreased rapidly during the first few days. After the animals had lost about 8% of their body N, the decrease in the rate of daily urinary excretion became relatively constant. Thus endogenous excretion of N could not be considered a constant until the animals were severely depleted. Although there was major loss of protein from the liver and part of the viscera during the first few days, the muscles and skin contributed the major part of the nitrogen lost in the urine during this period. The authors concluded that the muscles and skin represented the major part of the N reserves of

the body. They further noted a correlation to the protein: DNA and RNA : DNA ratios of various tissues, which suggested that the decrease in cellular protein was the result of a lowered rate of protein biosynthesis. The change in the daily urinary excretion of N could be explained as an attempt by the body to reach a new steady state, and the so-called labile protein reserve could be considered as part of the overall reserves of the body.

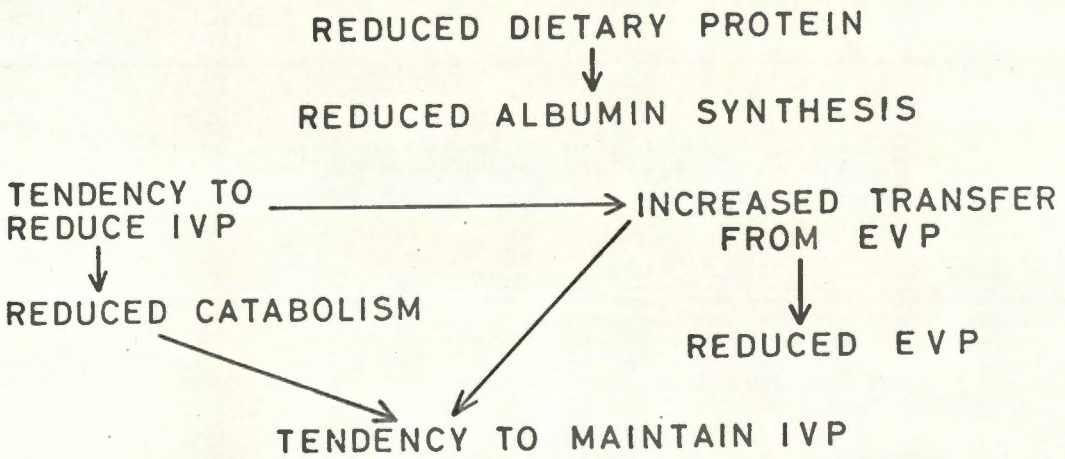
Using labelled amino acids, it has been found that the proteins of the various tissues were labelled with varying degrees of rapidity.(84) The proteins in the plasma, small intestinal mucosa, and the liver were rapidly labelled while those in the bones, skin and muscles were slowly labelled and contained only small amounts of the isotopic element per unit weight of tissue. The concept of varying rates of turnover in the different tissues is to-day an accepted fact and relative turnover rates of some tissues are shown in Fig. 20 Shoenheimer, in his book "The Dynamic State of Body Constituents", expresses the view that nearly all the proteins in the body are continually being synthesised and degraded.(116) This view was further supported by

Thompson and Ballou who, using tritium oxide, demonstrated that all the tissue proteins they studied were in a dynamic state but that some tissue proteins, e.g. collagen, were "uniquely inert", having half lives of 1,000 days while others, e.g. the liver and plasma proteins have shorter half lives.[117] These varying protein turnover rates answer the question as to why the liver should lose protein more rapidly during the early stages of a fast than does the carcass. It also indicates which tissues will be most affected if protein is withdrawn from the diet. The disorders of the pancreas, liver and GIT are well documented in kwashiorkor and the fall in those plasma proteins synthesised in the liver is one of the diagnostic criteria of the disease.

(b) REVIEW OF WORK DONE ON ALBUMIN TURNOVER IN PROTEIN DEPLETION STATES:

Studies of albumin turnover in protein depletion have been designed to elucidate three aspects of this condition. First is the problem of marginal deficiency. It is generally accepted that existing criteria for the recognition of protein malnutrition are crude and insensitive and give little indication of minor deficits.[16] While the plasma albumin concentration is low in developed

a. EFFECT OF REDUCED DIETARY PROTEIN



b. EFFECT OF PLASMAPHERESIS

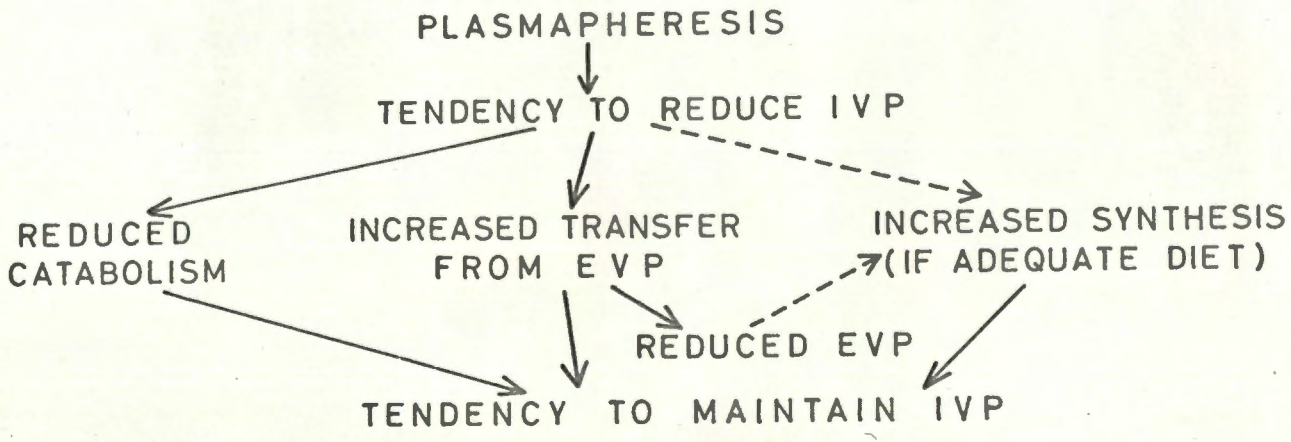


Fig. 21.

cases of PCM, it is accepted that considerable depletion can exist before this reduction occurs. Hoffenberg et al, using ^{131}I labelled albumin in adult human volunteers on low protein diets, demonstrated that a decrease in the catabolic rate of this protein occurred before its concentration dropped.(17)

The second application has been the elucidation of the body's adaptation to protein deficiency. Depletion can result from inadequate dietary supplies, from poor assimilation or absorption of protein or amino acids or from excessive loss from the urine or faeces; it can be produced experimentally by the technique of plasmapheresis. Hoffenberg (1964) has postulated two distinct responses to dietary protein deficiency on the one hand and to protein loss as exemplified by plasmapheresis on the other. These adaptive processes aim at maintenance of the intravascular albumin pool.(118) (Fig. 21)

The third object of albumin turnover studies has been an attempt at elucidating the regulatory mechanisms of albumin homeostasis. It is with these objects in mind that the literature on albumin metabolism has been reviewed.

(c) PLASMA ALBUMIN CONCENTRATION AND ALBUMIN POOL SIZES:

The plasma albumin concentration is reduced in established cases of PCM.[14] [83] In these cases there is a relationship between the plasma albumin concentration and the severity of the disease.[15] The plasma albumin pool is also reduced in PCM.[119] [120] The extravascular pool however, is difficult to measure as the validity of the measurement depends on the presence of a state of equilibrium, which cannot always be assumed under conditions of protein depletion. Nevertheless, there is quite strong evidence in support of a change in the distribution of albumin in PCM. Payne and Done, by injecting tritiated albumin into normal and protein depleted piglets, found a greater reduction in the EV protein pool than in the plasma protein pool.[121] Cohen and Hansen, in their studies on children with kwashiorkor, found that the EVP was reduced to 40% of normal, while the IVP was only reduced to 60%. During refeeding they increased by 100% and 50% respectively.[119] Picou and Waterlow found similar results and postulated that the relatively normal plasma SA curves found in cases of PCM by themselves [120] and by Garrow et al,[122] were due to changes in pool sizes. Freeman and Gordon found

a decrease in the EVP in rats on a protein-free diet.[123] Hoffenberg et al confirmed these findings in their adult volunteers on low protein diets.[17] This shift from the EVP to IVP has also been found in dogs and rabbits subjected to plasmapheresis (Yuile et al,[124] Wasserman et al [125] and Matthews et al[92]).

(d) ALBUMIN CATABOLIC RATE: (CR)

Measurement of the catabolic rate of a labelled protein by expressing urinary radioactivity in terms of plasma SA is regarded as valid even where pool masses are varying.[92] The first albumin turnover study on children suffering from PCM was by Gitlin et al (1958), who found the CR unchanged.[126] These studies were carried out during refeeding and while the circulating albumin mass was rapidly increasing. Cohen and Hansen (1962) studied the albumin CR in children with PCM maintained on a protein deficient diet for the duration of the study. The children were then put onto a therapeutic diet and re-studied after clinical and biochemical resolution of the disease. In this study there was a marked reduction of the CR in the first study which returned to normal when the children were cured.[119] At the same time Picou and Waterlow in Jamaica, working independently from the

Cape Town group, showed a similar depression of the albumin catabolic rate in children suffering from PCM.[120] In contrast to the Cape Town group, these authors gave their patients as much food as they could take during the first period of the study. These essentially similar results led Waterlow to question whether changes in protein metabolism vary with the level of protein intake at the time of the test or with the state of depletion; he concluded that both factors play a part in determining albumin catabolism.[127] Similar results have been obtained in rats on protein-free diets[123] and in rabbits on low protein diets.[17] Reduction of dietary protein is not the only cause of low albumin catabolic rates, since they are found in human subjects with proteinuria[128] or cirrhosis of the liver[129] as well as in experimental animals subject to plasmapheresis, where ad libitum feeding is permitted.[17] A fall in albumin catabolism as suggested by Bauman et al[128] and Friedberg[130] appears more closely related to reduction of the albumin pool than to protein intake.
In a series of papers based on infusions of albumin into rabbits, Rothchild and his colleagues found an increase in the CR while the SR remained unchanged.[131][132]

They concluded that albumin synthesis and degradation are not interdependent.

From this work it would appear that albumin catabolism responds to alterations in the plasma albumin or the extra - or intravascular pool sizes. Hoffenberg et al[17] found a significant reduction in the CR of albumin before any changes of pool size or concentration had occurred. This apparent discrepancy may be explained by the sensitivity of the control mechanism of the CR. The probable sequence of events is as follows:

The immediate effect of protein depletion is reduced albumin synthesis which would tend to diminish the intravascular pool. This tendency could be met by a lowering of catabolic rate and, at the same time, increased transfer of albumin from the extravascular pool - measures that would protect the intravascular pool. For a while, at least, the serum albumin concentration or mass could be maintained at normal levels but a lowered catabolic rate would reflect the threatening depletion.

Although it is not strictly within the scope of this thesis, it is interesting to note that most authors have found the gammaglobulin CR unchanged in states of protein depletion.[17] [119]

(e) ALBUMIN SYNTHESIS RATES:

Since steady state conditions cannot be assumed during periods of low protein intake, synthesis rates cannot be equated with catabolic rates. Indirect assessment of alterations in albumin synthesis have however, been derived from measurement of "synthesis + transfer" rates according to Matthews.[92] The decreased SR + transfer obtained by this method could theoretically be entirely due to transfer from the IVP to the EVP.[17] The evidence, as reviewed above, indicates that, if anything, the opposite occurs and that the results obtained may be even more significant than they appear. Cohen and Hansen,[119] Picou and Waterlow,[120] and Purves and Hansen[133] have all reported a diminution of the SR in children with PCM. Freeman and Gordon have described a similar fall of albumin SR in protein depleted rats.[123] Hoffenberg et al have recorded similar results in adult humans and in rabbits on low protein diets.[17]

When animals are plasmapheresed the SR of albumin increases.[17] [92] Matthews felt that this increased synthesis played the major part in restoring the albumin mass.[92] In Hoffenberg's series the increased SR and decreased CR appeared to play equal parts in this

restoration.[17] In cases of proteinuria an increase of the SR was found only where dietary protein was high[128]

Rothchild et al, by infusing dextran into rabbits, find a diminution of SR and CR which appears to be related to the hepatic interstitial albumin or colloid concentration.[131] The latter are greatly increased after dextran infusions. These authors postulate that these factors may control albumin synthesis.

The factors controlling the synthesis rate of albumin however, are far from clear. In contrast the CR is probably controlled by the plasma albumin concentration or mass of the intra - or extravascular pools. Waterlow has suggested that the SR is altered primarily by external factors, e.g. supply of amino acids, while the CR is regulated by a mechanism which attempts to compensate for changes imposed by the environment.[127]

PART 6.

THE KINETICS OF ALBUMIN METABOLISM IN EXPERIMENTAL PROTEIN DEPLETION AND REPLETION.

(a) INTRODUCTION:

The present state of our knowledge of albumin metabolism in protein deficiency states as reviewed in Part 5, may be summarised as follows:

The diminution of plasma albumin concentration, pool size and CR occurring in these states is well established. There is strong evidence to suggest that transfer from the extravascular to the intravascular albumin pool takes place. Less certainty exists over changes in SR of albumin although synthesis plus transfer is definitely diminished. The sequence in which the above changes occur is poorly understood. The main questions still unanswered are thus:

Is albumin synthesis diminished in protein deficiency states? In what order do the changes found in these states occur and how are they controlled?

It therefore seemed logical to apply the McFarlane technique [18] to the measurement of albumin SR in animals with experimental PCM. It was also decided to study early changes occurring during depletion by abruptly withdrawing all protein from the diets of rats previously fed on rat cubes (20% protein). While these experiments would shed considerable light on the kinetics of albumin metabolism, they were felt to be incomplete if the reverse sequence, i.e. those changes occurring during repletion, was left unstudied.

(b) MATERIALS:

i) Animals:

With certain exceptions, the animals used were similar to those described in Part 3. As it is customary to express the results of albumin turnover studies in the rat in terms of 300g body weight, most animals used weighed between 280 and 320g. When animals weighing less were used, e.g. those on the 5% protein diet, the results were adjusted accordingly.

Three dietary regimes were used. The control diet (NPD), the 5% protein diet (LPD) and the protein-free diet (OPD). The food consumption and weight gain of the rats were measured daily.

ii) ^{14}C Carbonate:

This was obtained as a sterile solution in the form of $Na_2\ ^{14}CO_3$ (SA 20-40 mc/m Mol.) from the Radiochemical Centre, Amersham, U.K. or from Phillips-Duphar, Amsterdam, Holland.

iii) Iodine Labelled Albumin:

The albumin to be labelled was fractionated by the ammonium sulphate salt precipitation method of Keckwick.(135) The iodine monochloride method of McFarlane was used for iodinating this albumin.(136) Full details are given in Appendix 3. Free iodine was measured by counting an aliquot of the ^{131}I albumin preparation before and after trichloracetic acid precipitation. Samples containing free iodine in excess of 1% were discarded. Cellulose acetate electrophoresis was used to ensure that the albumin was pure and that the label was attached to this protein. The labelled albumin was then sterilized by passing it through a Seitz filter.

