BIOCHEMICAL STUDIES ON THE SERUM PROTEINS OF PATIENTS SUFFERING FROM KWASHIORKOR.

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RESUMÉ.

This thesis describes the results of certain biochemical investigations on the serum proteins of patients suffering from the protein depleted state, kwashiorkor.

The thesis is divided into two parts, A and B.

PART A.

The historical background and main biochemical findings are described with special emphasis on disturbances in protein metabolism.

The serum protein pattern in untreated kwashiorkor and the serial changes occurring during treatment were investigated by the starch gel electrophoresis technique of Smithies.

The main conclusions were:-

1. The reduction in serum albumin concentration is quantitatively the most striking observation in the untreated patient. Certain other fractions, however, notably β globulin C (transferrin) and the haptoglobins are also markedly depleted. γ globulin concentrations varied from case to case.

2. Dramatic changes in the electrophoretic pattern occur during treatment starting as early as the third day after initiation of treatment.

In uncomplicated cases, the increase in concentrations

of/...

of albumin, transferrin and haptoglobin fractions run parallel.

In some cases, especially those associated with a megaloblastic type of anaemia, dramatic variations in the haptoglobin concentrations occurred. It is suggested that these changes indicate a haemolytic process of the type described as defective erythropoiesis.

In some patients fractions, normally not encountered or present in abnormal concentrations, developed during treatment.

It is concluded that the changes in the serum protein pattern during protein repletion are complex but that the protein-synthetic mechanisms remain intact in the depleted state even in the presence of complicating factors such as severe infection.

PART B.

In the second part of this thesis (part B) the amino acid composition of serum albumin from kwashiorkor was compared with that of normal children.

Serum albumin from both normals and patients with kwashiorkor was prepared by three different methods.

The albumin hydrolysates were examined by the ion exchange chromatographic technique of Moore and Stein.

No significant difference between the amino acid composition of albumin from normals and from patients with kwashiorkor could be demonstrated, with the possible exception of the amide

ammonia content.

The validity of the results are discussed and possible mechanisms for inducing alterations in the primary structure of proteins are considered.

It is concluded that, in the protein depleted state, replacement of essential by non-essential amino acids within a protein molecule does not operate as a mechanism for conservation of the essential amino acids.

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PART A.

STARCH GEL ELECTROPHORETIC STUDIES ON THE SERUM PROTEINS OF PATIENTS SUFFERING FROM KWASHIORKOR.

CHAPTER I.

HISTORICAL BACKGROUND AND BIOCHEMICAL FEATURES.

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CHAPTER I.

INTRODUCTION:

Kwashiorkor or infantile malnutrition occurs in all parts of the world where through local customs or socio-economic conditions a diet deficient in protein and relatively high in carbohydrate is consumed. It is characterised by growth failure, oedema, skin and hair changes, various neurological disturbances and low serum proteins. Variations in the clinical manifestations are encountered in different parts of the world but the basic syndrome remains the same.

Marasmus is the term used to describe a condition of general undernutrition resulting from a diet poor in caloric value and in protein. However a sharp distinction between these two syndromes is impossible as they probably represent the two ends of a wide spectrum with many intermediate forms.

HISTORICAL BACKGROUND:

The term kwashiorkor meaning "deprived child" was introduced by Dr. Cecily Williams [1], then working at the Childrens Hospital, Accra. She had already published an account of the clinical features of the illness two years earlier [2]. The almost inevitably fatal outcome of the condition was appreciated by the local inhabitants. Isolated reports of syndromes described under

a/...

a variety of names but resembling kwashiorkor are to be found in the early literature, reviewed by Trowell et al [3].

In 1906 Czerny and Keller [4] described the clinical features of a condition to which they gave the name of "Mehlnährschaden" meaning damage by cereal flours. There is little doubt, from their discription of the illness and its response to treatment [5, 6], that it resembled kwashiorkor as we recognise it today. Guillon [7], in 1913 and later Normet [8] (1926) gave a detailed account of "Annam swelling". Early reports from Africa were those of Mc Conner [9] (Uganda) where it was referred to as "oedema disease" and affected both adults and infants. Jamot [10] made similar observations in French Equatorial Africa during the first world War (1914-1918). The condition usually followed a period of fighting, again affecting both adults and infants. Oedema of the skin, depigmentation of the hair and a fatty liver at autopsy were the main findings.

Therefore by 1920 kwashiorkor as we recognise it today had been described in some form or another in most parts of the world. However the common aetiological background was unrecognised. Initially, especially in Africa, Helminth infestations were regarded as a major cause [11]. Williams [2] appreciated the importance of an insufficient diet and in her report to the Medical Department had no hesitation in labelling the condition as a deficiency disease. Stannus [12], disagreed with this view and because of the skin lesions, insisted that the condition is one of infantile

pellagra. This pellagra controversy raged for a long period of time and Stannus' views were generally accepted. Trowell [13], in 1937 reviewed the literature from Africa and also considered the condition to be one of infantile pellagra. As a result of his publication, a gift of nicotinic acid was received from the United States but proved completely ineffective in a therapeutic trial. At about this time Dr. Williams [14], then in Malaya, realised that the dermatosis was not an essential part of the illness as it occurred there. Trowell [15] in a further review of the literature considered the possibility of a nutritional oedema. At this time the first serum protein estimations were performed and the low serum albumin concentrations noted. The possibility of a protein deficiency syndrome was now realised although the role of associated vitamin deficiencies was undetermined.

It is interesting to note that Normet [8] in 1926 and in a subsequent paper [16] had observed the low blood urea values and small daily urinary nitrogen excretion. He was convinced that the condition was one of protein deficiency and advocated the intravenous injection of glycine as a specific form of therapy.

During this early period up to 1940, several important publications appeared from Central America [17, 18, 19, 20]. The clinical features were essentially the same as those described from Africa and the far East. Cofino Ubico and Kleé [21] also considered the oedema to be related to the hypoproteinaemia and

described the altered albumin globulin ratios.

The period from 1940 onwards saw a gradual reorientation of thinking although the pellagra issue was still not settled. Altman [22] in 1948 and subsequently Brock and Autret [23], although recognising certain similarities between the skin lesions of pellagra and kwashiorkor, realised that these lesions were not an essential part of the picture confirming the observations of Williams [14].

By 1952, as a result of the survey by Brock and Autret [23], the relationship between diets lacking in first class protein and kwashiorkor, had been firmly established. The observation that kwashiorkor did not occur amongst pastoral tribes in Africa, where the children received milk, was highly significant. An additional facet was recognised in that the diets on which kwashiorkor developed usually had a low protein - high calorie ratio. Diets deficient in both lead to the condition of marasmus or general undernutrition.

Reports on morbid anatomical and histological features obtained from autopsies and biopsy specimens were published by several workers. Davies [24] recognised that in addition to the fatty liver, degenerative changes were present in the pancreas, salivary glands and intestinal mucosa. At about the same time Veghelye [25, 26] noted the correlation between pancreatic atrophy and decreased enzyme production.

From 1950 onwards the problem of kwashiorkor was investigated along more specialised lines with emphasis on the biochemical disturbances. An attempt will now be made to summarise briefly the main lines of investigation.

AETIOLOGICAL FACTORS:

DIET:

It is evident from the work of Gopalan [27, 28], in India, that by the age of six months, the intake of protein by the infant, entirely breast fed, is insufficient for optimal growth. If breast feeding is continued for periods up to a year without supplementation with first class protein, the infant may already be protein depleted on a weight per age basis. Should weaning therefore occur suddenly at this time and breast milk replaced by a diet containing largely carbohydrate, the stage is set for the development of kwashiorkor. The age of maximal incidence is therefore closely related to the duration of breast feeding [3, 29, 30].

That the disease is indeed one of protein deficiency and not some other factor present in breast milk was demonstrated by Brock et al [31]. These workers demonstrated that a cure could be initiated by giving purified casein or synthetic mixtures of amino-acids. Confirmatory evidence was supplied by Behar et al [30] using vegetable protein mixtures with or without amino-acid supplements.

SPECIFIC AMINO-ACID DEFICIENCIES OR DEFICIENCY OF TOTAL NITROGEN:

Although the typical kwashiorkor diet is relatively deficient in sulfur containing amino-acids [33], [34], and tryptophan[35], these amino-acids are still present in amounts exceeding the minimum requirements as determined by Rose [36]. Collis et al [59] demonstrated that at least in Nigeria, the difference in amino-acid intake was quantitative rather than qualitative. The total protein requirements exceeded the sum of those for essential amino-acids and normal growth can be restored by a supply of glycine or even urea [37]. This fact was already appreciated by Normet [8] as far back as 1926.

AMINO-ACID BALANCE:

The problem of amino-acid balance of the diet and its effect on protein requirements was reviewed by Harper and Kumta [38]. The efficiency with which a protein can be utilised as a source of amino-acids for tissue synthesis depends very largely upon how closely the relative proportions of essential amino-acids in the protein coincide with those required by the body. Therefore if the amino-acid composition of a particular protein differs markedly from the ideal, utilisation will be inefficient and optimal growth will only occur at a much higher intake of the particular protein or through supplementation of the diet with well balanced protein.

Amino-acid imbalances occur when the percentages of one

or more of the amino-acids in a diet is so low that a decrease in protein utilisation occurs in addition to a drop in food consumption or an increased need for one or more amino-acids becomes evident.

These effects can be demonstrated in growing animals by the addition of small quantities of certain amino-acids to the diet when the animals are on a low protein diet of good biological value. For instance when rats are fed a diet of 6% fibrin supplemented with an amino-acid mixture lacking histidine severe growth depression results which is corrected by the addition of histidine. It is possible that imbalances may occur in certain kwashiorkor producing diets. For instance in Central America children develop kwashiorkor in spite of an adequate intake of nitrogen and essential amino-acids apart from lysine [39]. The addition of lysine and tryptophan to a diet of corn meal causes a marked improvement in nitrogen retention [39, 40]. However this issue is by no means settled and some of the differences of opinion might be explained by the fact that the composition of the diet varies from country to country whereas the basic syndrome produced is remarkably similar.

BIOCHEMICAL ALTERATIONS IN KWASHIORKOR.

CHANGES IN FLUID VOLUMES:

Total body water expressed as a percentage of body

weight is increased in kwashiorkor. Values of 80% compared with 62% in normal children are usually found [41, 42]. Part of the increase in body water is due to an expansion of the extracellular fluid compartment. Studies in Mexico revealed a mean extracellular fluid volume in children with kwashiorkor of 400 ml./Kg. body weight as against 250 ml. in controls [43]. Similar observations were made on adult malnutrition [44]. In adults there is also an increase in intracellular H_2O [45], but the results for children are rather conflicting probably as a result of technical problems [46,47].

ELECTROLYTE DISTURBANCES.

SODIUM:

Serum sodium concentrations vary widely in the acute stage probably depending on the state of hydration [48, 49, 50]. There is however evidence of sodium retention during the oedematous stage of the disease, while large losses may occur in the diuretic phase [51].

POTASSIUM:

Low serum potassium concentrations are commonly encountered in kwashiorkor and may be responsible for some of the sudden deaths [52]. Total body potassium may be reduced to one third of normal as demonstrated in isotopic studies using (K^{42}) [47].