(c) METHODS:

i) Catabolic Rate Measurement:

10 to 20 μc of sterilized iodinated albumin

were injected intravenously into the tail veins of rats. The rats had previously been put onto iodine water to block thyroidal uptake of the ^{131}I. After 10 minutes the animals were bled from the tail and the plasma radioactivity assayed for plasma volume and zero time readings. Plasma radioactivity and albumin concentration were measured daily on 0.25ml of blood. Whole body counting in the ring of 6-GM Pb 26 tubes was used to derive daily excretory loss of ^{131}I. A standard taken from the syringe used for the injections was used to calculate the dose. The CR was derived from assumed urinary excretion of ^{131}I (based on whole body counts) expressed as a function of the plasma albumin SA.

ii) Synthesis Rate Measurement:

With minor modifications the method of McFarlane was used. A mixture of ^{14}C - carbonate (250 μc) and ^{131}I or ^{125}I labelled albumin was injected into the tail veins of rats. The simultaneous injection of labelled protein enabled plasma volume measurements to be made. In addition, by comparing the radio-iodine activity of the 10 minute sample with one taken at the end of the experiment, 6 hours after injection, the

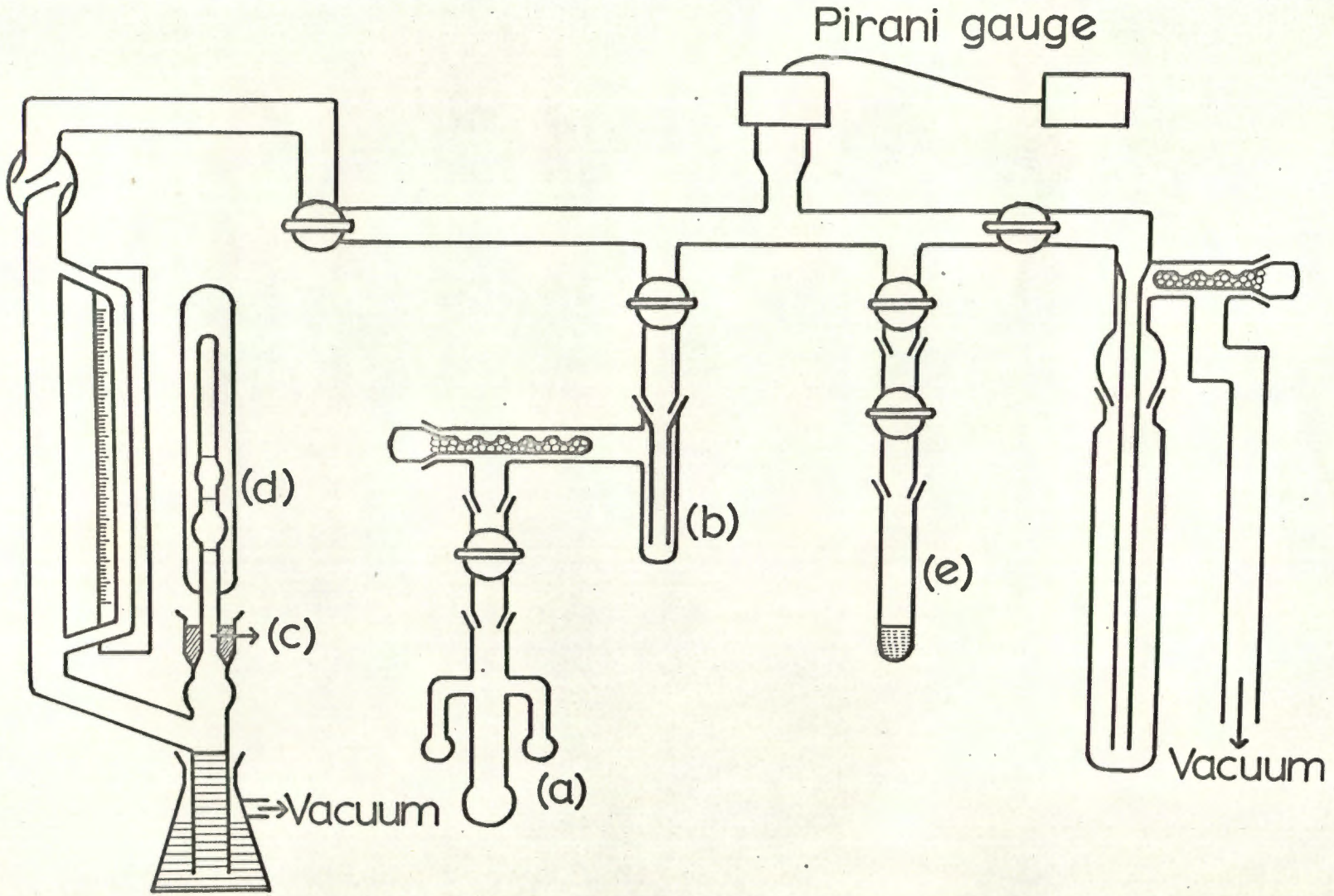
albumin SA at 6 hours could be corrected for t_o. Starting 3 hours after the injection of carbonate, the rats were bled at half-hourly intervals. 0.1ml of blood was taken from the tail into a pipette thus avoiding excessive blood loss. At 6 hours the animals were exsanguinated by cardiac puncture. Plasma was separated and stored frozen until assayed. Stable urea and albumin solutions were added to the samples to ensure adequate CO_2 volumes, appropriate corrections being made in the calculations.

iii) Plasma Fractionation:

Method for Measurement of Urea SA:

For determination of plasma urea SA, proteins were precipitated by addition of 0.66 -N H_2SO_4 and 10% sodium tungstate. This modification of the McFarlane technique while not affecting urea recovery, as checked by using ^{14}C urea, greatly reduced the time required for this technique. McFarlane used N-H_2SO_4 and tungstic acid (2ml 10% w/v sodium tungstate plus 7.6ml 0.1N-HCl plus 12.4ml H_2O) equal volumes of which were added to a similar volume of plasma. This mixture required heating to 100°C in order to precipitate the proteins.

Fig. 22. HIGH VACUUM GAS TRAIN.



(a) Reaction vessel. (b) Cold Finger. (c) Manometer cold trap. (d) Modified MacLeod Chamber. (e) Phenylethylamine trap.

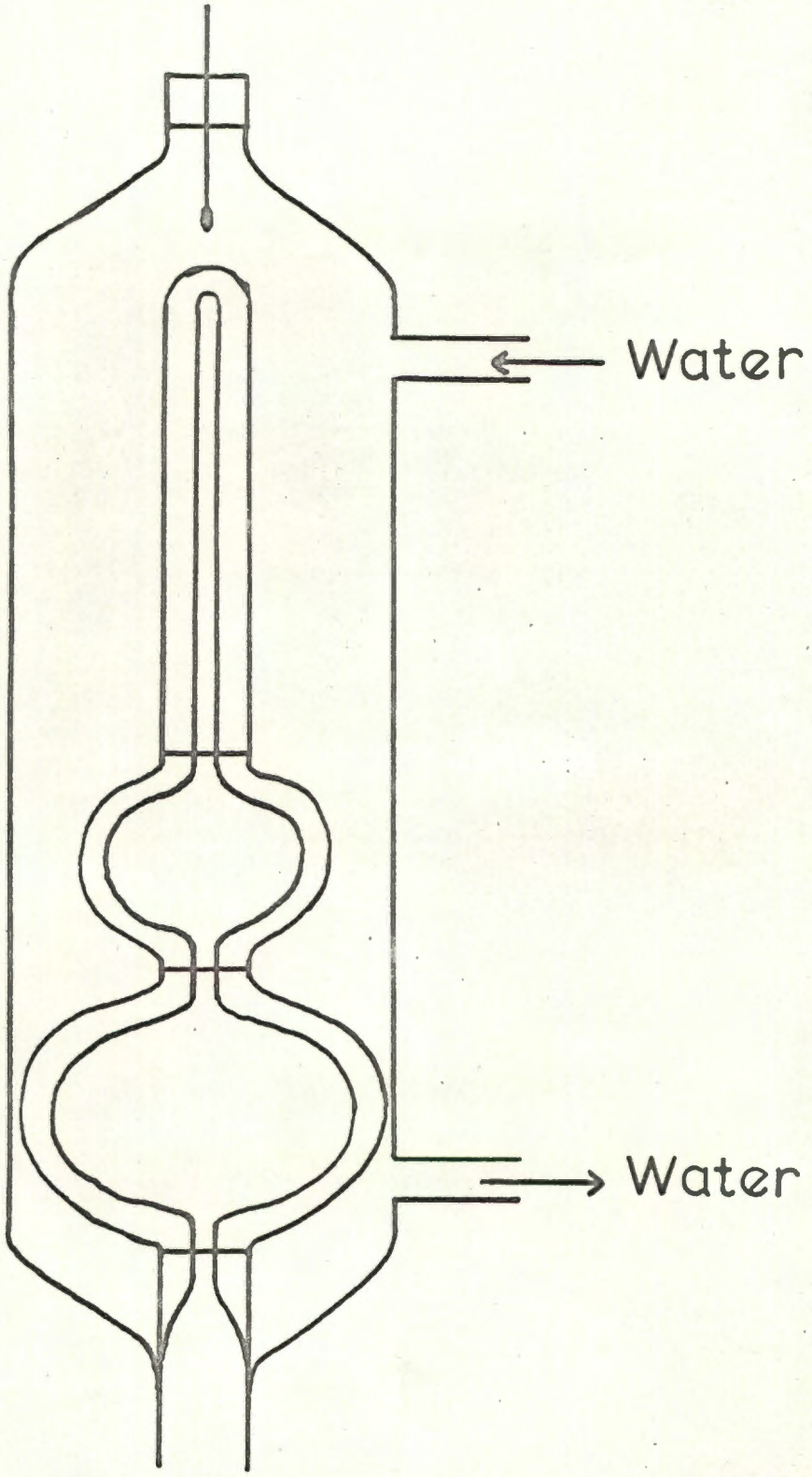


Fig. 23. Modified MacLeod Chamber.

The supernatant fluid (SNF) was reduced to dryness in order to remove any free $^{14}CO_2$. The residue was dissolved in CO_2-free water and neutralized with 0.1N NaOH. The sample was placed in a reaction vessel, urease and citric/tungstic acid being placed in the side arms. After evacuation of the reaction vessel on a high vacuum pump the urease was tipped into the solution which was then allowed to incubate for 30 minutes at 25°C. The CO_2 formed was liberated by tipping in the acid. The reaction vessel was connected to the high vacuum gas train. (Figs. 22 and 23) The gas train, based on that used by McFarlane, included the following modifications: Built-in phosphorus pentoxide traps removed any water vapour present in the system. The modified MacLeod chamber allowed for accurate manometric measurement of as little as 0.001mg of carbon. These modifications were introduced jointly by the author and by Dr. R. Hoffenberg. Using differential freezing with dry-ice-ethanol and liquid nitrogen, the CO_2 was separated from other gasses and was trapped and measured in the modified MacLeod chamber. After the volume of CO_2 had been measured, the gas was trapped in a tube containing phenylethylamine.[137] The radioactivity of the CO_2 was assayed in a Beckman liquid scintillation counter.

Method for Measurement of SA of the Guanidine Carbon of Albumin Arginine:

Albumin was fractionated from the plasma by acid-ethanol separation. After dialysis at $4^{o}C$ for 24 hours, the albumin was hydrolysed in sealed tubes with 6N-HCl (400ml/G albumin) for 20 hours at $110^{o}C$. The resultant hydrolysate was dried in order to remove excess acid and neutralized with deacidite FF resin in the CO_3^{--} form. The resin was filtered off and if more than 100mg of protein had been hydrolysed, the amino acid solution was poured onto a column of the same resin comprising equal quantities of Cl^- and OH^- forms. In the latter case by washing the column with water nearly pure arginine was eluted. The amino acid was exposed to activated arginase for 16 hours after which the reaction was stopped by the addition of 4M citric acid.

After any free CO_2 had been removed by reduction to dryness the urea residue was treated in the same way as were the plasma urea specimens. Albumin SA was determined in duplicate.

Stable urea was measured by an autoanalyser technique[138] and albumin by the method of Fernandez et al [78] or by electrophoresis.[79] Free plasma

amino acids were measured by the methods of Hamilton and Efron.[80] [81] Full details of the synthesis rate technique are found in the form of a laboratory manual in Appendix 4.

Synthesis rate was calculated according to McFarlane:

$$SR\ (\%\ IVP/hour) = \frac{K \times SA\ albumin\ arginine\ guanidine\ C\ at\ t_o}{SA\ urea\ C\ at\ t_o}$$

where K = observed slope of plasma urea SA curve (% hour). The urea value at t_o was derived by interpolation of its curve to zero time. Albumin SA was corrected for zero time by applying a factor based on the disappearance of simultaneously injected labelled albumin.

(d) EXPERIMENTAL DESIGN:

As the McFarlane technique had not been used in rats prior to this study, experiments were designed to validate the use of this method in our animals. This was done using 5 groups of 4 animals kept on NPD in which a steady state could be assumed so that synthesis should equal catabolism. The catabolic rates of the animals were measured individually over a period of 14 days after the intravenous injection of ^{131}I labelled albumin. At the end of this study ^{14}C - carbonate was injected and pooled plasma samples from each

of the 5 groups were used for SR determinations. As an additional control, the half life ($t\frac{1}{2}$) of the albumin prepared by the author was compared with that of albumin obtained from Dr. H. Gordon of the National Institute for Medical Research, Mill Hill, London. Dr. Gordon's albumin was labelled ^{131}I and the author's preparation with ^{125}I. A mixture of these two albumin preparations was injected into 5 animals and the plasma activities were measured over a period of 14 days.

Synthesis and catabolism were next measured by the above techniques in 5 rats fed LPD for 60 days and in 5 pair-fed control animals (NPD). This experiment was designed to demonstrate by direct measurement whether SR was diminished in PCM.

In order to determine the timing of metabolic adaptation to protein deprivation, 40 rats were assigned to a protein-free diet (OPD). Synthesis rate was measured daily for the first 8 days on this diet and again on the 10th and 12th day of protein deprivation. Each measurement was made on the pooled plasma of a group of 4 rats. On each day a control group of 4 animals, on NPD, was studied. The experiment was repeated to obtain duplicate values on days 3,4,6,7, and 10. The pooled plasmas obtained from this experiment were used for the measurement of the free plasma amino acids. (Appendix 2.)

TABLE X.