There/...

There is also evidence from studies on muscle that intracellular potassium is reduced with an increase in sodium [53]. This alteration in intracellular cation concentrations may have important implications as K^+ activates many intracellular enzymes which are inhibited by Na^+.

FAT METABOLISM.

Rather indirect estimates suggest that the total fat content of the body may be reduced to 5% as compared with 15% in normal infants. The fat content of the liver is usually increased and may account for 60% of the organs weight. The cause of the fatty liver has not been established but a lack of lipotrophic factors such as choline does not appear to be implicated [54].

PLASMA LIPIDS:

During the acute stage low levels of neutral fat, phospholipids and cholesterol with a low ratio of esterified to free cholesterol are found [55, 56]. Soon after treatment the levels increase with a marked elevation of neutral fat which subsequently returns to normal [55]. According to Mathew and Dean [57] this temporary increase is due to mobilisation (phanerosis) of the liver fat as a result of synthesis of lipoprotein complexes needed for transport of fat. This effect often manifests itself as

visible/...

visible lipaemia (personal observations).

CARBOHYDRATE METABOLISM.

Although fasting blood sugar levels are often low in kwashiorkor [58], this is not a constant finding and probably not an important cause of death. Slone et al [62] recently found average fasting blood sugar levels of 51 mg./100 ml. in these cases with a mild degree of glucose intolerance. These workers postulate that defective gluconeogenesis might be responsible. However this cannot be the sole factor as glycogen stores are usually well maintained which would suggest a defect in glycogenolysis in addition [60, 61].

PROTEIN AND AMINO-ACID DISTURBANCES.

In view of the aetiological considerations outlined before, it is only natural that protein and amino-acid metabolism should have been extensively investigated. The main observations have been dealt with in an extensive review by Waterlow et al [63].

CHANGES IN TOTAL BODY PROTEIN:

Although exact figures are difficult to obtain, there is no doubt a large deficit in total body protein when calculated from Total Body Solids as a percentage of body weight. To describe the pattern of protein depletion, Waterlow et al [63] introduced the

terms/...

TABLE I.

Nitrogen Content of Liver and Muscle in Malnourished Infants in Relation to DNA-Phosphorus (N/DNA-P).

Specimens.	N/DNA-P		
	A. Malnourished (mg/mg)	B. Controls (mg/mg)	Initial Nitrogen Content. (% of Normal.)
Autopsy			
Liver	81	110	73
Muscle	146	271	54
	A. Before treatment (mg/mg)	B. After treatment (mg/mg)	Initial Nitrogen Content. (% of Normal.)
Biopsy			
Liver	49	83	59
Muscle	237	343	68
Body weight (% of standard)	53	68	78

terms fixed and mobile body proteins. The former are proteins of connective tissue and brain while the latter are the intracellular proteins and plasma proteins.

When the nitrogen content of liver and muscle is expressed as a ratio to desoxyribonucleic acid (DNA) one can appreciate the magnitude of the deficit. Some observations along these lines are given in table I taken from Waterlow et al [63].

Creatinine/...

Creatinine excretion is a good index of muscle mass and will reflect indirectly changes in muscle mass in protein malnutrition [64]. Table II based on results of Standard et al [65] (kwashiorkor) and Stearn et al [66] (normals) indicate the changes occurring on treatment. Similar results were obtained by Arroyave et al [67].

PLASMA PROTEINS:

Being readily accessible, the serum proteins have been studied by a large number of workers. The main changes observed are a reduction in total protein concentration largely due to the low albumin levels. Globulin levels varied probably depending on the presence of co-existing infections.

Edozien [68] in a carefully conducted electrophoretic study in Ibadan made additional observations. In his series albumin and B globulin were the fractions most affected. He also points out that α 2 globulin fractions are significantly reduced when compared with normal controls of the same age group. The fall in α 2 and β globulin correlates well with low serum copper and iron binding capacities [69, 70].

The studies of Gitlin et al [71], Garrow and Waterlow [72] and Purves and Hansen [73] indicate that total exchangeable albumin is reduced by at least 50%. Some observations suggest a redistribution of the albumin pool with a contraction of the extravascular

compartment/...

TABLE II.

Daily Creatinine Output by Malnourished Babies at Intervals During Recovery, Compared with Normal Babies.

Subjects	Days after admission to hospital.	Creatinine output per 24 hr.	
		(mg/cm height)	(mg/kg weight)
Malnourished infants	Less than 20	0.79	9.4
	20-39	1.04	11.8
	40-59	1.48	14.3
	More than 60	1.86	15.0
Normal infants			
1 Year	-	-	13.5
2 Years	-	-	16.3

compartment [74, 75], but these findings could not be corroborated by other workers [71, 72, 73].

URINARY AND PLASMA AMINO-ACIDS.

URINARY AMINO-ACIDS:

An increased excretion of amino-acids in the urine of

patients/...

patients with kwashiorkor was found by several workers using both column chromatographic [76] and paper chromatographic methods[77,78,79].

Schendel et al [76] described a generalised amino-aciduria on admission and regarded this as of renal origin. Edozien et al[79] agreed with this view and also demonstrated an increased excretion of β aminoisobutyric acid and ethanolamine. Cheung [80] in an earlier study found no increase in excretion of total α amino nitrogen but noted abnormal patterns with high isoleucine to leucine and phenylalanine to tyrosine ratios.

PLASMA AMINO-ACIDS:

Plasma amino acid studies were also done by several workers [81, 82, 83, 79, 78]. In general there is a reduction in total plasma amino-acids affecting essential more than non-essential amino-acids. In a recent publication, Holt et al [84] described results obtained in a study of plasma amino-acids from patients with kwashiorkor from 9 different countries. Rather striking results were obtained in that the plasma aminogram in kwashiorkor was essentially the same in all nine countries irrespective of dietary variations and differed markedly from the normal. The reduction in essential amino-acids was confirmed but in addition, tyrosine and arginine levels were also well below normal. Patients were divided into four groups according to the degree of abnormality in the aminogram. This grading however did

not correlate with the clinical severity. Interesting theoretical considerations arise out of this study which will be referred to again.

CHANGES IN OVERALL NITROGEN METABOLISM.

NITROGEN ABSORPTION:

Nitrogen absorption in infants with protein malnutrition is only slightly less efficient than in normal babies when the source of protein is milk. In most studies 80-85% of ingested nitrogen was absorbed even in severely ill patients with diarrhoea [85, 86, 87]. When however dietary nitrogen was derived from certain vegetables only, such as corn and beans, nitrogen absorption was poor [81].

NITROGEN RETENTION:

Nitrogen retention was also highly efficient varying between 40-60% of ingested nitrogen [81, 49, 86], when cow's milk was the source of protein. At all levels of intake malnourished infants retain more nitrogen than normals [66], and as the nitrogen deficit is corrected, retention falls to normal values [86]. Even in infants who died, there was evidence of a positive nitrogen balance [86]. It is however important to bear in mind the observations of Allison [88] on protein depleted dogs, where, if protein depletion was continued for a long enough period an irreversible

stage was reached and the dogs could not retain nitrogen - with fatal results.

EFFECT OF CALORIE INTAKE ON NITROGEN UTILISATION.

If calorie intake is reduced to below normal requirements, nitrogen retention falls [89, 90]. However it also appears that the high calorie intake relative to nitrogen intake promotes specific changes such as the fatty liver and oedema. In marasmus where both calorie and nitrogen intake are low, these changes do not occur.

Supportive evidence comes from the experiments of Heard et al [91] who found that the addition of extra carbohydrate to a diet containing 5% protein, produced fatty livers, a fall in plasma albumin concentration and increased water content of the skin in weanling pigs. This observation might be related to the fact that in the fasting rat the feeding of carbohydrate increases the uptake of nitrogen by muscle but does not prevent losses of nitrogen from the liver [92].

PROTEIN TURNOVER STUDIES:

Initial studies by Gitlin et al [71] and Garrow and Waterlow [72] seemed to indicate that there was no difference in the turnover rate of albumin in the depleted state. In a subsequent investigation, using S^{35} methionine to study the halflife of total plasma proteins in dogs and human infants, Garrow [93]

demonstrated/...

demonstrated an increase in the halflife during the depleted state. These observations were confirmed by Yuile et al [94] in experiments with C^{14} labelled lysine in dogs. Conclusive evidence was provided by Purves and Hansen [73] in their studies with I^{131} labelled albumin on kwashiorkor. These workers demonstrated a doubling of the halflife during the acute stage. They could demonstrate no intestinal loss of protein by the technique of I^{131} labelled polyvinyl pyrrolidone and concluded that the reduced albumin concentrations were due to decreased synthesis of albumin.

There is experimental evidence to support this contention. Jeffay and Winzler [95] studied turnover rates of albumin, α 1, α 2, β and γ globulin in rats on different levels of protein intakes. The higher the protein intake the shorter the turnover rate of albumin whereas γ globulin turnover rates were not affected. The α and β globulin turnover rates were too short generally to be accurately evaluated.

REUTILISATION OF NITROGEN AND DISTRIBUTION OF PROTEIN SYNTHESIS.

The results obtained in the experiments by Yuile et al [94] and Garrow [93] could also be interpreted as indicating that there is more effective reutilisation of amino-acids in the depleted state.

Other evidence suggests that during the depleted state

the/...

the rates of protein synthesis at different sites in the body are altered with parenchymatous organs such as liver receiving priority over less vital ones such as skeletal muscle [96, 97, 98].

THE ANAEMIA OF KWASHIORKOR.

Anaemia is a common finding in kwashiorkor. The anaemia may be normochromic and normocytic [99], megaloblastic [100] or hypochromic [101]. Woodruff [102] considered the normocytic, normochromic variety to be due to protein deficiency per se. This view can however not be seriously entertained as response of the serum proteins to therapy is not accompanied by an equal rise in haemoglobin concentration.

A megaloblastic type of anaemia was found by Walt [100] in 15-20% of his cases. It is interesting to note that this form of anaemia is more common during the summer months. In his experience the severe anaemias are usually of this variety and these infants are prone to develop socalled "crisis" during which the haemoglobin concentrations may drop by 3-4 g.% in 24 hours. Treatment with folic acid rapidly corrects the megaloblastosis but the anaemia may not respond in the same way. MacIver and Back [103] in Jamaica found a much more dramatic response to folic acid but also drew attention to additional ascorbic acid deficiencies. Ascorbic acid plays an important part in folic acid metabolism and associated deficiencies of this vitamin may be responsible for the

sometimes poor response to folic acid.

Trowell and Simpkiss [101] report a good response to intramuscular iron in their cases but this has not been the experience of all workers.

It is evident therefore that the anaemia of kwashiorkor may have a complex aetiological background involving several haematinic principles in addition to the complicating factor of infection which so frequently is present.

ENZYME CHANGES IN KWASHIORKOR.

Enzyme changes in relation to nutritional status have recently been summarised by Waterlow [104]. Both serum enzymes, notably pseudo-cholinesterase and panreatic enzymes are decreased in the depleted state. However these enzyme levels return to normal rapidly on feeding. The dramatic increases in serum proteins and efficient nitrogen retention on refeeding, suggest that the decrease in enzyme activities is not a limiting factor in protein synthesis.