COMPARISON OF CAPE TOWN & MILL HILL

RAT	ALBUMIN PREPARATIONS	
	CAPE TOWN $t_{1/2}$	MILL HILL $t_{1/2}$
1	2·03	1·81
2	2·31	2·06
3	2·51	2·29
4	2·26	1·97
5	2·31	2·05
6	2·19	1·50

TABLE XI.

VALIDATION OF McFARLANE TECHNIQUE

GROUP	SYNTHESIS RATE mg./hr./300 G. RAT	CATABOLIC RATE mg./hr./300 G. RAT
1	12·8	12·9
2	11·7	10·7
3	11·2	10·4
4	11·6	11·9
5	11·1	10·0
MEAN	11·7	11·2

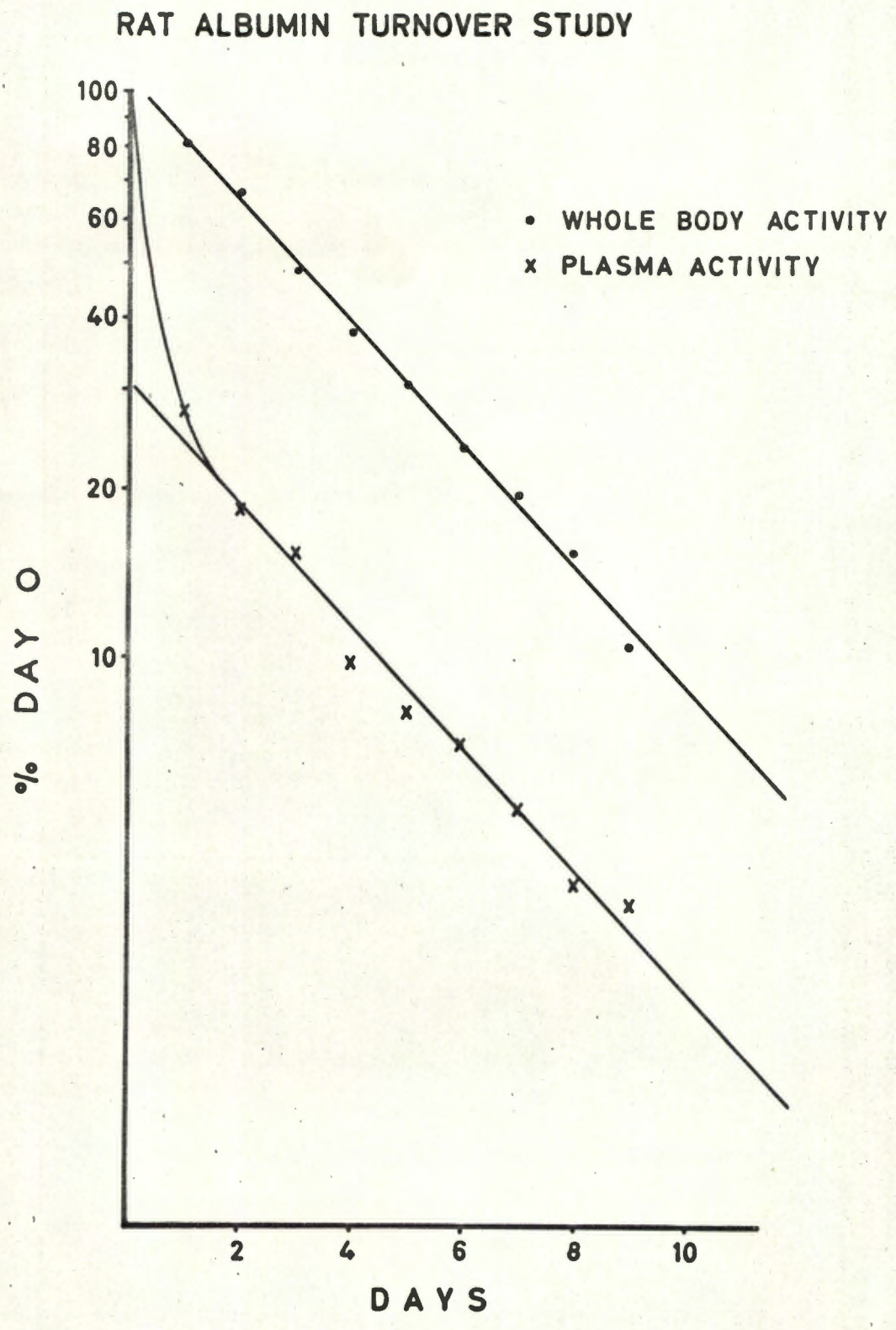

RAT ALBUMIN TURNOVER STUDY
100
80
60
40
20
10
% DAY O
• WHOLE BODY ACTIVITY
x PLASMA ACTIVITY
2
4
6
8
10
DAYS

Fig. 24.

The changes in catabolic rate during protein depletion were measured individually in 11 rats. These rats received intravenous ^{131}I albumin and catabolic rate was determined for 4 days before and 9 days after abrupt withdrawal of protein from the diet.

In order to study the changes occurring during repletion, a further group of 27 rats was fed OPD for 12 days, after which they were repleted (NPD). Prior to repletion the rats were depleted for 12 days as the results of the preceding experiment indicated that by this time gross changes in plasma albumin concentration, SR and CR had occurred. On each of days 1,3,4,5 and 7 of repletion, SR was measured on pooled plasma of 4 rats. Daily catabolic rates were determined on 7 rats for 3 days before and 12 days after repletion.

(e) RESULTS:

The results of the comparative study of the author's and Dr. Gordon's albumin preparations are shown in Table X. The local albumin preparation was found to compare very favourably with that obtained from Mill Hill. The results of ^{131}I albumin turnover studies in normal rats, (Table XI) a typical example of which is found in Fig. 24, compared well with those obtained by others.(123) (139)

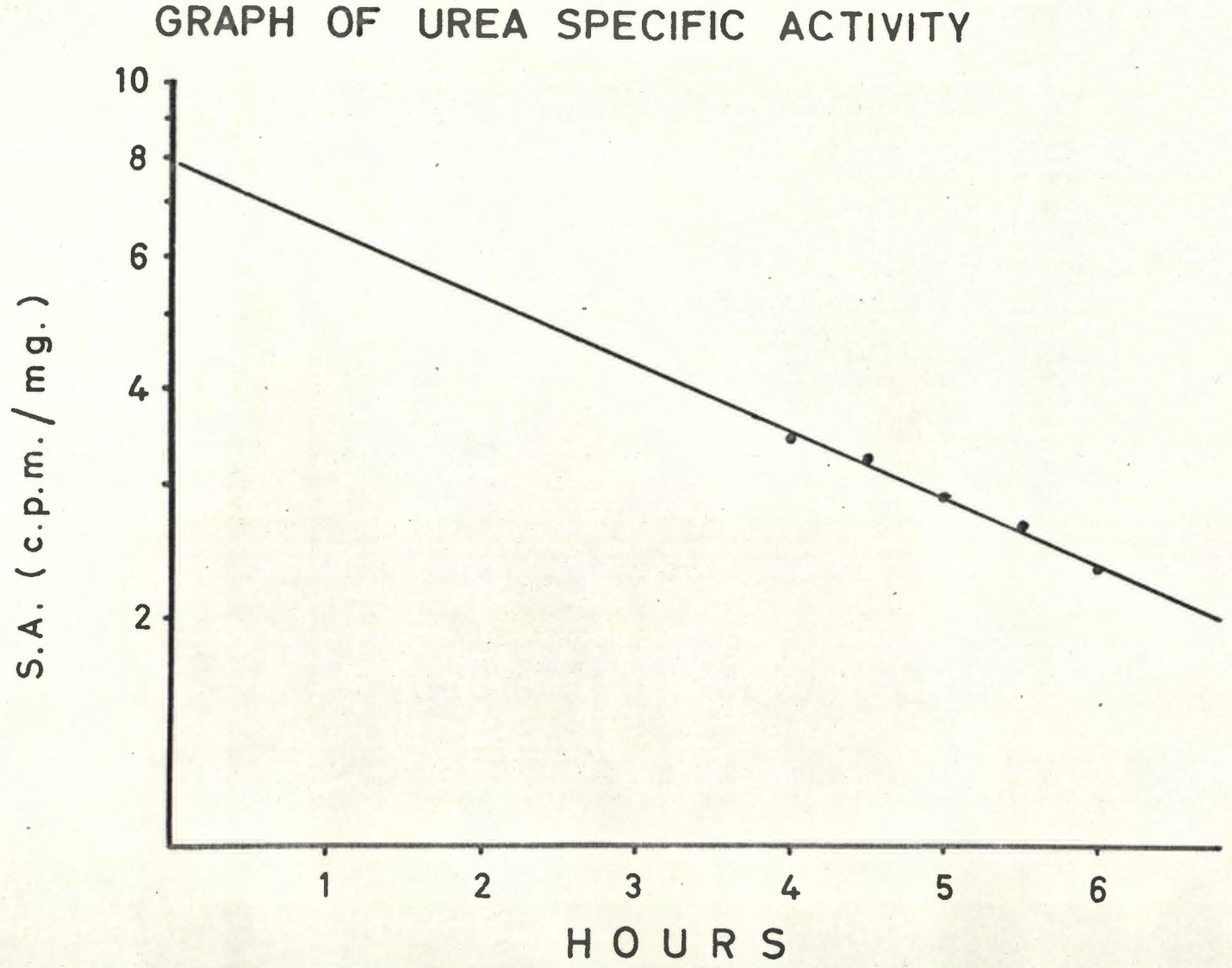

GRAPH OF UREA SPECIFIC ACTIVITY
S.A. (c.p.m./mg.)
10
8
6
4
2
1
2
3
4
5
6
HOURS

Fig. 25.

TABLE XII.

SYNTHESIS AND CATABOLISM RATES OF RATS ON 5% PROTEIN DIETS

RAT	SYNTHESIS RATE mg./hr./300 G. RAT	CATABOLIC RATE mg./hr./300 G. RAT
1	4·3	2·0
2	4·0	3·5
3	3·5	3·1
4	1·7	2·2
5	2·6	5·6
MEAN	3·2	3·3

TABLE XIII.

CHANGES IN ALBUMIN METABOLISM OCCURING DURING PROTEIN DEPLETION

DAYS ON NO PROTEIN DIET	PLASMA ALBUMIN (G. 100 ml.) MEAN ± S.D.	PLASMA VOLUME (ml. 300 G. Rat) MEAN ± S.D.	ALBUMIN SYNTHESIS RATE (mg. hour 300 G. Rat) MEAN ± S.D.	ALBUMIN CATABOLIC RATE (mg. hour 300 G. Rat) MEAN ± S.D.
0	3·4 ± 0·43 (20)	12·5 ± 3·45 (15)	11·7 ± 1·315 (14)	12·6 ± 2·168 (23)
1			13·6 (4)*	13·9 ± 2·9 (11)**
2			12·2 (4)	13·8 ± 3·1 (11)
3			14·4 (4) 12·8 (4)	12·5 ± 3·2 (11)
4	2·8 ± 0·38 (10)		10·9 (4) 12·3 (4)	12·7 ± 3·5 (11)
5			9·3 (4)	14·3 ± 3·2 (11)
6	2·6 ± 0·25 (10)		9·6 (4) 10·5 (4)	12·8 ± 2·8 (11)
7			6·1 (4) 7·3 (4)	10·3 ± 3·4 (11)
8	2·0 ± 0·24 (10)		7·2 (4)	9·1 ± 4·0 (11)
9				8·2 ± 3·1 (11)
10	1·9 ± 0·33 (10)		7·7 (4) 7·1 (4)	
11				
12		12·2 ± 3·76 (8)	4·0 (4)	

FIGURES IN BRACKETS REFER TO NUMBER OF DETERMINATIONS ON WHICH MEAN VALUES ARE BASED

* SERIAL SYNTHESIS RATE MEASUREMENTS WERE MADE ON SEPARATE GROUPS OF 4 RATS, PLASMAS FROM WHICH WERE POOLED

** SERIAL CATABOLIC RATE MEASUREMENTS WERE MADE ON 11 RATS STUDIED FOR THE WHOLE PERIOD

TABLE XIV.

CHANGES IN ALBUMIN METABOLISM OCCURING DURING PROTEIN REPLETION

DAYS OF REPLETION	PLASMA ALBUMIN (G. 100 ml.) MEAN ± S.D.	PLASMA VOLUME (ml. 300G. Rat) MEAN ± S.D.	ALBUMIN SYNTHESIS RATE (mg. hour 300G. Rat)	ALBUMIN CATABOLIC RATE (mg. hour 300G. Rat) MEAN ± S.D.
0	1·8 ± 0·37	12·2 ± 3·76 (8)	4·0 (4)*	4·9 ± 1·36 (7)**
1	2·0 ± 0·31		14·9 (4)	6·6 ± 1·66 (7)
2				9·6 ± 3·71 (7)
3	2·3 ± 0·47		19·6 (4)	8·7 ± 3·23 (7)
4			20·3 (4)	9·6 ± 2·75 (7)
5	2·2 ± 0·24		15·4 (4)	11·1 ± 3·88 (7)
6		12·2 ± 1·41 (5)		10·9 ± 2·97 (7)
7	2·4 ± 0·22		17·7 (4)	12·6 ± 3·00 (7)
8				11·9 ± 3·10 (7)
9	2·2 ± 0·26			13·8 ± 3·4 (7)
10				14·7 ± 5·0 (7)
11	2·4 ± 0·37			12·5 ± 3·20 (7)
12				12·9 ± 3·73 (7)
NORMAL VALUES		12·5 ± 3·45 (15)	11·7 ± 1·315 (14)	12·6 ± 2·168 (23)

FIGURES IN BRACKETS REFER TO NUMBER OF DETERMINATIONS ON WHICH MEAN VALUES ARE BASED

* SERIAL SYNTHESIS RATE MEASUREMENTS WERE MADE ON SEPARATE GROUPS OF 4 RATS, PLASMAS FROM WHICH WERE POOLED

** SERIAL CATABOLIC RATE MEASUREMENTS WERE MADE ON 7 RATS STUDIED FOR THE WHOLE PERIOD

TABLE XV.

CHANGES IN AMINO ACID CONCENTRATION AFTER ABRUPT WITHDRAWAL OF DIETARY PROTEIN

CONTROL(7)	VALINE μ mole./ml. 0·166 (0·130 - 0·221)	LEUCINE μ mole./ml. 0·146 (0·114 - 0·198)	ISOLEUCINE μ mole./ml. 0·107 (0·058 - 0·135)
DAYS ON OPD			
1	0·092	0·084	0·048
2	0·096	0·088	0·048
3	0·098	0·086	0·052
7	0·058	0·094	0·052
DAYS REPLETION			
1	0·153	0·155	0·070

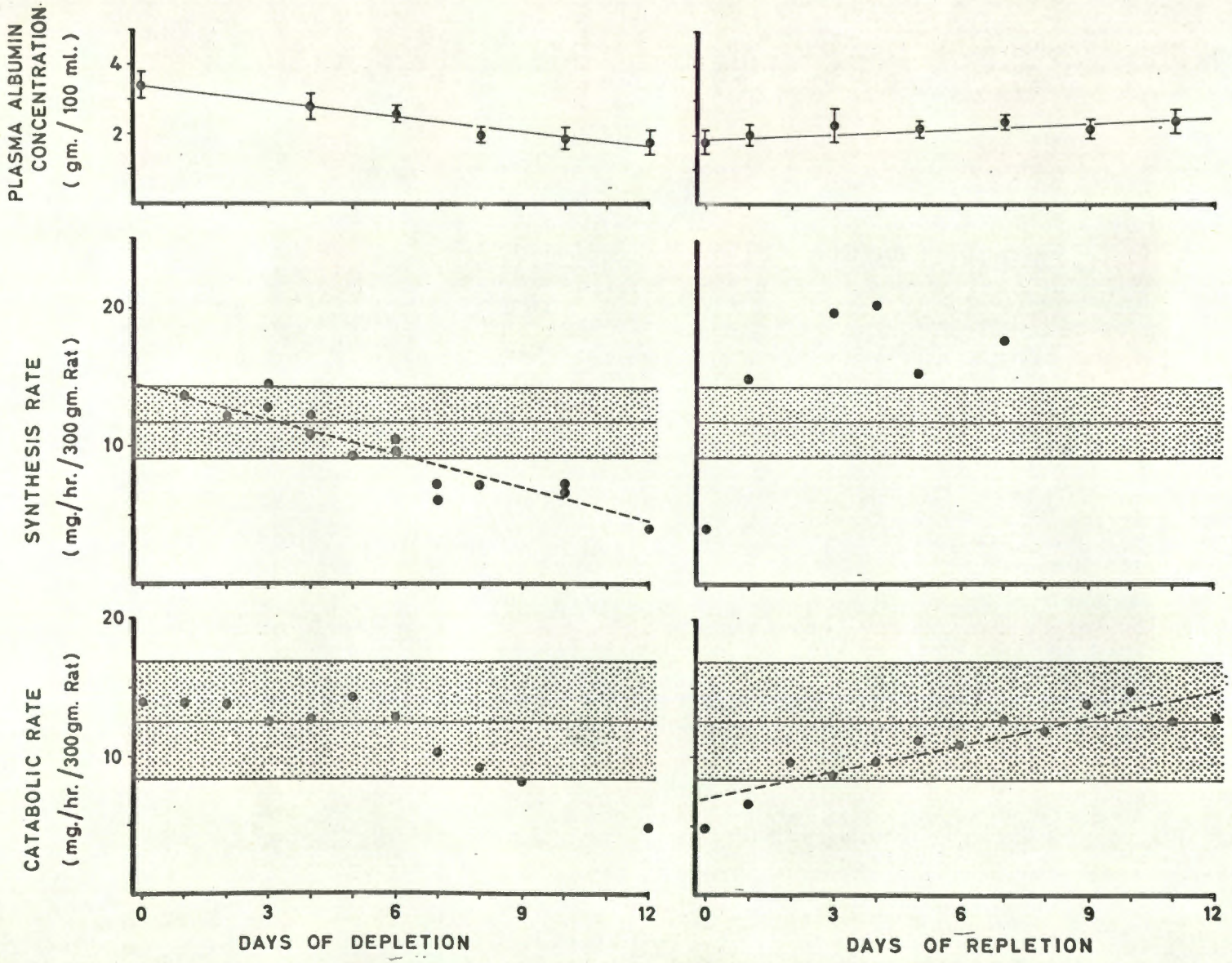


Fig. 26. Serial changes of albumin concentration SR and CR occurring during protein depletion and repletion.

The urea SA curves obtained from the blood taken at half-hourly intervals during SR experiments compared well with those obtained elsewhere. Fig. 25 shows a typical urea SA curve obtained by the author. When, as occasionally happened, the points failed to lie on or about a straight line, the experiment was repeated.

When catabolic and synthesis rates were compared in 5 groups of 4 rats on NPD in which a steady state could be assumed, excellent agreement was found (Table XI), thus validating the use of the McFarlane technique in the rat. Using the experimental model of PCM described in Part 3, SR and CR were both significantly diminished (Table XII) when compared to SR and CR of normal animals. (Tables XI and XIII). Once again, synthesis rate and catabolic rate tend to agree, indicating that the body has adjusted to the lowered protein intake.

The results of the serial studies of protein depletion and repletion are shown in Tables XIII and XIV and in Fig. 26. The concentration of the branch chain amino acids (characteristically reduced in kwashiorkor) are shown in Table XV. Following the abrupt withdrawal of protein from the diet, SR appears to fall off in a slow, gradual manner. The fact that the points fall about a straight line ($r = 0.84, p < 0.0005$) suggests that

TABLE XVI.

CHANGES IN 6:0 Hr. RATIO OCCURING DURING PROTEIN DEPLETION & REPLETION

DIET	DURATION (DAYS)	No. OF RATS	% OF 0Hr. RADIATION REMAINING 6 HOURS AFTER INJECTION MEAN ± S.D.
NPD		16	29·2 ± 6·4 (A)
DEPLETION			
OPD	0–8	12	26·0 ± 4·9 (B)
OPD	10–12	7	37·5 ± 5·7 (C)
LPD	60	8	75·0 ± 9·5 (D)
REPLETION			
NPD	1	2	23·1
NPD	2–10	10	16·9 ± 6·3 (E)

A vs. B - $p > 0{\cdot}1$

A vs. C - $0{\cdot}02 > p > 0{\cdot}01$

A vs. D - $p < 0{\cdot}001$

A vs. E - $p < 0{\cdot}001$

the gradual diminution in SR commences soon after protein withdrawal. Synthesis rate values do not, however, fall outside of 2 standard deviations from the mean until day 7. The catabolic rate is normal for the first 6 days after which it falls gradually (significance of deviation from mean values; day 7 $p \sim 0.02$; day 8 $p < 0.01$; day 9 $p < 0.001$). The levels of the branch chain amino acids, which are characteristically lowered in kwashiorkor, were significantly reduced after 1 day on OPD. Plasma albumin concentrations show a gradual fall similar to that of SR. The plasma volume remained unchanged during protein depletion.

On repletion the SR increased abruptly to normal or above normal values and remained high throughout the period of study. The CR increased gradually in a mirror-image of its fall on OPD, reaching the mean normal value for rats by the 7th day. Plasma volume, once again, remained unchanged.

Protein depletion appears to be associated with an alteration in the 6/0 hour ratio of injected ^{131}I albumin (Table XVI). After 10 days of OPD, a significantly slower disappearance of labelled protein was noted. The same change, though more marked, was found in rats fed LPD for 60 days. On repletion the disappearance of ^{131}I

seemed even more rapid than normal. An explanation for these changes is offered under "Discussion".

(f) DISCUSSION:

The validation of the McFarlane technique in rats has been an essential part of this project. The good agreement between SR and CR measurements in animals on a normal protein diet (Table XI), appears strongly to validate the use of the ^{14}C carbonate technique. The agreement in these animals becomes even more remarkable when the time periods over which measurements were made (6 hours for SR, 10-15 days for CR) and other differences between the two techniques are taken into consideration.

There appears to be no reason why the McFarlane technique should not be applied to SR measurements on animals in an unsteady state. If there was significant reutilization of urea carbon in PCM, the results obtained by the McFarlane technique would be falsely low. However, while there is significant reutilization of urea nitrogen in PCM, there is no evidence that urea carbon reutilization is significantly increased.(140) Animals on OPD or LPD showed a consistent alteration of the 6/0 hour ratio of injected ^{131}I albumin, indicating a slower disappearance rate of intravascular albumin. Since the albumin SA at

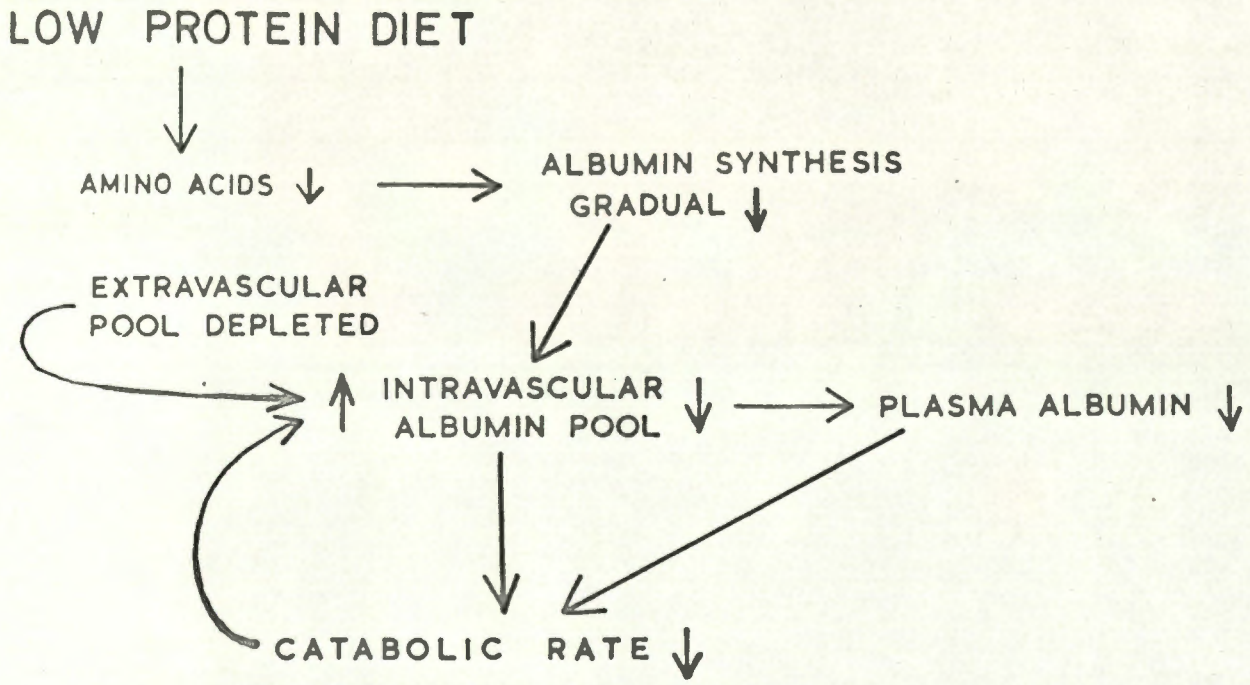
zero time is based on this correction, the lower SR found in rats on LPD or OPD (in the latter case after 8 days), could partially be explained by this. For the first 8 days on OPD however, the 6/0 hour ratio remains unchanged while the SR falls significantly. This slower rate of disappearance of intravascular albumin may result from diminished catabolism or decreased exchange to the extravascular pool. From the preceding review (Part 5), it is apparent that both factors obtain in protein deprivation. On protein repletion (after the first day) the 6/0 hour ratio changes once more, this time indicating a more rapid loss of protein than is normally found. This is thought to be due to increased catabolism and increased exchange of protein from the intravascular to the extravascular pool. There is strong evidence to suggest that both these factors occur during repletion.(119) Even if the correction factor obtained from the 6/0 hour ratio of normal rats was used in the repletion study, the SR results obtained would fall within the mean normal range from the first day of repletion.

While there has been no doubt as to the validity of CR estimations based on ^{131}I albumin turnover studies, less certainty exists over SR values derived by this method in unsteady states. Accordingly, SR and CR were

studied independently on rats fed LPD for 60 days to verify the results obtained by others using ^{131}I albumin for both measurements. The results of this study (Table XII), prove conclusively that both synthesis and catabolism are diminished in experimental PCM.

Once it had been established that both SR and CR were diminished in PCM, it was decided to study the timing of these changes. It is evident from Tables XIII and XIV and from Fig. 26, that after abrupt withdrawal of dietary protein (OPD), SR diminishes before catabolic rate. Based on this study the sequence of events occurring after withdrawal of protein from the diet appears to be as follows: Withdrawal of dietary protein leads to a decrease in concentration of certain essential amino acids. This is followed by a reduction of SR. Catabolism continues at a normal rate so that a gradual fall in plasma albumin concentration and pool size occurs. At some critical level the catabolic rate falls precipitously, probably in an attempt to conserve the albumin pool in the face of impending depletion.

An additional measure aiming at conservation of the plasma albumin concentration or mass is the transfer of extravascular albumin to the intravascular pool. When this occurs, however, is uncertain.



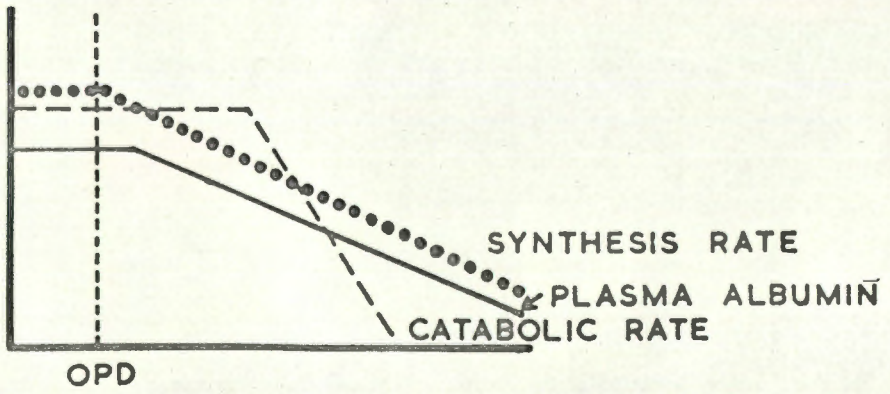


Fig. 27. Diagrammatic representation of changes occurring during protein depletion.