Certain changes in tissue enzymes have been described but the pattern is confusing, both in experimental animals and human beings. In general there appears to be little evidence of selective preservation of enzymes especially in the liver. However as pointed out by Lehninger [105] and Green [106], enzyme activity depends on structural integrity of the subcellular components,

TABLE III.

Activity of Some Enzymes in Biopsy Specimens from the Livers of Malnourished Infants Before and After Treatment.

Enzyme and Units	Before treatment	After treatment
I. Cholinesterase Q_{CO_2}	6.8	8.9
Dehydrogenases Q_{CO_2}		
Lactic	54	52
malic	237	173
glutamic	6.5	8.4
Transaminase Q_{CO_2}	299	280
Cytochrome reductase Q_{O_2}	38	31
Succinoxidase Q_{O_2}	21	27
II. Xanthine oxidase, µmoles/gm protein/hr	2.6	6.9
D-amino acid oxidase, µmoles/gm protein/hr	150	432
Glycollic acid oxidase, µmoles/gm protein/hr	471	506
DPNH-dehydrogenase, mmoles/gm protein/hr	18.5	15.4
Malic dehydrogenase, mmoles/gm protein/hr	116	106
Transaminase, mmoles/gm protein/hr	82	71
III. Catalase, ml O_2/mg N/hr	3.2	1.4
Cholinesterase units/mg wet weight/hr	0.06	0.3
Alkaline phosphatase µg p/mg wet weight/hr	4.13	2.12

mitochondria and microsomes and their relationship to cofactors. Therefore a certain irreversible stage may be reached when the functional integrity of the cell is seriously interfered with after prolonged protein depletion. Some liver enzyme changes are given in Table III taken from Waterlow et al[63] page 63.

CHAPTER II.

MOTIVATION

AND

OUTLINE OF PROJECT.

CHAPTER II.

MOTIVATION AND PROJECT.

It is evident from this brief outline of the biochemical disturbances in kwashiorkor that one is dealing with a complex syndrome. The importance of a deficient intake of first class dietary protein seems clearly established. It is also obvious that kwashiorkor is not an ideal model for the investigation of biochemical changes produced by pure protein depletion. No doubt the variations in clinical and biochemical manifestations encountered in different areas could be determined by many factors.

a. Exact composition of the diet:

The staple diet varies in different parts of the world and even in different parts of the same country. This dietary pattern is difficult to evaluate as even individual families may show certain preferences. Co-existing deficiencies of vitamins especially nicotinic acid and folic acid may affect the protein depleted infant adversely and may be more pronounced in some areas.

b. The role of infection:

The onset of the full blown syndrome of kwashiorkor is frequently precipitated by an infection developing in an infant already marginally depleted. In South Africa gastro-enteritis which is prevalent during summer is a potent cause and largely explains the seasonal incidence. In tropical countries parasitic

infestations/...

infestations might similarly alter the course of the disease.

c. Socio-economic factors:

Although these factors are not normally considered to fall within the realm of the biochemist, they may be of paramount importance in determining the time period between the onset of the illness and admission to hospital or medical attendance and initiation of therapy. These variations make assessment of the individual case extremely difficult as no reliable indices of the degree of protein depletion are available.

In spite of the complexity of the aetiological background it was felt that some useful information could be obtained from a more detailed study of the serum proteins as an example of the labile body proteins.

In most of the reported studies, the methods used were rather crude and possessed insufficient resolving power to define the detailed structure of a complex mixture of proteins such as found in blood serum. Serial studies were performed by a few workers but again the methods did not permit evaluation of changes in the finer fractions of the serum spectrum.

The development by Smithies [107] of the starch gel electrophoresis technique provided a method with high resolving power, allowing several samples to be examined under identical conditions. Although this technique suffers from the disadvantage

of not lending itself readily to quantitative evaluation, it appeared to us suitable for a comparative study of the serial changes occurring during treatment of kwashiorkor. We were particularly interested in the following problems:-

1. <u>The pattern of serum protein depletion in untreated kwashiorkor.</u>

The severe reduction in serum albumin is quantitatively the most striking change in untreated kwashiorkor [54, 68, 109, 110]. Changes in globulin fractions are less well defined. Hellyer and Went [111] and Edozien [68] recorded reduced β and α 2 globulin levels whereas Scrimshaw [110] noted an increase in α 2 globulin concentration. It is now recognised that each of the conventional globulin fractions consists of a large number of sub fractions, some of which have important carrier functions [112]. This aspect seemed to us important as little is known about the priority of any of these fractions in the protein depleted state.

2. <u>The order and rate at which newly formed proteins are produced during protein depletion;</u>

Certain studies along these lines have been made [111,113,114]. Usually determinations were performed at long intervals and day to day changes on treatment could not be observed. No information is therefore available as to the relative order of restoration of the serum protein pattern and its relationship to the turnover rates of

individual fractions.

3. <u>The time taken for an apparently normal pattern to be established</u>.

It is well known that nitrogen retention during the initial stages of treatment is higher in kwashiorkor than in normal infants. The retention gradually returns to normal levels on treatment but this period of depletion of total body protein may take as long as 100 days [73]. Most workers found a rapid increase in serum protein concentration on treatment. We do not however know whether this applies to all fractions or whether some fractions may remain reduced in concentration for long periods of time.

4. <u>The appearance of fractions not normally encountered during the course of the illness</u>.

Although there is no direct experimental evidence or observations on human material to support this suggestion, the electrophoretograms of El Gholmy et al [114] obtained during treatment showed a rather unusual α globulin pattern which returned to normal after recovery. These authors do not comment on these changes. It seemed to us important to obtain more evidence on this point in order to determine whether, during a state of rapid protein depletion, certain incomplete or abnormal fractions may be produced which could be detected by a sensitive technique.

5. <u>The effect of infection and specific therapy on these parameters.</u>

Although as outlined before nitrogen retention is efficient even in severely ill patients no specific information on serum protein patterns is available. Some of the children admitted in a collapsed state receive steroid therapy while others are treated with blood transfusions. The effect of these could be studied at the same time.

CHAPTER III.

STARCH GEL ELECTROPHORETIC STUDIES ON KWASHIORKOR.

METHODOLOGY AND CASE MATERIAL.

CHAPTER III.

STARCH GEL ELECTROPHORETIC STUDIES ON KWASHIORKOR.

METHODOLOGY.

INTRODUCTION:

The technique of starch gel electrophoresis was first described by Smithies in 1955 [107, 108]. This method comprises zone electrophoresis with a starch gel as the supporting medium and combines the convenience of protein detection by staining procedures, with high resolving power, in many cases superior to the moving boundary method of Tiselius [115]. Additional advantages are:-

a. Adsorption effects are minimal.
b. Boundary anomalies encountered in the moving boundary technique do not occur.
c. Only micro quantities of serum are required.
d. Convenient in that several samples can be examined in the same gel under identical conditions. Serial changes can therefore readily be detected.

GENERAL PRINCIPLE OF THE METHOD:

Soluble starch is produced by hydrolysing potato starch

under/...

under rigidly controlled conditions. A starch gel is then prepared by heating the desired amount of starch in a buffer of correct composition. The gel which forms is poured into a suitable tray and allowed to cool. Samples for analysis are introduced into vertical slits in the gel at right angles to its long axis. Electrical contact is made to the ends of the gel with filter paper wads, soaked in suitable buffer which dip into vessels containing the same solution. Filter paper bridges in turn connect these vessels to the electrode containing chambers. Current is passed through the gel for the desired period of time. The gel, after removal from the tray, is then sliced along its length in a horisontal plane and stained in a suitable dye.

Originally electrophoresis was conducted with the gel in a horizontal plane - fig. 1. This horizontal starch gel procedure suffered from one serious disadvantage. In order to prevent electrodecantation the sample had to be introduced into the slits absorbed onto inert supporting substances which lessened the resolving power of the technique. In 1959 Smithies [116] described an improved technique which enabled samples to be introduced without the aid of supporting substances. Electrodecantation was prevented by carrying out electrophoresis in a vertical plane.

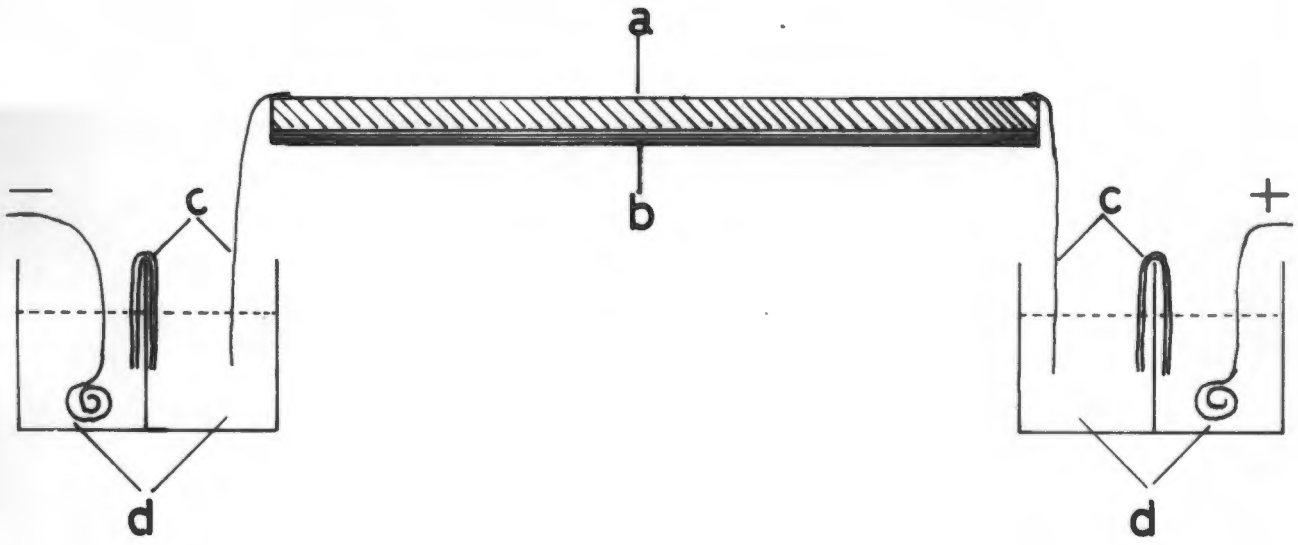
Methodology:

The method used was essentially as described by Smithies

with/...

FIG. 1

EXPERIMENTAL ARRANGEMENT OF HORIZONTAL STARCH GEL ELECTROPHORESIS APPARATUS.



a. STARCH GEL.

b SUPPORTING TRAY.

c FILTER PAPER CONNECTIONS.

d BUFFER SOLUTIONS.

with certain minor modifications.

Apparatus:

The different components of the vertical starch gel electrophoresis apparatus were made from perspex using the dimensions specified by Smithies. The parts with important dimensions are indicated in figs. 2 - 6.

Plastic Tray to contain the gel - fig. 2.

This consists of a flat plastic tray $\frac{1}{4}$" deep recessed at either end to produce a thick layer of gel which would trap hydrolytic end products and impurities in the gel in addition to providing a big surface for optimal electrical contact. The tray is fitted with removable endplates which can be secured by means of screws.

Lid with slot former - fig. 3.

This consists of a flat perspex sheet $\frac{1}{8}$" in thickness designed to fit the tray exactly. A slot former is inserted into the lid at right angles to the long axis of the tray 4" from the one end. The slot former was made out of perspex sheet 1/32". Each projecting slot former was 3/16" high and $\frac{1}{2}$" wide and separated by a gap of 3/16". It was so designed to leave a narrow unbroken strip of gel on either side. The slots should also not extend through the whole thickness of the gel.

Slicing Tray for cutting the gel - fig. 4.

The slicing tray consists of a perspex tray $\frac{1}{8}$" deep and slightly narrower than the gel tray to ensure a tight fit of the removed gel.

Cutting Device - fig. 5.

Smithies [116] recommends the use of a dermatome knife for cutting the gel in half. In our experience gels are easily damaged by this procedure as the gel tends to lift out of the cutting tray during the process causing irregularities of the cut surface. A cutting device illustrated in fig. 5 and consisting of a length of spring-steel wire 5/1000" in diameter supported in a suitable holder of perspex by means of two screws whereby the tension can be adjusted gives uniformly satisfactory cut surfaces.

Electrode/...

FIG. 2.

GEL TRAY WITH REMOVABLE ENDPLATES.

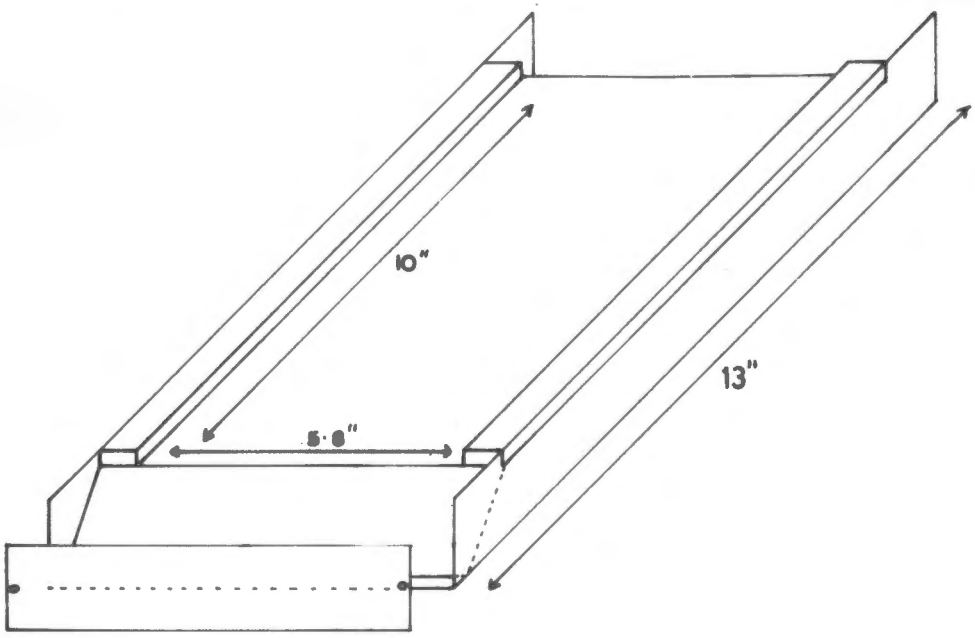


FIG. 3.

LID WITH SLOT FORMER

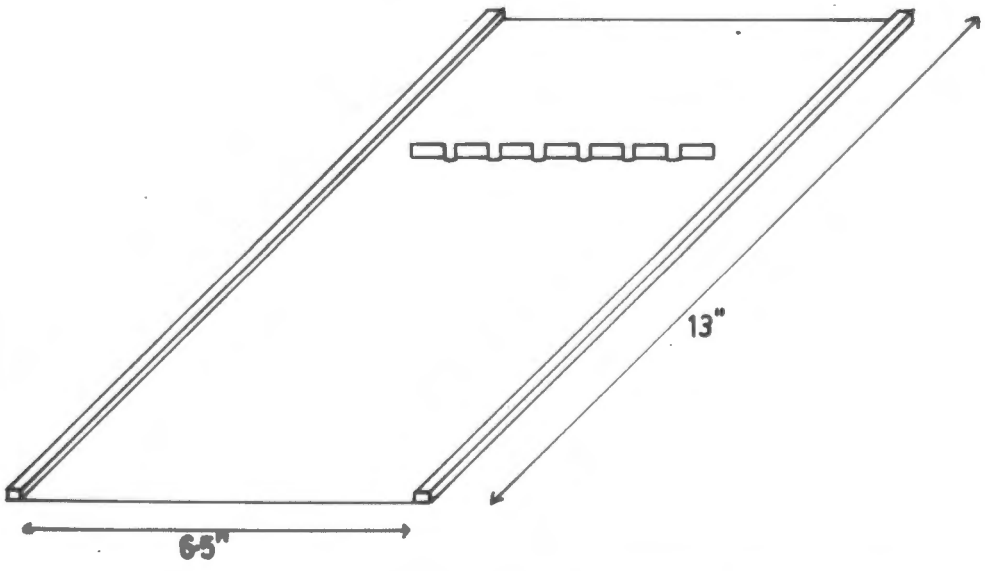


FIG. 4

TRAY FOR CUTTING GEL.

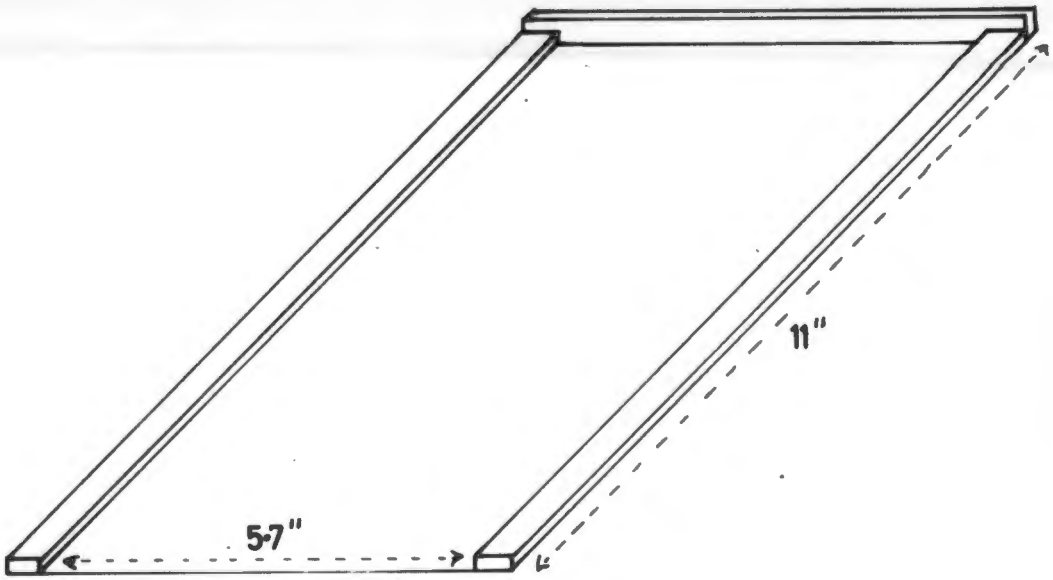


FIG. 5.

DEVICE USED FOR SLICING GEL.

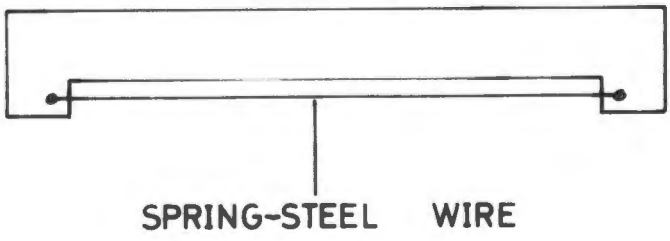
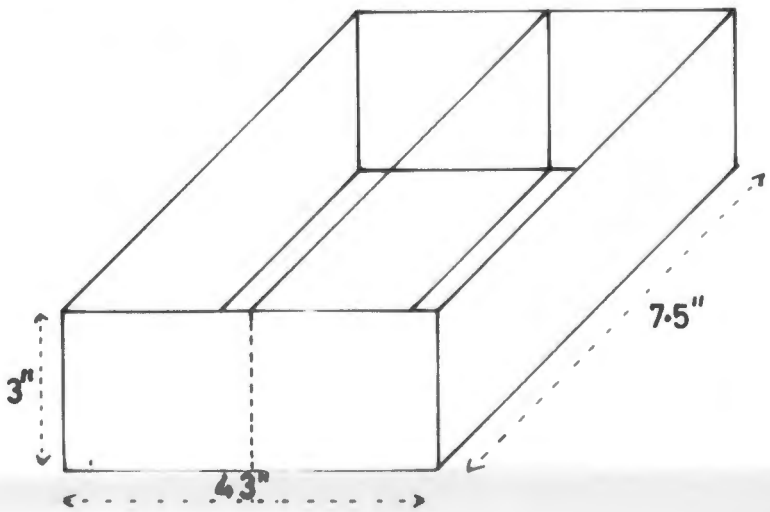


FIG. 6.

VESSELS FOR BUFFER SOLUTIONS AND ELECTRODES



Electrode vessels - fig. 6.

These had the dimensions indicated in the figure.

Electrodes:

Reversible Ag/AgCl electrodes were used to minimise pH changes in the buffer during electrophoresis. These were prepared from silver wire 1 millimeter in diameter by electrolysis in a solution containing 0.5 M. NaCl and 0.5 M. HCl for 24 hours at 40 m.a. Each electrode was 20 feet in length and rolled into a coil.

The gel was prepared in a round bottom flask of 2 litre capacity fitted with a B34 ground glass joint. A vacuum could be applied by means of suitable connections to a vacuum pump.

REAGENTS.

Stock buffer for preparation of the gel.

A stock buffer was prepared to contain 0.5 M. H_3BO_3 and 0.2 M. NaOH per litre keeping the ratio H_3BO_3 : NaOH at 10 : 3/4 v/v 1/2 This buffer is diluted to the desired concentration for the preparation of each gel.

Bridge Buffer Solution.

The buffer used in the electrode compartments were prepared to contain 0.3 M H_3BO_3 and 0.06 M NaOH per litre with a final pH of 8.1 $\pm$ 0.05.

NaCl 10% W/V.

For use in the electrode vessel housing the anode.

Hydrolysed Starch.

"Starch Hydrolysed" was obtained from the Connaught Laboratories, Toronto, Canada.

PROCEDURE:

Before commencing the preparation of the gel, the gel tray is assembled by screwing the end plates into position. The under-surface of the lid is lightly smeared with mineral oil except for the slot former and put in an oven at 65°.

The required amount of starch is transferred to the flask followed by the addition of the specified quantity of suitably diluted buffer. The optimal starch and buffer concentrations are given for each batch of starch by the manufacturers. 550 ml. of buffer per gel were used in our apparatus.

After proper mixing of the starch-buffer mixture, the contents of the flask are heated over an open flame with constant swirling until just short of boiling when a homogeneous almost completely transparent solution is obtained. Negative pressure is applied through proper connections to a suction pump and the contents of the flask allowed to boil vigorously for 1-2 minutes. The deaerated gel is then transferred to the gel tray filling the latter completely. The preheated cover is carefully lowered into position taking care not to trap any air bubbles between the cover and the surface of the gel. The lid is kept in position by means of weights on the corners and the gel allowed to cool to room temperature.