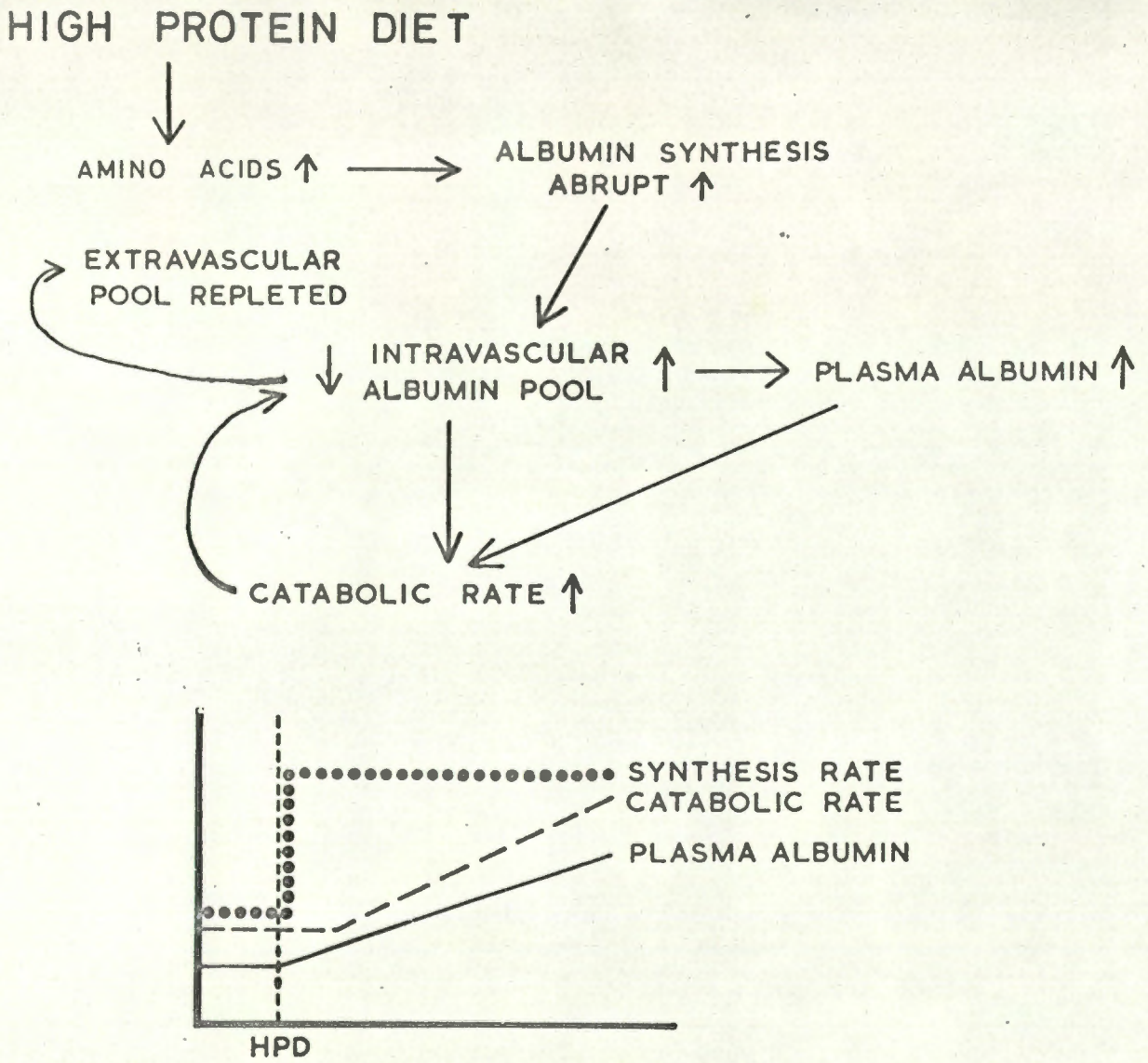


Fig. 28. Diagrammatic representation of changes occurring during protein repletion.

The above sequence of events is diagrammatically illustrated in Fig. 27.

On repletion the changes are even more marked. The SR increases abruptly to normal or above normal levels. The plasma albumin concentration and pool size increase until the need for compensatory lowering of the CR no longer obtains and normal values are re-established. (Fig. 28)

James et al [141] have recently reported on direct measurement of CR and indirect measurement of SR in infantile malnutrition. By studying infants in three consecutive dietary periods, e.g. HPD,LPD,HPD, before and after recovery from malnutrition, they concluded that rates of albumin synthesis were primarily affected when protein intake was changed. Catabolism, they felt, responded more slowly. Waterlow [127] has suggested that SR is primarily altered by external factors, e.g. supply of amino acids, while CR is regulated by a mechanism which attempts to compensate for changes imposed by the environment.

The results of the depletion and repletion studies (Fig. 26) strongly support this hypothesis and seem to demonstrate conclusively that SR and CR are separately and independently controlled.

In conclusion, the aims of this thesis have been to demonstrate by direct measurement, the diminution of albumin SR in experimental PCM; to study the sequence of the changes in albumin SR, CR and concentration in this disease and to elucidate, as far as possible, the control mechanisms of these processes. These aims, as set out in Part 1, have to a large extent been fulfilled. The diminution of albumin synthesis in experimental PCM has been demonstrated, the hypothetical sequence of events (Part 1) has been tested and found to be correct and separate control mechanisms for SR and CR have been shown to exist.

THE KINETICS OF ALBUMIN METABOLISM IN EXPERIMENTAL PROTEIN DEPLETION AND REPLETION.

S U M M A R Y.

This study has aimed at elucidating the changes of albumin metabolism occurring in PCM. In particular, it has aimed at defining the sequence in which these changes occur. To date this has not been possible due to the lack of a method for direct measurement of albumin synthesis. There is good evidence that catabolism of albumin is slowed in PCM, but less certainty exists about alterations in synthesis rate. The sequence in which these changes take place, and the factors regulating albumin metabolism, have thus remained hypothetical. It has been postulated that reduction or withdrawal of dietary protein causes a diminution of albumin SR, presumably through decreased availability of amino acids. Catabolism continues at a normal rate, so that a gradual fall in plasma albumin concentration and pool size occurs. At some critical level reduction in catabolic rate (CR) follows, probably

as an attempt to conserve the albumin pool in face of impending depletion. If adequate dietary protein is now made available, SR increases abruptly. Plasma albumin concentration and pool size gradually rise, until the need for compensatory lowering of CR no longer obtains and normal values are re-established. The work testing this hypothesis, and finding it to be essentially a correct one, has been recorded in this thesis.

Synthesis rate was measured directly by the method of McFarlane and Reeve while CR was measured by the standard ^{131}I labelled albumin technique.

After reviewing the literature, it was decided to produce in the rat an experimental model of PCM. Male albino rats were fed 3 low protein diets consisting of 12, 8,& 5 percent mixed protein. In addition, a protein-free diet was used. Weight-matched control animals were fed a diet containing 20% of the same mixed protein, in amounts iso-caloric to those consumed by rats on the low protein diets. The rats fed a diet containing 5% mixed protein developed clinical, biochemical and histological disorders similar to those seen in children suffering from kwashiorkor. Growth was markedly decreased, oedema occurred in most animals, and they developed significant hypoalbuminaemia, a plasma amino acid profile similar to

that seen in kwashiorkor, and histological evidence of periportal fatty infiltration in the liver.

Synthesis rate and catabolic rate were first measured in rats on a normal protein diet in which steady state conditions could be assumed to exist so that SR should equal catabolic rate. The extremely good agreement obtained validated the use of the McFarlane technique.

A similar agreement noted in rats fed the 5% protein diet, both SR and CR being greatly diminished, indicated adaptation by these rats to the lowered protein intake.

Serial studies of plasma amino acid concentration, albumin concentration, pool size, SR and CR were made after abrupt withdrawal of protein from the diet and after re-introduction of dietary protein. The levels of the branch chain amino acids, characteristically lowered in kwashiorkor, were reduced after 1 day on the protein-free diet. Starting soon after dietary protein deprivation the SR gradually decreased, reaching 2 standard deviations from the mean on the 7th day. The plasma albumin concentration and pool size diminished in a similar manner. Catabolic rate was maintained for the first 6 days after which it fell abruptly reaching 2 standard deviations from the mean on the 9th day.

Repletion resulted in an abrupt increase in amino acid

concentration and SR to normal or above normal levels. The plasma albumin concentration and pool size increased gradually while the catabolic rate rose in a similar manner, mirroring its fall noted during depletion.

These findings seem to demonstrate clearly that synthesis and catabolism of plasma albumin are separately and independently controlled and that synthesis rate is probably altered by external factors, e.g. supply of amino acids, while CR is regulated by a mechanism which attempts to compensate for changes imposed by the environment.

APPENDIX 1.

METHODS USED FOR QUANTITATIVE MEASUREMENT OF PLASMA PROTEINS.[78]

REAGENTS:

(1) NaCl 0.9% (w/v)

(2) Hydrochloric acid - Ethanol

(3) Sodium acetate - ethanol

(4) NaOH 3% (w/v)

(5) Biuret reagent

(6) Protein Standard (30mg/ml)

PREPARATION OF REAGENTS:

Hydrochloric acid - Ethanol

Add 1.0ml concentrated HCl to 600 ethanol. Denatured ethanol (95% ethanol, 5% methanol) may be used.

Sodium acetate - ethanol

Sodium acetate will not dissolve in ethanol. Dissolve 2.7218g. 0.2M sodium acetate (C_2H_3 Na O_2. $3H_2O$) in 5ml methanol. When completely dissolved make up to 100ml with ethanol.

Biuret reagent

(1) Dissolve 17.3g Cu SO_4 5 H_2O in 100ml hot distilled water. (2) Dissolve 173g sodium citrate and 100g anhydrous Na_2CO_3 in 800ml water with heating. When cool pour second mixture into first while stirring. Dilute to 1 L.

METHOD FOR ALBUMIN DETERMINATION: (Duplicates of samples and standard must be done).

(1) Put 0.2ml serum + 0.8ml 0.9% NaCl into a centrifuge tube. Add 9.0ml HCl - ethanol. Mix well. Stopper tightly to prevent evaporation and incubate at 37°C for 30 minutes.

(2) Centrifuge. Transfer 5ml of supernatant fluid (SNF) to a test tube.

(3) Add 0.5ml sodium acetate - ethanol solution to the 5ml SNF. Mix well. Stopper tubes and let them stand at room temperature for 10 minutes.

(4) Centrifuge. Discard SNF.

(5) Invert tubes on filter paper and allow to drain.

(6) Dissolve precipitate in 5.0ml 3% NaOH and add 1.0ml biuret reagent.

(7) Prepare reagent blank: 5.0ml NaOH + 1ml biuret reagent.

(8) Prepare Standard: 0.1ml protein standard solution, 4.9ml 3% NaOH, 1.0ml biuret reagent.

(9) After 15 minutes read colour intensity of albumin, standard and blank on Klett colorimeter with 54 filter against distilled water.

METHOD FOR TOTAL PROTEIN DETERMINATION: (Duplicates of samples and standard must be done).

(1) Mix 0.1ml serum or plasma with 4.9ml 3% Na OH.

(2) Add 1.0ml biuret reagent.

(3) Prepare blank and standard as for albumin determination.

(4) Turbidity of the specimens is measured by reading a mixture of 0.1ml plasma and 5.9ml 3% Na OH.

(5) Read as in albumin determination method.

CALCULATIONS:

From reading subtract blank (and where done, turbidity). This gives values Ap. for plasma sample and As for standard (Std).

g. albumin (or protein) / 100ml plasma =

$$\frac{Ap}{As} \times \text{g. protein in Std} \times \frac{100}{0.1}$$

METHOD FOR SERUM PROTEIN ELECTROPHORESIS ON CELLULOSE ACETATE:

APPARATUS:

(1) Perspex cells (each cell holds 6 strips)

(2) Power pack

(3) "Oxoid" cellulose acetate strips 2.5 x 18cm

(4) Plastic ruler

(5) Micro-pipettes

(6) Blotting paper

(7) Filter paper strips 2 inch width

(8) 5 Plastic dishes with lids

(9) 2 pairs blunt-tipped forceps

(10) Scissors

REAGENTS:

(1) Barbitone Buffer (pH 8.6 0.06M)

For impregnating strips and filling cell

Barbitone 1.533g.

Sodium diethyl barbitone 10.633g.

5% thymol in isopropyl alcohol 5ml

Redistilled water to 1 litre

(2) Ponceau S Stain

0.2% Ponceau S (Gurr) in 3% aqueous trichloracetic acid. Filter after making up.

(3) 10% aqueous acetic acid

(4) Liquid paraffin for clearing strips prior to scanning

PROCEDURE:

(1) Filling Tank

Put cotton wool plugs into holes in partitions. Fill tank with barbitone buffer to depth of ± 2cm in all compartments. The buffer should not cover the partitions. Make levels equal in all compartments by tilting the tank so that buffer flows over the partitions. Wait until an equal level is attained in all compartments then lower back into position. Buffer should be replaced and the tank cleaned periodically.

Cut filter paper strips to fit the bridge. Wet paper and apply to bridge. The dependent edge of the filter paper strip should project into the buffer solution. Change frequently.

(2) Cellulose Acetate Strips

Never handle strips with fingers!

Cut strips in half

(3) Impregnation of Strips

Float strips on surface of buffer. Allow buffer to soak into strip before immersing completely.

Remove strips and blot lightly with clean blotting paper. Place strips across bridge and pull them taut. When strips are in place turn on current (0.4 MA/cm width) for 10 minutes before applying sample.

(4) Application of Sample

Place ruler $\pm \frac{1}{4}$ of the way from the cathode. Apply sample from pipette by leaning the latter against the ruler and turning it back and forwards across the strip. Apply sparingly (1 to 10 μL). Run current for $1\frac{1}{2}$ hours. Check and readjust amperage periodically throughout run. As the strips warm the resistance drops and the current rises.

(5) Removal of Strips

Turn off current. Remove strips and place in staining solution. Allow to stain for $\pm$ 20 minutes. Wash out all unbound colour with dilute acetic acid. Dry strips between two layers of blotting paper.

(6) Scanning of Strips

Soak strips in liquid paraffin in order to render them transparent. Scan on chromoscan which measures the areas under the peaks by auto-planimetry.

APPENDIX 2.

METHOD USED FOR MEASURING FREE PLASMA AMINO ACID CONCENTRATION.

APPARATUS:

(1) Technicon amino acid analyser

(2) Column 127 x 0.62cms

(3) Chromobeads type B (Technicon) resin

REAGENTS:

(1) Salicylsulphonic acid crystals

(2) Ninhydrin

(3) Buffer pH. 2.875

(4) Buffer pH. 3.800

(5) Buffer pH. 4.700

PREPARATION OF REAGENTS:

Ninhydrin:

(1) Dissolve 45g ninhydrin and 3.375g hydrindantin in 4.5L of methylcellusolve in a dark bottle. Mix for 15 minutes.

(2) Add 900ml of sodium acetate buffer (sodium acetate 656g, glacial acid 200ml, water to 2L) and 3,600ml of water to the ninhydrin solution.

(3) Nitrogen should be bubbled through this mixture during mixing and for at least 30 minutes after the addition of the water.

pH 2.875 Buffer:

(1) Add 14.71g sodium citrate (0.05M, 0.150N with respect to Na^+) 25ml of 2N (standardised) NaOH and 5.0ml of thiodiglycol (Pierce Chemical Co.) to 900ml of water.

(2) Adjust pH to 2.875 with 6-N HCl and add 10ml of Brij 35 solution (Atlas Chemical Industries Incorp.)

(3) Make up to 1L with deionised water and if necessary readjust pH to 2.875 with 6-N HCl

pH 3.800 Buffer:

This buffer is made up in precisely the same manner as the pH 2.875 buffer, but the final pH adjustment is to pH 3.800.

pH 4.700 Buffer:

(1) Dissolve 471.30g of sodium citrate in 5.4L of water

(2) Adjust pH to 4.7 with 6-N HCl. Add 60ml Brij solution and make up to 6L with deionised water

NB.

All water must be glass distilled and then deionised.

All buffers must be stored at $4^{o}C$ and checked immediately before use.

METHOD:

(1) Fasting, unhaemolysed plasma is deproteinised by the addition of a spatula tip of salicylsulphonic acid crystals. After centrifugation the SNF is removed for assay.

(2) 0.2N NaOH is pumped through the column for 30 minutes followed by sodium citrate buffer pH 2.875 for a further 90 minutes.

(3) Apply 0.5ml of internal standard (Norleucine 0.1 µM/ml) and wash down sides of column with 2ml of buffer pH2.875. Pump norleucine and buffer into column with nitrogen at 100 pounds per square inch (psi)

(4) When the norleucine and buffer have been pumped into the column, 0.2 -0.5ml of deproteinised plasma are applied in the same way.

(5) Fill column to the brim with buffer and connect it to autograd.

(6) Nine chambered autograd is filled with buffers as follows:

Chamber	1	75ml	pH 2.875	buffer
Chamber	2	75ml	pH 2.875	buffer
Chamber	3	75ml	pH 2.875	buffer
Chamber	4	75ml	pH 2.875	buffer
Chamber	5	40ml	pH 2.875	buffer
		35ml	pH 3.800	buffer
Chamber	6	6ml	pH 2.875	buffer
		9ml	pH 3.800	buffer
		60ml	pH 4.700	buffer
Chamber	7	75ml	pH 4.700	buffer
Chamber	8	75ml	pH 4.700	buffer
Chamber	9	75ml	pH 4.700	buffer

(7) Turn on autograd pump and stirrer and pump buffers through column at 30ml/hour and 200 psi.

(8) Set temperature of column at 37°C for the first 90 minutes after which it is reset to 60°C for the remaining 19 hours of the run.

(9) Colour development is with ninhydrin under nitrogen, the amino acids passing through an oil bath at 95°C before being cooled and entering the flow cuvettes of the colorimeter.

(10) Set colorimeters to read at 440 um and 570 um wavelengths.

(11) Calculate results after method of Moore and Stein.(143)

APPENDIX 3.

A. PREPARATION OF PURE RAT ALBUMIN FOR IODINATION.

AMMONIUM SULPHATE PRECIPITATION OF ALBUMIN.[135]

REAGENTS:

(1) Normal saline (0.9% w/v)

(2) Saturated ammonium sulphate (saturated at 37°C)

(3) Dilute acetic acid (10%)

METHOD:

(1) To x ml of plasma add 2x ml of normal saline.

(2) Add an equal volume (3x) of saturated ammonium sulphate pH5.6. Mix and allow to stand for 15 minutes for globulin precipitate to form. Spin hard till SNF clear.

(3) Transfer SNF into 100ml beaker.

(4) Using expanded scale on pH meter, add acetic acid dropwise mixing continuously until pH 4.7. The albumin precipitates out at this pH. If the pH drops below pH 4.5 the alpha and beta-globulins precipitate out.

(5) Spin hard. Discard SNF.

(6) Dissolve albumin in as little water as possible.

(7) Dialyse overnight at 4°C against distilled water.

(8) Measure albumin concentration (Appendix 1) and check purity by electrophoresis.

(9) If any plasma protein fraction other than albumin is present reprecipitation after iodination is essential.

B. METHOD FOR THE IODINATION OF ALBUMIN:(136)

REAGENTS:

(1) ± 15mg albumin in less than 1ml solution

(2) Iodine monochloride (I Cl)

(3) Glycine buffer

(4) 1N NaOH

(5) 2M NaCl

(6) ^{131}I or ^{125}I

(7) Deacidite FF resin (Cl^- form)

(8) 20% trichloracetic acid

(9) ± 5ml plasma

PREPARATION OF REAGENTS:

Iodine monochloride

(1) Dissolve 150mg sodium iodide in 8ml 6-N HCl

(2) Forcibly inject 108mg sodium iodate monohydrate in 2ml distilled water.

(3) Dilute with distilled water to 40ml and shake with 5ml carbon tetrachloride (CCl_4)

(4) If the CCl_4 has a faint red colour repeat the treatment with CCl_4

(5) Remove residual CCl_4 by aerating with moist air for 1 hour and make up to 45.0ml with distilled water.

This solution is 0.0337 with respect to ICl and $\pm$ N to HCl.

(6) Before use dilute 1 volume of the above ICl solution with 9 volumes of 2M NaCl

Glycine Buffer

Dissolve 7.5g glycine in 75ml distilled water. Add 25ml of 1N NaCl.

Chloride resin column

Prepare column of Deacidite FF resin Cl^- form $\pm$ 5cm by 1cm. Run through 100ml of 0.9% NaCl.

METHOD:

(1) Put 1.8ml glycine buffer + 0.2ml 1N NaOH in a tube

(2) Put 0.3ml of dilute ICl into another tube and add ^{131}I or ^{125}I

(3) Take 15mg albumin (usually $\pm$ 0.5ml) and add glycine buffer dropwise until pH8.5. (1 - 2 drops are usually necessary)

(4) Bring pH of radioactive iodine solution to 8.5 with glycine buffer (requires ± 12 drops). The yellow colour of the ICl solution disappears just before pH 8.5.

(5) Take radio-iodine solution into a pasteur pipette and squirt vigorously into albumin solution (do not squirt more than once).

(6) If the labelled albumin is to be used for a turnover study the albumin is reprecipitated after the addition of 2 to 5ml of plasma thus ensuring a high degree of purity and at the same time removing the free iodine present.

(7) If the labelled albumin is to be used for plasma volume measurement only the free iodine may be removed by passing the solution through a column. (Steps 8 - 10)

(8) Make a column of Cl resin (this may be prepared in advance).

(9) Pass 1ml of plasma through column.

(10) Load labelled protein solution onto column collecting effluent in a clean tube.

(11) The efficiency of labelling may be tested by TCA precipitation. Take 0.01ml of iodinated albumin, add 1ml of saline and 1ml of plasma. Mix well and count in well-counter.

(12) Add 2ml 20% TCA and spin.

(13) Take 2ml SNF and count.

(14) Free radio-iodine expressed as a percentage of total radio-iodine present is calculated as follows:

$$\frac{\text{Activity in 2ml SNF x 2 x 100}}{\text{Activity of sample before addition of TCA}}$$

All samples containing free iodine in excess of 1% are discarded.

(15) Run labelled iodine and a plasma sample Electrophorectically on cellulose acetate strips.

(16) Stain strips. Cut the strip with the labelled albumin (which should only have one band) into pieces corresponding to the protein fractions of the plasma strip. Count.

(17) The label should be in the albumin only.

C. STERILIZATION OF LABELLED ALBUMIN:

APPARATUS:

(1) Seitz Filter

(2) Vacuum System

(3) Sterilized Bottle

METHOD:

(1) Put 1.0ml plasma onto Seitz filter, soaking pad thoroughly. Turn on vacuum. Put 1ml saline (0.9%) onto pad.

(2) Pour sample onto filter. When the sample has passed through the filter, wash with known volume of saline.

(3) Using sterile technique transfer sample to sterile bottle at the same time noting its volume.

(4) Count 0.01ml aliquot of sample and dilute the labelled albumin solution so that the activity required for injection is in $\pm$ 0.5ml.

APPENDIX 4.

METHOD USED FOR MEASURING ALBUMIN SYNTHESIS RATE.(18)(101)(142)

METHOD FOR DETERMINING SPECIFIC ACTIVITY OF GUANIDINE-C OF ARGININE OF ALBUMIN.

REAGENTS:

(1) 10% TCA:- 1 in 5 dilution of 50% TCA (stored in cold)

(2) Activated Arginase:-

Manganese/Maleic buffer: 8.90g Na Maleate
11.15g $MnSO_4 . 4H_2O$

Dissolve Na Maleate in ± 200ml dist. H_2O. Bring pH to 9.7 - 9.8. Add this solution to $MnSO_4 . 4H_2O$ and make up to 500ml. Bring pH to 7. Add 500mg arginase to 500ml buffer (1mg/ml). Incubate at 37°C for 3 hours. Then store in frig. Make fresh every 2 months.

(3) 1 N NaOH:- 4g NaOH pellets 100ml

(4) CO_2-Free H_2O:- Boil dist. H_2O till ½ its original volume - store tightly stoppered.

(5) CO_2-Free 1 N NaOH:- Make solution using 10ml 10 N NaOH to 90ml CO_2-free H_2O. Make fresh for each run.

(6) 4 M Citric Acid:- 84g citric acid made up to 100ml.

(7) Phosphate Buffer:-41.75g Na Pyrophosphate
5.7ml Orthophosphoric acid
Make up to 500ml.

(8) Urease:- 1mg/ml solution in Phosphate buffer. Keep in frig. (Make fresh each month)

(9) Tungstic Acid:- 10ml 10% Na Tungstate
3.8ml 1 N HCl
Make up to 100ml

(10) Citric/Tungstic:- 1:1 4 M Citric Acid : Tungstic Acid

(11) Stable Urea:- 1mg/ml solution of dehydrated urea in dist. water.

(12) 10% Na Tungstate:- 10g Na Tungstate make up to 100ml with distilled water.

(13) 2/3 N H_2SO_4:- 18.7ml conc. H_2SO_4/litre.

(14) RESINS.

OH Form:- Chloride form of Deacidite FF is washed in Buchner filter with H_2O until clear. Then washed with $\pm$ 2L 2 N NaOH. Then wash with H_2O and check whether eluate is Cl-free (using $AgNO_3$). If not Cl-free wash with more NaOH and repeat. When Cl-free, wash with H_2O till pH7.

HCO_3 Form:- Chloride form converted to OH-form Then washed with 0.5 N $NaHCO_3$ ($\pm$ 2L) and then with H_2O till pH7.

OH/Cl Form:- 1:1 OH and Cl forms of resin (approximate).

(15) Phenylethylamine/methanol:- 1:1 Phenylethylamine/methanol. Keep in dark bottle.

(16) Scintillation Mixture:- 7.5g PPO + POPOP → $2\frac{1}{2}$ l. Toluene. Keep in dark bottle.

STEPS:

A. Extraction of albumin from serum (Alcohol - TCA)

B. Acid hydrolysis of albumin.

C. Separation of arginine.

Neutralization of hydrolysate with or without purification (needed if 100mg albumin).

D. Incubation with arginase.

E. Incubation with urease.

F. Estimation of CO_2 specific activity.

A. EXTRACTION OF ALBUMIN:

(All samples are done in duplicate)

(1) Take 1ml plasma and add 100mg of stable albumin. Total volume xml.

Add equal vol. (X) of 10% Trichloroacetic acid (TCA) Mix and centrifuge. Pour off supernatant.

(2) Add equal vol. (i.e. X) of 5% TCA to precipitate. Mix thoroughly and centrifuge. Pour off supernatant.

(3) Add 3 vols. (i.e. 3X)oof pure absolute Ethanol. Mix very thoroughly - centrifuge till SNF clear. Soak dialysis tubing in dist. H_2O.

(4) Put supernatant into dialysis tubing and dialyse overnight against dist. H_2O (at least several hundred volumes) in cold (4^oC).

(5) Concentrate alb. sample to $\pm$ original volume of serum (rotary evaporation).

(6) Estimate protein conc. by Biuret reaction (total protein) and measure volume - Y mg/ml in Z ml. Total protein = YZ mg.

BREAK POINT - store in cold.

B. ACID HYDROLYSIS OF PROTEIN:

(1) Add 100ml 6N HCl for 250mg protein.

NOTE: For solutions of protein calculate the volume of conc. HCl required to give the correct volume of 6N HCl taking into account the volume of protein solution. Conc. HCl = 11.65 N. Work out amt. protein in sample. X = YZmg.

Use 100ml 6N HCl for 250mg alb.

X x $\frac{100}{250}$ ml 6N HCl for X mg alb.

(X x 0.4)ml = (b)

Conc. HCl = 11.65 N.

(X x 0.4)ml 6N HCl = (X x 0.4) x $\frac{6}{11.65}$ ml conc. HCl

= (X x 0.4) x 0.515ml conc. HCl (a)

∴ Desired vol. 6N HCl to be added = (b)

∴ Add water - volume = (b) - (a + z)

Add conc. HCl - volume = (a)

(2) Hydrolyse in sealed tubes in oven or oil-bath at 107-110° for ± 20 hours.

BREAK POINT - store in cold.

(3) After hydrolysis carefully transfer solution to a round-bottom flask.

(4) Evaporate off HCl on rotary evaporator to dryness.

(5) Wash once with dist. water.

(6) Evaporate to dryness again.

BREAK POINT - store in cold.

C. SEPARATION OF ARGININE:
Neutralization with HCO_3 Resin.

(1) Dissolve amino acid residue in dist. water (3-4ml).

(2) Bring solution to pH 7 by slowly adding solid,

washed Deacidite FF in the HCO_3 form. Test with pH paper.

NOTE: CO_2 bubbles form when pH 7 is nearly reached.

(3) Prepare a resin column by putting in a small quantity ($\pm$ 5mm layer) of the HCO_3 resin - wash with dist. H_2O to pH 7.

(4) Put resin-protein mixture through this column with pasteur pipette.

(5) Rinse flask with $\pm$ 10ml dist. water and put on column.

(6) Collect eluate in round-bottom flask.

(7) If less than 100mg albumin was hydrolysed add arginase as in D. If more than 100mg albumin was hydrolysed proceed as follows:

Purification of Arginine.

(8) Prepare a resin column (9 x 1cm) by pouring on Deacidite FF resin containing equal volumes of OH and Cl forms of the resin. Wash with dist. H_2O to pH 7 (requires prolonged washing).

(9) Dry sample to 1-2ml, put sample on the column and allow it to run in before washing.

(10) Wash with dist. water collecting 4ml aliquots of the eluate (about 6 tubes). (Arginine usually comes off in tubes 1 - 3).

(11) Do a spot test for Arginine.

(i) Put 1 drop from each tube on a strip of filter paper - dry in oven.

(ii) Spray with Ninhydrin - dry in oven.

Arginine stains purple or blue purple.

(12) Combine Arginine eluates in round-bottom flask. Wash Arginine tubes with eluates from less concentrated tubes on either side, and add these washings to round-bottom flask.

D. INCUBATION WITH ARGINASE.

(1) Add 1 - 3ml activated arginase (2ml for 100mg protein; 3ml for 100mg).