INSERTION OF THE SAMPLES:

The lid should be removed with great care as the small slots in the gel are easily damaged. As advised by Smithies [116], the lid should first be gently freed from the gel on both sides of the slots. By supporting both hands on the underlying bench the cover can then be removed by steady upward pressure avoiding sudden jerky movements.

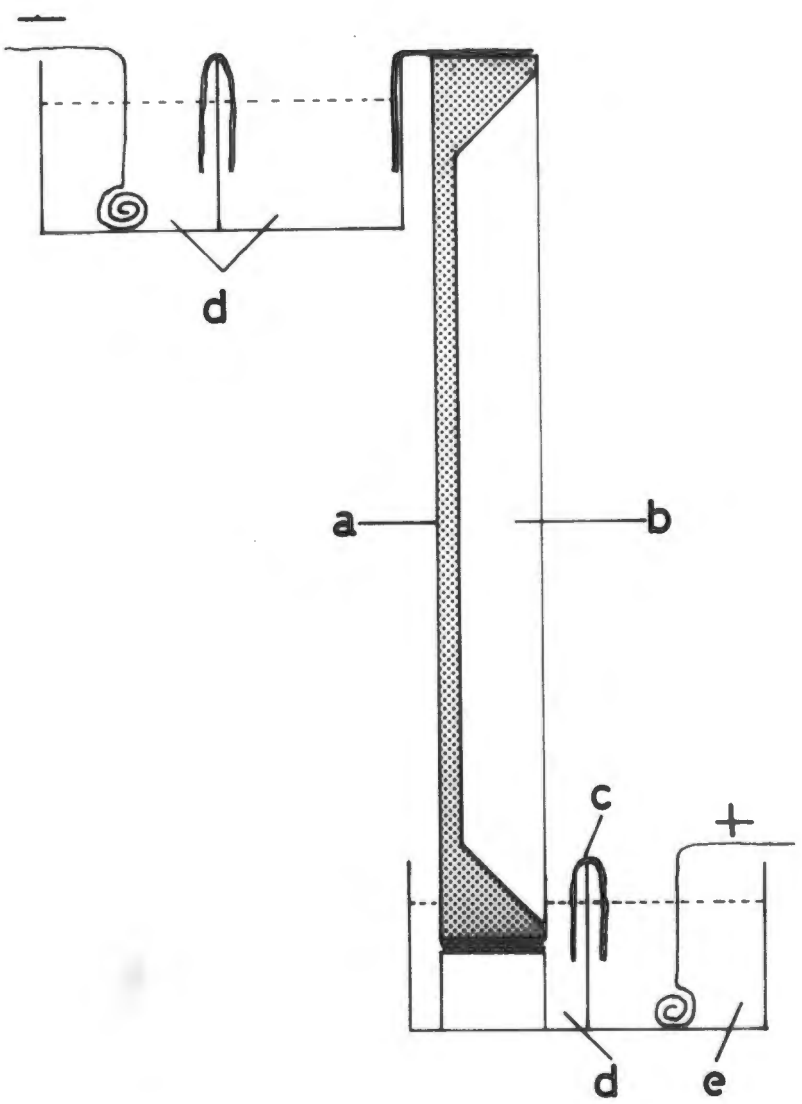
The exposed surface of the gel is completely covered with Parafilm (a waterproof plastic material obtained from Lindsay and Williams, London) except for an area half an inch on either side of the slots.

The samples are introduced into the slots with a finely drawn out Pasteur pipette taking care not to damage the gel. The slots should be filled completely so that no air bubbles remain trapped. Admixture of adjacent samples is avoided by the ridges

of/...

FIG. 7.

EXPERIMENTAL ARRANGEMENT OF VERTICAL STARCH GEL ELECTROPHORESIS APPARATUS.



a STARCH GEL

b SUPPORTING TRAY

c FILTER PAPER CONNECTIONS

d BORATE BUFFER SOLUTION

e NaCL 10% W/V

of gel formed between the slots by the circular saw cuts in the lid. The samples and surrounding exposed gel are then covered by melted petroleum jelly at approximately 45^{0} allowing the layer of jelly to overlap the edges of parafilm sheet. In this way the entire surface of the gel is effectively sealed off. After removal of the end plates the apparatus is mounted vertically as shown in fig. 7.

POWER SUPPLY AND ELECTRICAL CONNECTIONS:

Electrophoresis was continued for 16-18 hours at constant voltage using a potential gradient of 4-5 volts/cm. length of the gel. This could be achieved at a current of 13-14 m.a. which increased to 16 m.a. as the gel temperature rose slightly during the experiment.

CUTTING AND STAINING OF THE GEL:

At the completion of the electrophoretic run the layer of petroleum jelly is gently scraped away with a spatula. The parafilm is easily peeled off leaving the exposed gel surface. The ends of the gel are trimmed square marking one side of the gel for subsequent identification of the samples. The gel is bisected through the line of the slots to avoid tearing of the gel at this point during removal and slicing.

The two halfs of the gel are then sliced individually in a horizontal plane along the long axis.

Staining is performed for 2 minutes in a saturated solution of Amido Schwarz (Mercks) in Methanol : dist H_2O : Glacial acetic acid 50 : 50 : 10 v/v/v. After staining the gel is decolourised for 6-8 hours in a several changes of solvent of similar composition.

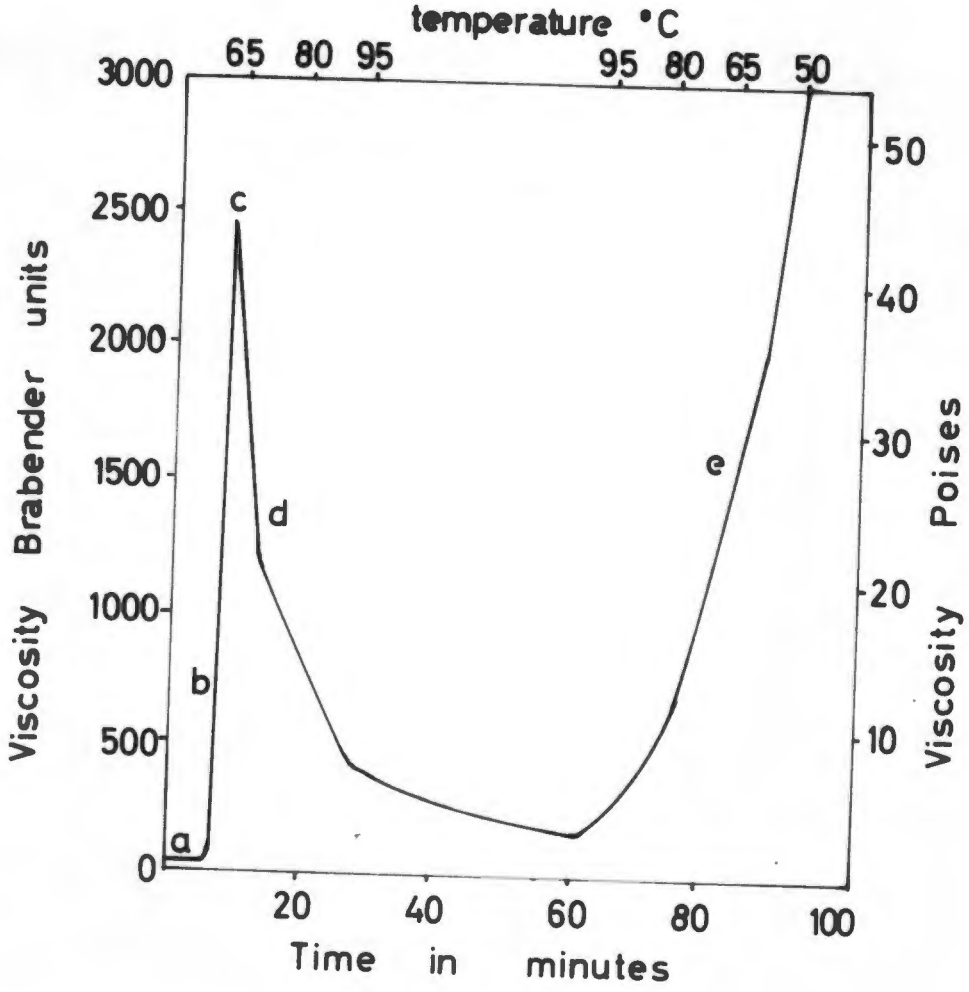
PHOTOGRAPHY:

Stained gels were usually photographed as soon as possible after decolorisation but may be stored in solvent for long periods without deterioration in quality. Photographs were taken on Kodak 35 mm. Microphile film with tungsten lamp illumination. No filters were used. Development was carried out in Kodak D 76 developer diluted 1 : 1 for 6 minutes. Excessive

contrast/...

FIG. 8

CHANGES IN VISCOSITY DURING THE PREPARATION OF A BORATE BUFFER STARCH GEL.



a Low initial viscosity.

b Sudden increase at "gel point".

c Maximum viscosity.

d Fall in viscosity on continued heating.

e Viscosity increase when gel sets.

contrast should be avoided as printing difficulties may arise. Colour photographs were taken on Kodachrome reversal daylight film by available light in the laboratory.

PROBLEMS ENCOUNTERED WITH THE TECHNIQUE.

1. Excessively high viscosity of the gels.

When gels are not properly prepared, they may become extremely viscous, causing serious difficulties during deaeration and subsequent pouring of the gel. As Smithies [117] points out this complication is usually the result of insufficient heating of the gel. Viscosity measurements [117] during the preparation of gels indicate that at a certain temperature an optimal low viscosity is reached and heating should continue until this stage (fig. 8). From practical experience this point is reached when the gel becomes uniformly transparent just before actual boiling.

2. Poor Resolution:

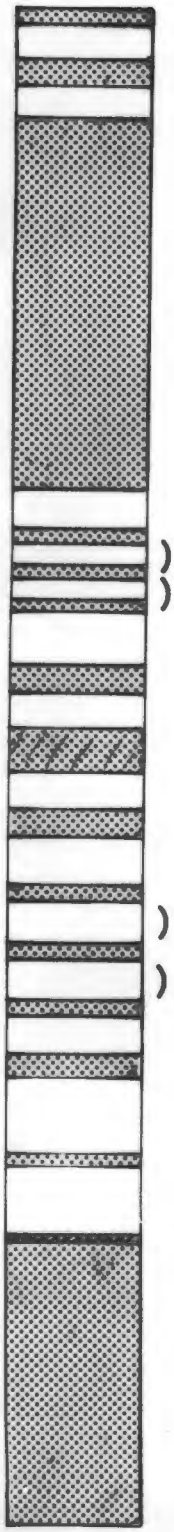
The proper starch concentration and molarity of the gel buffer should be established by each worker although guidance is given by the manufacturer for each batch of starch.

The starch concentration is selected at which the migration distance of B globulin C is approximately 2.5 times that of the slow α 2 fraction. For identification of the fractions see fig. 9. Keeping the starch concentration constant, the molarity of the borate buffer is varied until the front and back of the

albumin/...

FIG. 9.

KEY TO THE STARCH GEL ELECTROPHORETOGRAM OF NORMAL HUMAN SERUM PROTEINS



UNIDENTIFIED COMPONENT
OROSOMUCOID

ALBUMIN

POST ALBUMINS

α_2 GLOBULIN D + HAPTOGLOBIN 1-1

β GLOBULIN C (TRANSFERRIN)

$\alpha_2\beta$ GLOBULIN A

HAPTOGLOBINS 2-I AND 2-2

Sα_2 MACROGLOBULIN

β LIPOPROTEIN (T COMPONENT)

SITE OF APPLICATION OF SERUM

γ GLOBULIN

albumin zone are equally sharp. Under these conditions optimal resolution can be expected [117].

Distorted Patterns:

Distorted patterns are produced most commonly as a result of the following errors:-

(a) Trapping of air bubbles in the sample slots.

This may occur when air bubbles are introduced at the time of insertion of the sample. The same effect will also be produced when the sample slots are not completely sealed. The effect produced is demonstrated in fig. 10.

(b) Seepage of buffer underneath the covering layer of parafilm.

The surface of the gel should be covered efficiently to prevent buffer from the filter paper connections to the top of the gel from running in between the gel and the covering layer. This will produce uneven voltage potentials between the surface of the gel and the deeper layers causing distortion of the pattern at this site (fig. 11).

INTERPRETATION OF THE STARCH GEL ELECTROPHORETOGRAM.

As the starch gel technique resolves the serum protein spectrum into many more components than usually demonstrated by conventional forms of zone electrophoresis, the nomenclature used to define these fractions had to be revised. Fig. 12 shows a

starch/...

FIG. 10

Distorted gel pattern produced by air bubble in sample slot.

(Sample No. 7)

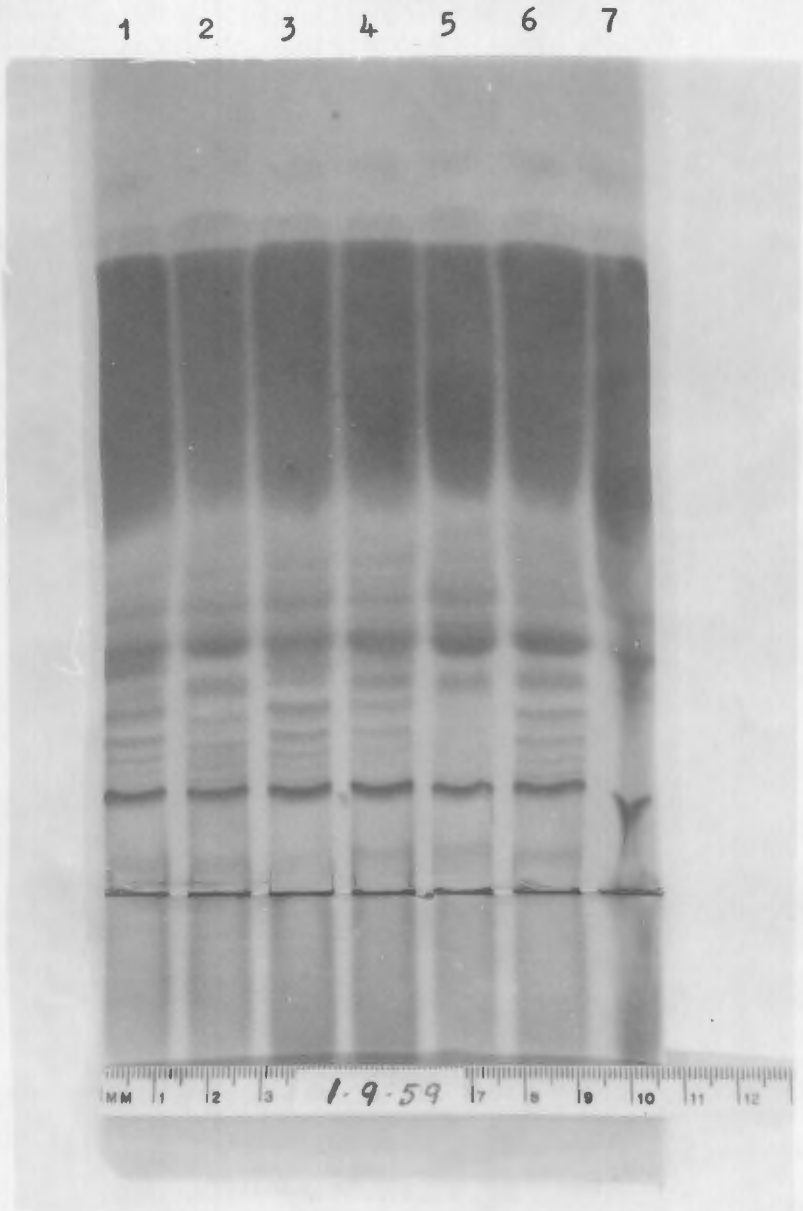
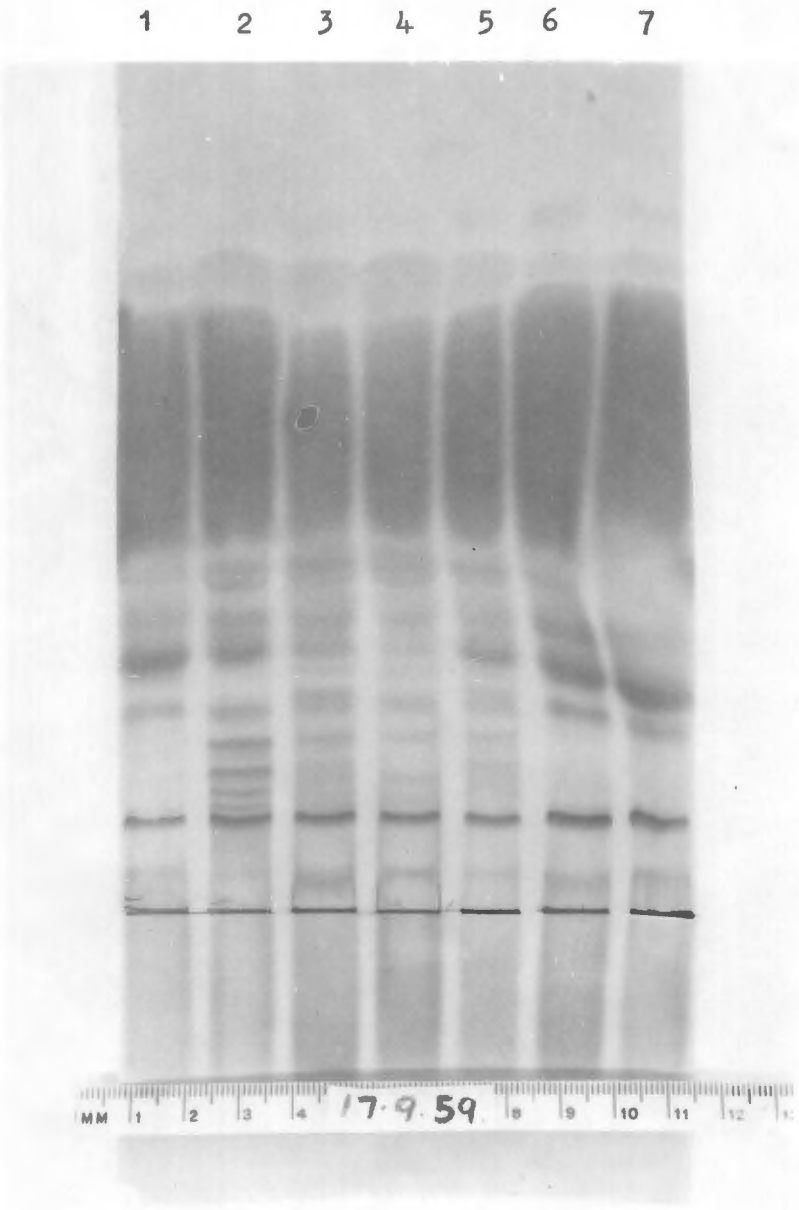


FIG. 11.

Distorted gel pattern produced by seepage of buffer underneath the covering layer of parafilm.

(Samples Nos. 6 and 7)



starch gel electrophoretogram of two normal children 1-3 and 4-7 with next to it a key to the identification of the fractions based on the two dimensional experiments of Poulik and Smithies[112]. It is evident that certain differences exist between the patterns from the two children especially in the post albumin zone, the haptoglobin sone and the fraction labelled α 2 B globulin A. By means of this technique genetically determined differences between the serum proteins of individuals can be detected.

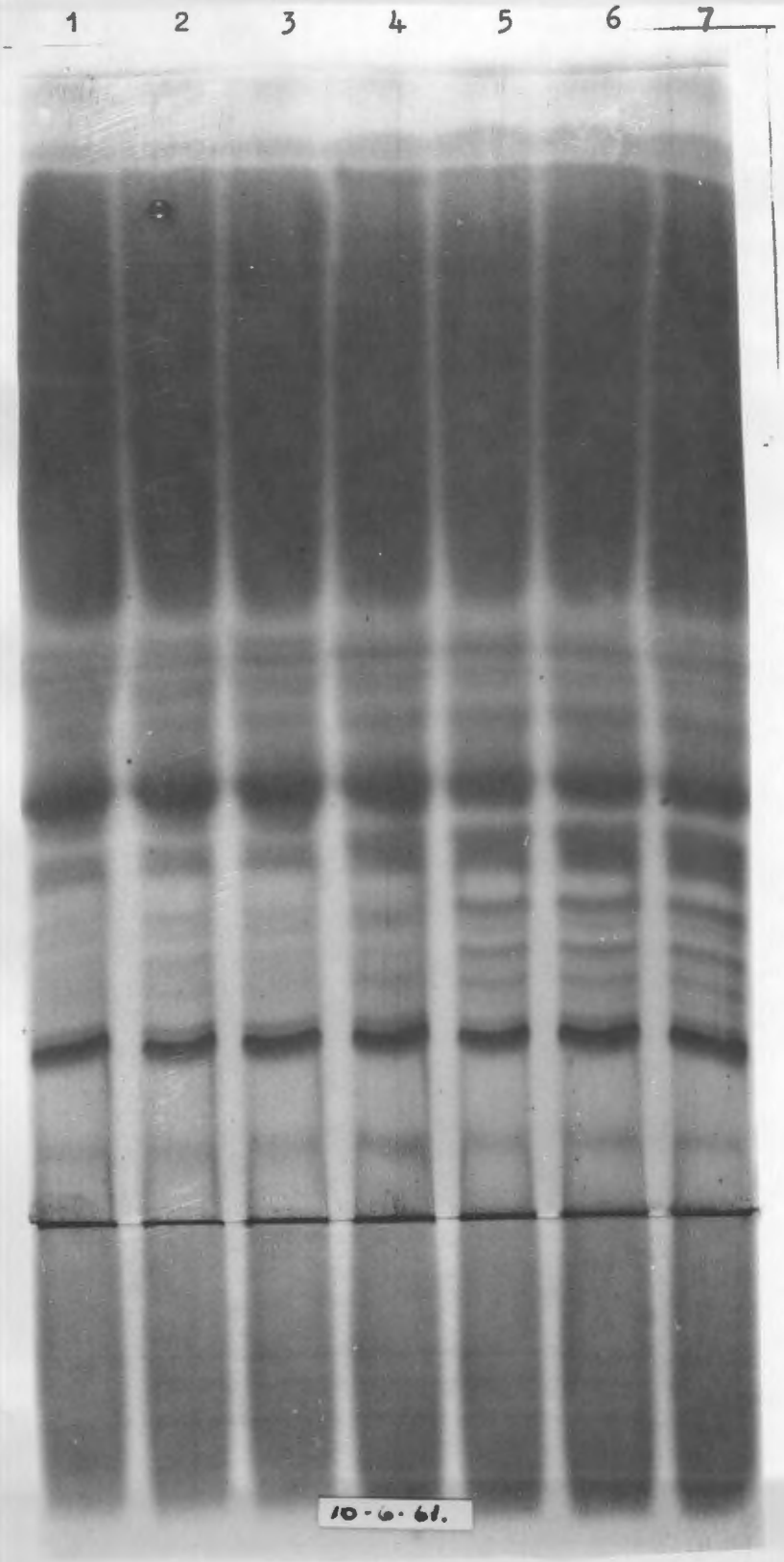
Such differences have now been demonstrated in the haptoglobins [108], transferrins [118], postalbumins [116], and caeruloplasmin [119, 120]. In interpreting the electrophoretogram these genetic differences must be considered and it is therefore difficult to define a "normal pattern" for a particular person or to describe deviations from the normal unless complete genetic typing is performed. The biological significance of some of these minor fractions have not been determined and the significance of changes cannot be evaluated fully from a functional point of view.