(2) Bring sample to pH 9 (pH paper) with 1N NaOH. Usually 1-2 drops only).

(3) Incubate for $\pm$ 16 hours at 37°C.

E. INCUBATION WITH UREASE.

(1) Bring sample to pH 1-2 (pH paper) with 4M Citric Acid (usually 7-8 drops).

(2) Evaporate to dryness on rotary evaporator.

(3) Re-dissolve in dist. H_2O ($\pm$ 1ml) + 0.5ml phosphate buffer,

(4) Bring solution to pH 7 (pH paper) with CO_2-free 1N NaOH (Usually several ml.).

BREAK POINT - Put Sample in Test Tube in Deep Freeze.

(5) Put sample in reaction vessel.
Put 1ml of urease solution onto one side-arm of reaction vessel (mark urease arm).

(6) Put 1ml of citric acid/tungstic acid into other side-arm.

(7) Cool vessel in CO_2-ethanol and evacuate with warming till no more free CO_2.

(8) Disconnect vessel from pump with tap closed.

(9) Tip in urease from side-arm.

(10) Incubate for $\frac{1}{2}$ hour or longer at $25^{\circ}C$.

F. ESTIMATION OF CO_2 SPECIFIC ACTIVITY.

(1) After $\frac{1}{2}$ hour tip citric acid/tungstic acid from other side-arm. (Note: more than $\frac{1}{2}$ hour incubation with urease is permissible but citric/tungstic must be tipped in immediately before connecting reaction vessel to gas train, as this releases CO_2 and leaks may occur).

(2) Evacuate gas-line completely ($<$ .005mm Hg).

(3) Attach reaction vessel to gas-line.

(4) Remove excess H_2O from arms of reaction vessel with hairdrier (hot air).

(5) Freeze reaction vessel in CO_2-alcohol.

(6) Evacuate cold finger.

(7) Immerse cold finger in liquid N_2.

(8) CLOSE PUMP TAP.

(9) Turn tap to allow CO_2 from reaction vessel to cold finger.

(10) Keeping cold finger in liquid N_2, OPEN PUMP TAP and pump out any other gas from cold finger. Shut cold finger tap; immerse cold finger in water at room temperature.

(11) Open manometer tap.

(12) Surround manometer cold finger with liquid N_2. (Keep adding liquid N_2 until step (15) complete).

(13) Immerse reaction vessel cold finger in CO_2-alcohol. CLOSE PUMP TAP.

(14) Open cold finger tap to allow CO_2 into manometer.

(15) When Pirani gauge register $<$.005mm Hg close reaction vessel cold finger tap. OPEN PUMP TAP. When good vacuum registered, close manometer bulb tap. Bring Hg into top of bulb. De-freeze manometer bulb with warm water.

(16) Read pressure difference on manometer when manometer cold finger is at room temperature. Bring Hg down into flask.

(17) Attach scintillation tube (containing 2ml. phenylethylamine-methanol) to gas-line.

(18) Immerse scintillation tube in liquid N_2 and evacuate tube completely.

(19) CLOSE PUMP TAP.

(20) Open manometer tap to allow transfer of CO_2 to scintillator.

(21) When Pirani gauge measures .005mm Hg close scintillation tap and remove tube.

(22) Thaw scintillation tube slowly to prevent bubbling of phenylethylamine.

(23) Transfer to counting vial containing 8ml scintillation fluid. Screw on top of counting vial very firmly

METHOD FOR DETERMINING SA UREA CARBON.

(1) Take 1 part blood or plasma + 5 parts stable urea solution (1mg/ml) + 2.5 parts 10% Na Tungstate + 2.5 parts $^2/_3$N H_2SO_4. Mix by inversion and centrifuge.

(2) Take supernatant into round-bottom flask and evaporate to dryness.

(3) Re-dissolve in dist. H_2O ($\pm$ 1ml) + 0.5ml phosphate buffer. Bring pH to 7 with CO_2-free 1N NaOH. Put into small test tube ready for gas train.

BIBLIOGRAPHY.

1. CZERNY, A. and KELLER A. (1906)
"Des Kindes Ernährung, Ernährungsstörungen und Ernahrungstherapie," 1st ed., p. 62. F. Deuticke, Vienna.

2. CORREA, J.P. (1908) Rev. méd. Yucatan, 3, 86.

3. GUILLON, as quoted by NORMET, L. (1926) La "bouffissure d'Annam" Bull. Soc. Path. exot., 19, 207.

4. McCONNEL, R.E. (1918)
Uganda Ann. Med. San. Report. Appendix 2. Government Printer, Entebbe.

5. WILLIAMS, C.D. (1931)
Gold Coast Annual Report p. 93 Medical Department, Accra.

6. WILLIAMS, C.D. (1935)
Kwashiorkor: A nutritional disease of children associated with a maize diet. Lancet, 2, 1151.

7. TROWELL, H.C. (1932)
A clinical study of pneumonia among Africans in Nairobi. E. Afr. med. J., 9, 258.

8. GILLAN, R.U. (1934)
Investigation into certain cases of oedema occurring among Kikuyu children and adults. E. Afr. med. J., 11, 88.

9. STANNUS, H.S. (1934)
Nutritional disease of childhood associated with maize diet and pellagra. Arch. Dis. Childh., 9, 115.

10. LOEWENTHAL, J.A. (1935)
Letter. E. Afr. med. J., 12, 29.

11. SEQUIRA, J.H. (1936)
An early description of "Gillan's oedema" E. Afr. med. J., 13, 52.

12. BROCK, J.F. and AUTRET, M. (1952)
Kwashiorkor in Africa. World Health Organization Monograph Series No. 8, W.H.O., Geneva.

13. JELLIFFE, D.B. (1959)
Protein-calorie malnutrition in tropical pre-school children (a review of recent knowledge) J. Paediat., 54, 227.

14. TROWELL, H.C., DAVIES, J.N. and DEAN R.F.A. (1954)
Kwashiorkor, Edward Arnold, London.

15. SCHENDEL, H.E., HANSEN, J.D.L. and BROCK, J.F. (1962)
The biochemical assessment of protein nutritiona; status. 1. Marginal hypo-albuminaemia. 2. Serum Cholinesterase. S. Afr. J. Lab. Clin. Med., 8, 23.

16. WATERLOW. J.C. (1963)
The assessment of marginal protein malnutrition. Proc. Nutr. Soc., 22, 66.

17. HOFFENBERG, R., BLACK, E. and BROCK, J.F. (1966)
Albumin and gamma-globulin tracer studies in protein depletion states. J. Clin. Invest., 45, 143.

18. McFARLANE, A.S. (1963)
Measurement of synthesis rates of liver-produced plasma proteins. Biochem. J., 89, 277.

19. KOHMAN, E.A. (1920)
The experimental production of edema as related to protein deficiency. Amer. J. Physiol., 51, 378.

20. PLATT, B.S., HEARD, C.R.C. and STEWART, R.J.C. (1964)
In Mammalian Protein Metabolism. Ed. Munro, H.N. and Allison, J.B., Vol. 2, p. 445, Academic Press, New York and London.

21. BALFOUR, B.M. (1952)
Growth, nitrogen balance and histological picture of organs of rats fed on Gambian diets. Rept. 2nd Inter-African Conf. on Nutr., Gambia. p. 120.

22. WOODNOTT, J. (1961)
A simple technique for passing an oesophageal tube in a rat. J Animal Tech. Ass. 12, 59.

23. PLATT, B.S., HALDER, K. and DOELL, B.H. (1962)
Pathology of acute experimental protein malnutrition in the force-fed rat. Proc. Nutr. Soc., 21, vi.

24. NJAA, L.R. (1965)
Weight maintenance and protein intake of the young rat. Brit. J. Nutr. 19 443.

25. HALDER, K and PLATT, B.S. (1962)
The effects on the new-born rat of force-feeding carbohydrate for 3 days during pregnancy. Proc. Nutr. Soc., 21, XXXII.

26. BHUYAN, U.N., NAYAK, N.C., DEO, M.G. and RAMALINGASWAMI, V. (1965)
Effect of dietary protein on carbon tetrachloride induced hepatic fibrogenesis in albino rats. J. Lab. Invest., 14, 184.

27. BEST, G.H., HARTROFT, W.S., LUCAS, C.C. and RIDOUT, J.H. (1955)
Effects of dietary protein, lipotropic factors and re-alimentation on total hepatic lipids and their distribution. Brit. Med. J., 2, 194.

28. WIDDOWSON, E.M. and McCANCE, R.A. (1956)
The effects of chronic undernutrition and total starvation on growing adult rats. Brit. J. Nutr., 10, 363.

29. ROSE, W.C., OESTERLING M.G. and WOMAK, M. (1948)

Comparative growth on diets containing ten and nineteen amino acids with further observations upon the role of glutamic and aspartic acids. J. Biol. Chem., 176, 753.

30 SIDRANSKY, H. and FARBER, E. (1958)

Chemical pathology of acute amino acids deficiencies. 1. Morphologic changes in immature rats fed threonine, methionine or histidine-devoid diets. AMA. Arch. Path., 66 , 119.

31. SHILS, M.E., FRIEDLAND, I. and STEWART, W.B. (1954)

Rapid development of portal fatty liver in rats consuming various plant materials. Proc. Soc. Exp. Biol and Med., 87, 473.

32. MILLER, D.S. and PAYNE, P.R. (1961)

The effect of caloric intake on net dietary-protein value. Proc. Nutr. Soc., 20, xlviii.

33. MORRISON, A.B. and NARAYANA RAO, M. (1967)

Some relationships between proteins and calories. World Rev. Nutr. Diet. 7, 204.

34. DEO, M.G., SOOD, S.K. and RAMALINGASWAMI, V. (1965)

Experimental protein deficiency. AMA Arch. Path. 80, 14.

35. WIDDOWSON, E.M., DICKERSON, J.W.T., and McCANCE, R.A. (1960)

Severe undernutrition in growing and adult animals. Brit. J. Nutr. 14, 457.

36. BROCK, J.F. and HANSEN, J.D.L. (1965)

Body composition and appraise of nutriture in Symposia of the Society for the Study

of Human Biology, Vol. VII Human Body Composition: Approaches and Applications ed. J. Brozek. Pergamon Press, Oxford, Part II, p. 255.

37. TROWELL, H.C. (1947)
Tropical macrocytic anaemia and nutritional macrocytic anaemia. S. Afr. J. med. Sci., 12, 21.

38. KNOWLES, C.B. (1957)
Protein malnutrition in the pig. Proc. Nutr. Soc., 16, ix.

39. HEARD, C.R.C., PLATT, B.S. and STEWART, R.J.C. (1958)
The effects on pigs of a low protein diet with and without additional carbohydrate. Proc. Nutr. Soc. 17, xli.

40. AL RABII (1962)
As quoted by Platt B.S., Heard, C.R.C. and Stewart (1964) in Mammalian Protein Metabolism ed. Munro, H.N. and Allison J.B. Vol. 2, p 445, Academic Press, New York and London.

41. BROCK, J.F. (1961)
Recent Advances in Human Nutrition, Churchill, London.

42. WIEMER, H.E., (1961)
The effects of protein depletion and repletion on the concentration and distribution of serum proteins and protein bound carbohydrates of the adult rat. Ann. N.Y. Acad. Sci., 94, 225.

43. RAMALINGWASWAMI, V., VICKERY, A.L. Jnr, STANBURY, J.B. and HEGSTED, D.M. (1965)
Some effects of protein deficiency on the rat thyroid. Endoc. 77, 87.

44. McCANCE, R.A. and WIDDOWSON, E.M. (1966)
Protein deficiencies and calorie deficiecies. Lancet 2, 158.

45. FOLLIS, R.H. (1957)
A kwashiorkor-like syndrome observed in monkeys fed maize.
Proc. Soc. Exp. Biol. Med. 96, 523.

46. RAMALINGASWAMI, V. (1964)
Perspectives in malnutrition.
Nature 201, 546.

47. FRIEND, C.J., HEARD, C.R.C, PLATT, B.S., STEWART, R.J.C. and TURNER, M.R. (1961)
The effect of dietary protein deficiency on transport of vitamin A in the blood and its storage in the liver.
Brit. J. Nutr. 15, 231.

48. WESTALL, R.G., ROITMAN, E., de la PENA, C., RASMUSSEN, H., CRAVIOTO, J., GOMEZ, F., and HOLT, L.E. Jnr. (1958)
The plasma amino acids in malnutrition.
Arch. Dis. Childh., 33, 499.

49. HOLT, L.E., SNYDERMAN, S.E., NORTON, P.M., ROITMAN, E. and FRENCH, J. (1963)
The plasma aminogram in kwashiorkor.
Lancet 2, 1343.

50. ARROYAVE, G., WILSON D., de FUNES, C. and BEHAR, M. (1962)
The free amino acids in blood plasma of children with kwashiorkor and marasmus.
Amer. J. Clin. Nutr. 11, 517.

51. VIS, H. (1963) Aspects et mecanismes des hyperamino-acidurie de l'enfance. Brussels.

52. EDOZIEN, J.C. and OBASI, M.E. (1965)
Protein and amino acid metabolism in kwashiorkor. Clin. Sci. 29, 1.

53. SAUNDERS, S.J., TRUSWELL, A.S., BARBAZAT, G.O., WITTMAN, W. and HANSEN, J.D.L. (1967)
Plasma-free amino acids in protein calorie malnutrition. Reappraisal of its diagnostic value. Lancet 2, 795.

54. SQUIRES, B.T. (1962)
The buccal mucosa in pigs on a protein deficient diet with and without added carbohydrate. Proc. Nutr. Soc., 21,xxxiii

55. DEO, M.G. and RAMALINGASWAMI, V. (1965)
Reaction of the small intestine to induced malnutrition in rhesus monkeys - a study of cell population kinetics in the jejunum. Gastroenterol. 49, 150.

56. SIRSAT, As quoted by Ramalingaswami, V., (1964) Perspectives in malnutrition. Nature 201, 546.

57. VEGHELYI, P.V. and KEMÉNY, T.T. (1962)
In "The Exocrine Pancreas," Ciba Foundation Symposium p. 329. Churchill, London.

58. FRIEDMAN, S.M. and FRIEDMAN, C.L. (1946)
The effect of low protein diet on the structure of the pancreas. Canadian M. Assoc. J., 55, 15.

59. MONTGOMERY, M.L. ENTEMAN, C., CHAIKOFF, I.L. and NELSON, C. (1941)
The role of the external secretion of the pancreas in lipid metabolism. J. Biol. Chem. 137, 693.

60. LINDAN, O. (1952)
Experiments on dietary liver injury. Rept. 2nd Inter-African Conf. on Nutr. Gambia. p. 129.

61. SIDRANSKY, H. and CLARK, S. (1961)
Chemical Pathology of acute amino acid deficiencies. A.M.A. Arch. Pathol. 72, 468.