QUANTITATIVE EVALUATION OF STARCH GEL ELECTROPHORETOGRAMS.

Although this technique has the advantage of high resolving power, quantitative evaluation of the separated fractions presents serious problems. The principles of certain procedures which have been used will be briefly outlined.

FIG. 12.

Starch gel electrophoresis diagrams of the serum proteins from two normal children, (Samples 1 - 3 and 4 - 7).



Albumin

Post Albumins

Transferrin

Haptoglobins

α_2 Globulin

γ Globulin

1. Quantitative measurement after elution of the protein from serial sections of the gel.

Protein can be eluted by repeated freezing and thawing of the unstained gel [108]. The protein content in serial sections of the gel can be measured by a suitable method. This however becomes impractical for technical reasons when large numbers of samples have to be analysed. In addition recoveries are not quantitative.

2. Densitometric Scanning of the stained gel by transmitted light.

Starch gel strips can be made transparent by immersing the stained strip in boiling acetic acid 10% v/v for 30 seconds. Photometric evaluations can be performed in a suitable apparatus by supporting the gel between two sheets of thin "plexiglass" [121].

3. Densitometric Scanning of a photograph of the stained gel.

A tracing of the stained gel can also be obtained through densitometric recording of the pattern by scanning of the positive image obtained from a contact print of the original negative made on photographic film [122]. This technique has been used succesfully in agar gel electrophoresis where the pattern is less complex.

The disadvantage of all these procedures, apart from being time consuming, is that a highly complex record is obtained which becomes extremely difficult to interpret. Minor variations in day to day technique may affect the tracing and as quantitation depends

on/...

on measuring the areas under the peaks for each fraction, absolute quantitative measurement is still not possible.

It was therefore decided to evaluate the serial changes by visual inspection of the stained gels. Where possible, consecutive specimens from the same patient were examined in the same gel whereby day to day changes could readily be observed. An arbitrary system of grading whereby concentrations of the fractions are recorded by numbers e.g. 0-4 would be meaningless as the absolute concentrations at zero time are unknown.

Although precise quantitative measurements could therefore not be made, changes in concentrations are readily detected in serial studies and it was felt that most of the information sought could be obtained in this way.

CASE MATERIAL.

Case material was obtained from patients attending the local childrens hospital. Patients were assessed clinically and graded according to severity as mild, moderate and severe by Dr. P.M. Smythe, fulltime senior paediatrician to the hospital. The main criteria used for clinical evaluation were:-

1. Oedema.
2. Skin and hair changes.
3. Muscle tone and wasting.

4./...

4. Mental state.
5. The presence of jaundice which was regarded as a grave prognestic sign.
6. The presence and severity of complications such as infections.

Muscle tone is regarded as an important index. If a child is unable to sit on its own the condition is severe.

Another serious finding is the presence of extensive "weeping" skin lesions as this poses problems with regard to fluid and electrolyte losses.

As a result of shortage of beds all patients could not be admitted to hospital. A high percentage of patients with clinically obvious kwashiorkor is treated as outpatients. A number of specimens from outpatients were examined initially but follow up studies in these cases were almost impossible. It was also realised that the examination of a single specimen of serum yielded very little information.

Most of the cases studied were therefore inpatients and usually severely ill children often with complications.

CONTROLS:

In view of the genetic differences already mentioned, the selection of proper controls presented certain difficulties as comparison between normals and patients with kwashiorkor are not entirely valid unless full genetic typing of each patients' serum

is performed. Samples of blood were however collected from normal infants in the same age group admitted for elective surgery and from patients in convalescent homes recovering from attacks of acute poliomyelitis. Controls from adult normals were obtained from laboratory staff and from patients admitted to a general hospital for elective surgery. In addition sera from a number of patients suffering from diseases associated with altered serum protein patterns such as cirrhosis of the liver and the nephrotic syndrome were examined.

These controls were studied to exclude major differences between adults and children or distinctive abnormalities in kwashiorkor as compared to normal. It was soon realised that changing patterns or deviations from the normal were best evaluated by using the recovered phase electrophoretic pattern as the normal for each patient. In so doing each patient acted as its own control.

MANAGEMENT OF PATIENTS ADMITTED TO HOSPITAL.

As a general regime, each child was given half strength Darrow's Solution and 2.5% glucose by mouth for 24 hours, followed by half cream skimmed milk for 2 days; 2.5 oz. of each of these foods being given daily per lb. of body weight. A mixed diet was given from the 10th day. Vitamins and potassium supplements were added and broad spectrum antibiotics to combat infections. Some

children received blood transfusions, and in a few children cortisone was given to counteract a severe state of collapse. The effect of intravenous albumin was studied in three children.

As a routine procedure blood cultures, stool cultures and tuberculin reactions were performed on all children. Where indicated X-ray examination of the lungs was performed.

COLLECTION OF SPECIMENS:

On the day of admission or first attendance to the Outpatient Department, a specimen of venous blood was collected from which an aliquot was taken for routine biochemical analysis and blood culture. The serum of the remainder of the specimen was immediately separated by centrifugation and stored at 4^{o} C until it could be studied. Subsequent specimens were collected daily or on alternate days from heel or fingerpricks until no further changes in the electrophoretic patterns were evident. All samples were examined spectroscopically for haemolysis which was recorded. Grossly haemolysed samples were discarded. In three cases oedema fluid from the foot was collected for investigation.

Albumin-globulin ratios could not be determined on the small samples of blood obtained from finger or heel pricks but where possible total protein concentrations were measured by the biuret method [123].

CHAPTER IV.

RESULTS OF STARCH GEL ELECTROPHORETIC STUDIES ON PATIENTS WITH KWASHIORKOR.

TABLE IV.

Summary of main clinical and biochemical findings in cases of kwashiorkor studied by starch gel electrophoresis.

Patient	Age Years	Race	Sex	Severity	Infection Stool culture. Blood culture. Tuberculin.	Hb g/100 ml.	Serum Chemistry. (mgs/100m) Urea	(g./100 ml) Total Protein	Albumin	Globulin	Remarks.
H.A.	$1\frac{10}{12}$	C	F	Mild	Cultures -ve	10	7.3	3.5	2.0	1.5	Uncomplicated recovery.
M.M.				"	" "		6.8	4.3	2.8	1.5	Uneventful recovery. Received I.V. Albumin.
E.D.	$\frac{12}{12}$	C	M	Moderate	" "			3.28	1.56	1.72	Uneventful recovery.
M.B.	$1\frac{4}{12}$	C	F	"	" "	10		3.7	1.7	2.0	Stormy clinical course. Persistant oedema. Received cortisone to combat state of collapse.
T.W.	$1\frac{7}{12}$	C	M	"	" "	8		4	2.1	1.9	Uneventful recovery.
D.E.	2	C	F	"	" "	10	11.4	4.66	2.0	2.66	Relapsed case. Cerebral atrophy. Put onto maize diet after normal pattern had been established.
D.G.	2	N	M	"	" "	7	12	3.78	2.04	1.74	Uneventful speedy recovery.
K.A.	1	C	F	"	" "	8	16.7	3.5	1.66	1.84	Uneventful recovery apart from febrile episode. Received I.V. Albumin.
W.K.	$1\frac{10}{12}$	C	F	Severe Marasmic type	" "	8.5		2.76	1.16	1.60	Good response to treatment. Persistant anaemia with megaloblastic bonemarrow.
W.de.V.	$1\frac{8}{12}$	C	M	Severe	" "	11.4		3.68	1.68	2.00	Cortisone treatment. Dramatic recovery.
M.A.	1	C	F	"	Herpes Stomatitis Salmonella, Stool.	8.5	11.3	3.97	1.92	2.05	Uneventful recovery.

M.K./...

Patient	Age Years	Race	Sex	Severity	Infection. Stool culture. Blood culture. Tuberculin.	Hb g/100 ml.	Serum Chemistry. (mgs/100m) Urea	(g./100 ml) Total Protein	Albumin	Globulin	Remarks.
M.K.	5	C	F	Severe	Blood Culture +ve Stool Culture +ve	6.5	9.8	3.72	1.00	2.72	Satisfactory response to treatment.
G.J.	$1\frac{1}{12}$	C	M	"	Culture -ve	11.5	7.9	3.88	1.98	1.90	Slow response clinically with febrile episodes. I.V. cortisone.
A.N.	$\frac{12}{12}$	N	M	"	Stool Culture +ve	11.0	8.5	3.68	1.94	1.74	Developed kwashiorkor after measles. Continuous pyrexia. Died of septicaemia.
S.A.	$1\frac{7}{12}$	C	F	Purpura	Culture -ve	6.0	14.7	4.15	1.49	2.66	Clinical response satisfactory. Resistant anaemia & megaloblastic bonemarrow.
F.S.	$2\frac{3}{12}$	C	F	Severe	Staph. Aureus Pneumonia	6.8	16	3.32	1.74	1.58	Fluctuating clinical course & pyrexia. Recovered.
C.A.	1	C	F	"	Culture -ve Pneumonia	6.5	10	4.34	1.89	2.45	Cerebral atrophy. Persistent oedema. Poor clinical response. Received blood transfusion. Recovered.
O.A.				"	Culture -ve			3.5	1.8	1.7	Satisfactory clinical response. Received I.V. Albumin.
C.J.	$\frac{11}{12}$	C	F	"	S. typhi-murium-stool	9		3.4	-	-	Marasmic kwashiorkor. Received blood transfusion. Died 48 hrs. after admission.
D.M.	2	C	M	"	Culture -ve	7.5	13.8	3.10	1.57	1.53	Jaundiced. Sudden deterioration on 3rd day. Died.
N.K.	3	C	M	"		4	-	2.8	-	-	Received blood transfusion. Died unexpectedly on 3rd day after initial improvement.