62. HALDER, K. Ph.D. Thesis, London University.

63. WANG, C.F., HEGSTEAD, D.M., LAPI, A, ZAMCHECK, N. and BLACK, M.B. (1949)
Progressive changes in liver composition, body fluids and liver cytology during protein depletion in the rat and the effect of choline upon these changes.
J. Lab. Clin. Med., 34, 953.

64. SVOBODA, D. and HIGGINSON, J. (1964)
Ultrastructural changes produced by protein and related deficiencies in the rat liver.
Am. J. Path., 45, 353.

65. BHUYAN, U.N., NAYAK, N.C., DEO, M.G. and RAMALINGASWAMI, V. (1965)
Effect of dietary protein on carbon tetrachloride induced hepatic fibrogenesis in albino rats.
J. Lab. Invest., 14, 184.

66. SHOENTAL, R. and MAGEE, P.N. (1957)
Chronic liver changes in rats after a single dose of lasiocarpine, a pyrrolizidine (senecio) alkaloid.
J. Path. Bact., 74, 305.

67. LEWIS, B., WITTMANN, W., KRUT, L.H., HANSEN, J.D.L., and BROCK, J.F. (1966)
Free fatty acid flux through plasma in protein malnutrition of infants.
Clin. Sci., 30, 371.

68. LOMBARDY, B. (1965)
Pathogenesis of fatty liver.
Fed. Proc. 24, 1200.

69. FARBER, E. (1966)
On the pathogenesis of fatty liver.
Gastroenterology 50, 137.

70. ADDIS, T., POO, L.J. and LEW, W. (1936)
The quantities of proteins lost by the various organs and tissues of the body during a fast. J. Biol. Chem., 115, 111.

71. WAINO, W., EICHEL, B, EICHEL, H., PERSON, P., ESTES, F. and ALLISON, J.B. (1953)
Oxidative enzymes of liver in protein depletion. J. Nutr. 49, 465.

72. SREBNICK, H.H. and NELSON, M.M. (1962)
Anterior pituitary function in male rats deprived of dietary protein. Endocrinology, 70, 723.

73. PIMSTONE, B., WITTMANN, W, HANSEN, J.D.L. and MURRAY, P. (1966)
Growth hormone and kwashiorkor. Lancet, 2, 779.

74. HAIST, R.E. (1944)
Factors affecting insulin content of pancreas. Physiol. Rev., 24, 409.

75. RUBINSTEIN, A. (1967)
Personal communication.

76. LEATHEM, J.H. (1958) Hormones and protein nutrition. Recent Progr. Hormone Res. 14, 141.

77. PLOTNIKOVA, Ju.I. (1959)
Probl. Endokrinol i Gormonoterap. 5, 34; see Abstr. World Med. (1960) 27, 680.

78. FERNANDEZ, A., SOBEL, C., and GOLDINBERG, H. (1966)
An improved method for determination of serum albumin and globulin. Clin. Chem. 12, 194.

79. SMITH, I. (1960) Chromatographic and electrophoretic techniques. Vol. II.

80. HAMILTON, P.B. (1963)
Ion exchange chromatography of amino acids. Anal. Chem. 35, 2055.

81. EFRON, M.S. (1966)
Proceedings of Technicon symposium on automation in analytical chemistry. New York. p. 637.

82. McCANCE, R.A. and WIDDOWSON, E.M. (1966)
Protein deficiencies and calorie deficiencies. Lancet 2, 158.

83. VITERI, F., BEHAR, M. and ARROYAVE, G.
Clinical Aspects of Protein Malnutrition. In Mammalian Protein Metabolism, ed. Munro, H.N. and Allison, J.B.(1964) Vol. 2, p. 523.

84. NEUBERGER, A. and RICHARDS, F.F.
Protein Biosynthesis in Mammalian Tissues. Part II. Studies on Turnover in the Whole Animal. In Mammalian Protein Metabolism, ed. Munro, H.N. and Allison, J.B. (1964) Vol. 1,

85. REEVE, E.B. (1964)
The measurement of the synthetic rates of liver synthesized proteins in animals. In Radioisotope techniques in the study of protein metabolism. International Atomic Energy Agency, Vienna.

86. VEAL, N. and VETTER, H. (1958)
Radioisotope techniques in clinical research and diagnosis. pub. Butterworths, London.

87. STERLING, K. (1951)
Turnover rate of serum albumin in man as measured by ^{131}I-tagged albumin. J. Clin. Invest., 30, 1228.

88. MATTHEWS, C.M.E. (1957)
The theory of tracer experiments with ^{131}I - labelled plasma proteins. Phys. in Med. Biol., 2, 36.

89. CAMPBELL, R.M., CUTHBERTSON, D.P., MATTHEWS, C.M.E. and McFARLANE, A.S. (1956)
Behaviour of ^{14}C and ^{131}I-labelled plasma proteins in the rat.
Int. J. Appl. Rad., 1, 66.

90. McFARLANE, A.S. (1964)
Metabolism of Plasma Proteins.
In Mammalian Protein Metabolism ed. Munro, H.N. and Allison, J.B.
Pub. Academic Press, New York.

91. REEVE, E.B. and ROBERTS, J.E. (1959)
The catabolism of plasma albumin in the rabbit. Its rate and regulation.
J. Gen. Physiol. 43, 445.

92. MATTHEWS, C.M.E. (1961)
Effects of plasmapheresis on albumin pools in rabbits. J. Clin. Invest.
40, 603.

93. STEINBOCK, H.L. and TARVER, H. (1954)
Plasma protein V. The effect of the protein content of the diet on turnover. J. Biol. Chem. 209, 127.

94. WU, H. and BISHOP, C.W. (1959)
Pattern of N^{15} excretion in man following administration of N^{15} labelled glycine.
J. Appl. Physiol., 14, 1.

95. HUMPHREY, J.H. and SULITZENU, B.D. (1957)
The use of ^{14}C - amino acids to study sites and rates of antibody synthesis in living hyper-immune rabbits.
Biochem. J. 68, 146.

96. SWICK, R.W. (1959) Proc. 2nd U.N. Intern. Conf. Peaceful Uses of Atomic Energy.
Geneva, 25, 115.

97. WEISMANN, S., TSCHUDY, D.A., BACCHUS, H. and EUBANKS, M. (1961)
Use of precursor product relationships in determining serum albumin half life.
J. Lab. Clin. Med., 57, 136.

98. REEVE, E.B., PEARSON, J.R. and MARTZ, D.C. (1963)
Plasma protein synthesis in the liver: method for measurement of albumin formation in vivo. Science, 139, 914.

99. DELLUVA, A.M. and WILSON, D.W. (1946)
A study with isotopic carbon of the assimilation of carbon dioxide in the rat. J. Biol. Chem. 166, 739.

100. SWICK, R.W. (1958) Measurement of protein turnover in rat liver. J. Biol. Chem. 231, 751.

101. McFARLANE, A.S., IRONS, L., KOJ, A. and REGOCCZI (1965)
The measurement of synthesis rates of albumin and fibrinogen in rabbits. Biochem. J., 95, 536.

102. PETERS, T. (1957)
Serum albumin precursors in cytoplasmic particles. J. Biol. Chem. 229, 659.

103. GORDON, A.H. and HUMPHREY, J.H. (1961)
Measurement of intracellular albumin in rat liver. Biochem. J. 78, 551.

104. GREEN, H. and ANKER, H.S. (1955)
Kinetics of amino acid incorporation into serum proteins. J. Gen. Physiol. 38, 283.

105. HOFFENBERG, R., KIRSCH, R.E. and BLACK, E. (1967)
Albumin metabolism in rabbits and rats on low protein diet. In "Radioisotope techniques in the study of protein metabolism." International Atomic Energy Agency, Vienna. (in press).

106. HALLER, A. (1754) Lectures in Physiology. Vol. II p.122. Innys and Richardson, London.

107. VOIT, C. (1866) Z. Biol, 2, 6.

108. MARTIN, C.J. and ROBINSON, R. (1922)
The minimum nitrogen expenditure of man and the biological value of different proteins for human nutrition. Biochem. J. 16, 407.

109. CAMPBELL, R.M. and KOSTERLITZ (1948)
The relationship between losses in labile liver cytoplasm and urinary nitrogen excretion.
Biochem. J., 43, 416.

110. SWANSON, P.P. (1959)
In "Protein and Amino Acid Nutrition". (A. A. Albanese, ed.) p. 195, Academic Press, New York.

111. McCOLLUM, E.V. and STEINBOCK, H. (1912)
Wisconsin Univ. Agr. Expt. Sta. Res. Bull. 21, 69.

112. CUTHBERTSON, D.P. and MUNRO, H.N. (1939)
The relationship of carbohydrate metabolism to protein metabolism.
Biochem. J. 33, 128.

113. GAEBLER, O.H. (1933)
Further studies of anterior pituitary extracts. J. Expt. Med., 57, 349.

114. KOCHAKIAN, C.D. and MURLIN, J.R. (1935)
Effect of male hormone on protein and energy metabolism in castrated dogs.
J. Nutr. 10, 437.

115. ALLISON, J.B. and WANNEMACHER, R.W. Jnr. (1965)
The concept and significance of labile and overall protein reserves of the body. Amer. J. Clin. Nutr., 16, 445.

116. SCHOENHEIMER, R. (1942)
"The Dynamic State of Body Constituents". Harvard Univ. Press, Cambridge, Massachusetts.

117. THOMPSON, R.C. and BALLOU, J.E. (1956)
Studies of metabolic turnover with tritium as a tracer: v. the predominantly non-dynamic state of body constituents in the rat.
J. Biol. Chem. 223, 795.

118. HOFFENBERG, R. (1964)

Applications of radioactive protein studies in nutritional disorders. In "Radioisotope techniques in the study of protein metabolism". International Atomic Energy Agency, Vienna.

119. COHEN, S., and HANSEN, J.D. L. (1962)

Metabolism of albumin and gamma-globulin in kwashiorkor. Clin. Sci., 23, 351.

120. PICOU, D. and WATERLOW, J.C. (1962)

The effect of malnutrition on the metabolism of plasma albumin. Clin. Sci., 22, 459.

121. PAYNE, P.R. and DONE, J. (1959)

The equilibration of water and of serum albumin labelled with tritium in the body fluids of pigs maintained on normal, low-protein and low-protein plus carbohydrate diets. Proc. Nutr. Soc. 18, 6.

122. GARROW, J.S. and WATERLOW, J.C. (1959)

Observations on Evans Blue as a tracer for human plasma albumin. Clin. Sci., 18, 35.

123. FREEMAN, T. and GORDON, A.H. (1964)

Metabolism of albumin and gamma-globulin in protein deficient rats. Clin. Sci., 26, 17.

124. YUILE, C.L., LUCAS, F.V., NEUBECKER, R.D., COCHRANE, C.G. and WHIPPLE, G.H. (1959)

Depletion of reserve protein from extravascular extracellular fluid. ^{14}C labelling of plasma proteins in dogs after plasmapheresis. J. Exp. Med., 109, 165.

125. WASSERMAN, K., JOSEPH, J.D. and MAYERSON, H.S. (1956)
Kinetics of vascular and extra-vascular protein exchange in unbled and bled dogs.
Amer. J. Physiol., 184, 175.

126. GITLIN, D., CRAVIOTO, J., FRENK, S., MONTANO, E.L., RAMOS-GLAVAN, R., GOMEZ, F. and JANEWAY, C.A. (1958)
Albumin metabolism in children with protein malnutrition.
J. Clin. Invest., 37, 682.

127. WATERLOW, J.C. (1962)
Protein malnutrition and albumin breakdown. Lancet, 2, 1279.

128. BAUMAN, A., ROTHSCHILD, M.A., YALLOW, R.S. and BERSON, S.A. (1955)
Distribution and metabolism of ^{131}I-labelled human albumin in congestive heart failure with and without proteinuria.
J. Clin. Invest., 34, 1359.

129. BERSON, S.A., YALLOW, R.S., SCHREIBER, S.S. and POST, J. (1953)
Tracer experiments with ^{131}I-labelled human serum albumin: distribution and degradation studies.
J. Clin. Invest. 32, 746.

130. FRIEDBERG, W. (1963) Relation between pool size and catabolism of albumin in the mouse.
Amer. J. Physiol., 204, 501.

131. ROTHSCHILD, M.A., ORATZ, M., WIMER, E. and SCHREIBER, S.S. (1961)
Studies on albumin synthesis; the effects of dextran and cortisone on albumin metabolism in rabbits studied with albumin ^{131}I.
J. Clin. Invest., 40, 545.

132. ROTHSCHILD, M.A., ORATZ, M., EVANS, C. and SCHREIBER, S.S. (1964)
Alterations in albumin metabolism after serum and albumin infusions. J. Clin. Invest., 43, 1874.

133. PURVES, L.R., AND HANSEN, J.D.L. (1962)
The nature of the hypoalbuminaemia of kwashiorkor. S. Afr. Med. J., 36, 1047.

134. WATERLOW, J.C., CRAVIOTO, J. and STEPHEN, J.C. (1960)
Protein malnutrition in man. Advances in Prot. Chem., 15, 131.

135. KEKWICK, R.A. and MACKAY, M.E. (1954)
Separation of protein fractions from human plasma with ether. M.R.C. of Great Britain special report series No. 286.

136. McFARLANE, A.S. (1958)
Efficient trace-labelling of proteins with iodine. Nature, 182, 53.

137. WOELLER, F.H. (1961)
Liquid scintillation counting of ^{14}C ^{2} with Phenethylamine. Analyt. Biochem., 2, 508.

138. MARSH, W.H., FINGERHUT, B. and MILLER, H. (1956)
Studies of the diacetyl reaction for determination of urea. Clin. Chem., 2, 291.

139. GORDON, A.H. and HUMPHREY, J.H. (1960)
Methods of measuring rates of synthesis of albumin by isolated perfused rat liver. Biochem. J., 75, 240.

140. RICHARDS, P., METCALFE-GIBSON, A., WARD, E.E., WRONG, O., and HOUGHTON, B.J. (1967)
Utilization of ammonia nitrogen for protein synthesis in man, and the effect of protein restriction and and anaemia. Lancet 2, 845.

141. JAMES, W.P.T., HAY, A.M. and WATERLOW, J.C. (1967)
Studies on albumin metabolism in infantile malnutrition. In "Radioisotope techniques in the study of protein metabolism.
International Atomic Energy Agency, Vienna, (in press).

142. REGOECZI, E., IRONS, L., KOJ., and McFARLANE, A.S. (1965)
Isotopic studies of urea metabolism in rabbits. Biochem. J. 95, 521.

143. MOORE, S. and STEIN, W.H. (1954)
Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins.
J. Biol. Chem. 211, 893-906.