N = Native.

C = Cape Coloured.

CHAPTER IV.

RESULTS.

Case Material:

The main clinical and biochemical findings of the patients on admission to hospital are given in table IV. It is evident that most of the cases were severely ill as judged clinically. Certain observations may be made at this stage.

Most of the patients were anaemic with an average haemoglobin value of 8.3 g./100 ml. The low blood urea values observed by previous workers are confirmed in the present study where the concentrations were mostly below 15 mgs./100 ml.

Serum Protein Values on Admission:

The serum protein concentrations on admission are given in table V and represent the average value obtained in 27 patients studied by starch gel electrophoresis on at least one occasion. These are compared with values obtained in different parts of the world. The values after recovery represent the average of eleven patients before discharge from hospital. The serum protein concentrations in the cases of recovered kwashiorkor almost certainly do not represent the mean concentration for the population group as marginal protein deficiency must be exceedingly common in an area with such a high incidence of kwashiorkor. These values should rather be regarded as the mean theoretical normal on an adequate diet.

TABLE/...

TABLE V.

Serum protein concentrations in cases of untreated kwashiorkor in different parts of the world compared with normals or recovered infants.

Country.	Nutritional State.	Serum Proteins g./100 ml.		
		Total Protein.	Albumin.	Globulin.
Guetemala [124]	Before Treatment	4.87	2.09	2.78
Jamaica [111]	Before Treatment	4.6	1.8	2.8
West Africa [49]	Before Treatment	4.28	1.5	2.78
Mexico [125]	Before Treatment	4.36	1.64	2.72
Egypt [114]	Before Treatment	3.92	1.42	2.50
New Guinea [113]	Before Treatment	4.20	1.16	3.04
Cape Town, S.A. (present author)	Before Treatment	3.8	1.7	2.1
Guetemala	Normal	7.31	4.11	3.20
Jamaica	Normal	7.0	3.1	3.9
Jamaica	After Treatment	7.3	3.5	3.8
West Africa	After Treatment	7.04	3.25	3.79
Mexico	Normal	6.87	3.62	3.24
Egypt	After treatment	6.80	3.08	3.72
Cape Town, S.A. (present author)	After treatment	7.11	4.27	2.84

The/...

The finding of slightly lower total serum protein values on admission in the present series of cases can probably be explained by the fact that these form a selected group representing mostly the severely ill infants. The total globulin concentrations are lower both in the untreated cases and after recovery than the values found in other parts of the world while albumin levels after recovery are higher in the present series. The differences probably reflect the lower incidence of chronic parasitic infestations in this area.

STARCH GEL ELECTROPHORESIS RESULTS.

Some of the results presented here have already been published in the form of a preliminary communication [126].

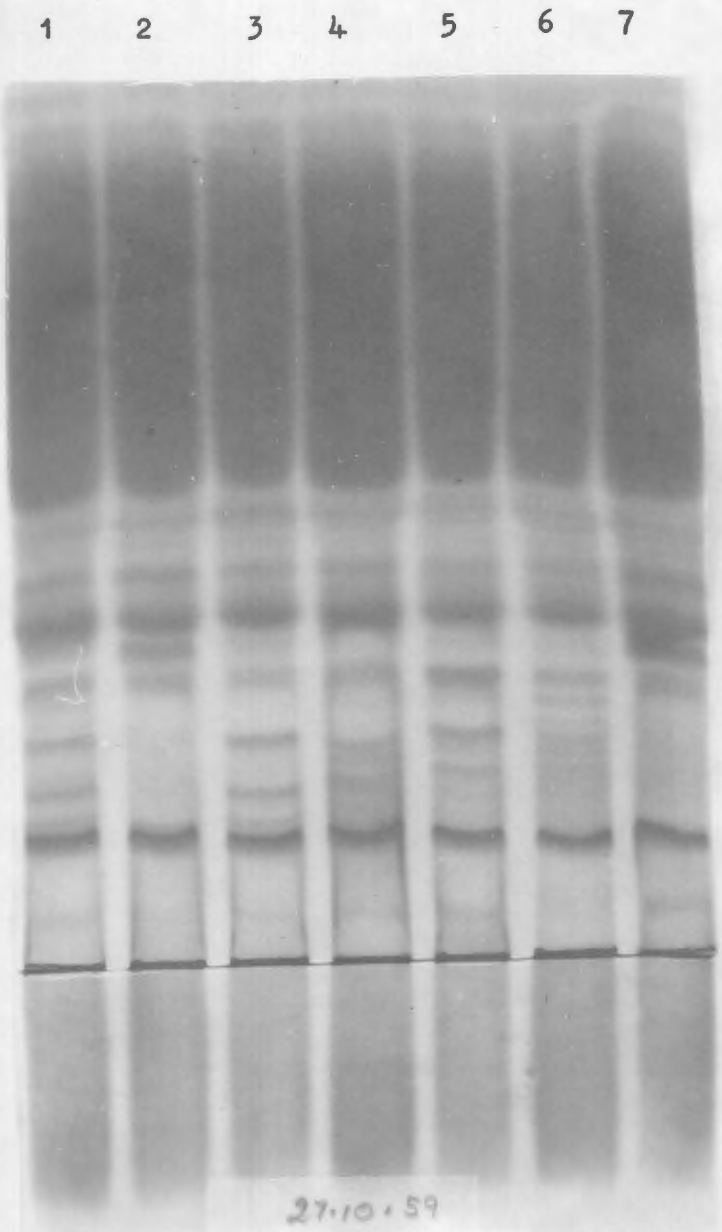
(a) Electrophoretic pattern in normal children.

The electrophoretic patterns of seven normal children are shown in fig. 13. It will be noted that significant differences are present in the "haptoglobin area" of the samples which are determined by the genetic type to which the individuals belong. The absence of any fractions in this area in specimens no. 1 and 7 indicate that these belong to the group Hp. 1-1. The double band in the transferrin area (labelled β globulin C) indicates a genetic variant of this fraction in sample no. 7. Comparisons between normals and abnormals should take these variations into

account/...

FIG. 13.

Starch gel electrophoresis diagrams of the serum proteins from seven normal children.



account and complications may arise in the untreated case where some fractions are reduced to a degree where accurate genetic typing becomes almost impossible. As pointed out before differences are best evaluated by comparing the acute stage pattern with that obtained after recovery.

(b) Starch gel electrophoretic pattern in untreated kwashiorkor.

Fig. 14 demonstrates the differences between a case of severe untreated kwashiorkor (patient MK., table IV) and patients after recovery, where sample no. 4 represented the untreated patient. It is evident that all the fractions including γ globulin are markedly reduced. Especially striking is the complete absence of demonstrable haptoglobins and virtual absence of β globulin C in this patient. These fractions returned rapidly after initiation of treatment proving that the initial absence on starch gel electrophoresis was not genetically determined.

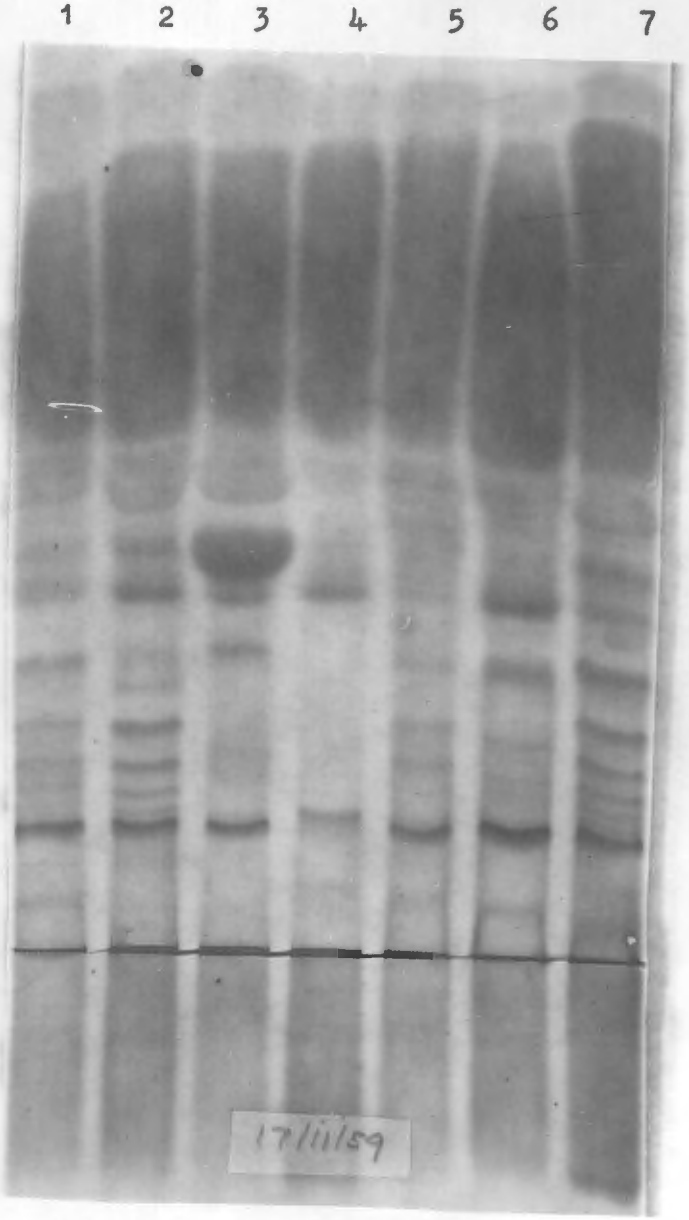
The pattern in untreated kwashiorkor showed quite marked variations in different patients. These differences affected certain fractions more than others. For instance in some cases albumin was markedly reduced while the haptoglobins and β globulin C were present in relatively higher concentrations. γ globulin concentrations varied widely as judged by the intensity of staining in this area. These variations will be discussed again when serial changes on treatment are demonstrated. Specimen no. 2 represents a sample of serum from patient A.N. (table IV) collected

one/...

FIG. 14.

Starch gel electrophoresis diagrams of the serum proteins from six treated patients compared with one untreated patient suffering from kwashiorkor.

(Sample No. 4)



one day before death and will be referred to again.

SERIAL ELECTROPHORETIC CHANGES ON TREATMENT.

Day to day changes in the electrophoretic patterns likewise showed a lack of uniformity. Representative examples will be demonstrated in the following photographs of starch gel electrophoretograms obtained on serum from different patients.

In fig. 15a, b, the changes in the electrophoretogram which developed during treatment of a moderately severe case of kwashiorkor (T.W. table IV) are demonstrated. On the day of admission albumin β globulin C and $\alpha 2$ - B globulin A are significantly reduced while haptoglobins are just detectable in the stained gel. It will be noticed that the post albumin fractions are clearly shown on the day of admission. On the third day after admission changes are already evident in the albumin, β globulin C and $\alpha 2$ B globulin A. Haptoglobin fractions appear rather dramatically on the 6th day. The last two samples represent the patterns obtained on oedema fluid from the foot collected on alternate days. The main fractions present appear to be albumin, β globulin, C, Sα 2 globulin and γ globulin. Total protein concentrations of the oedema fluid were 0.46 and 0.5 g./100 ml. respectively.

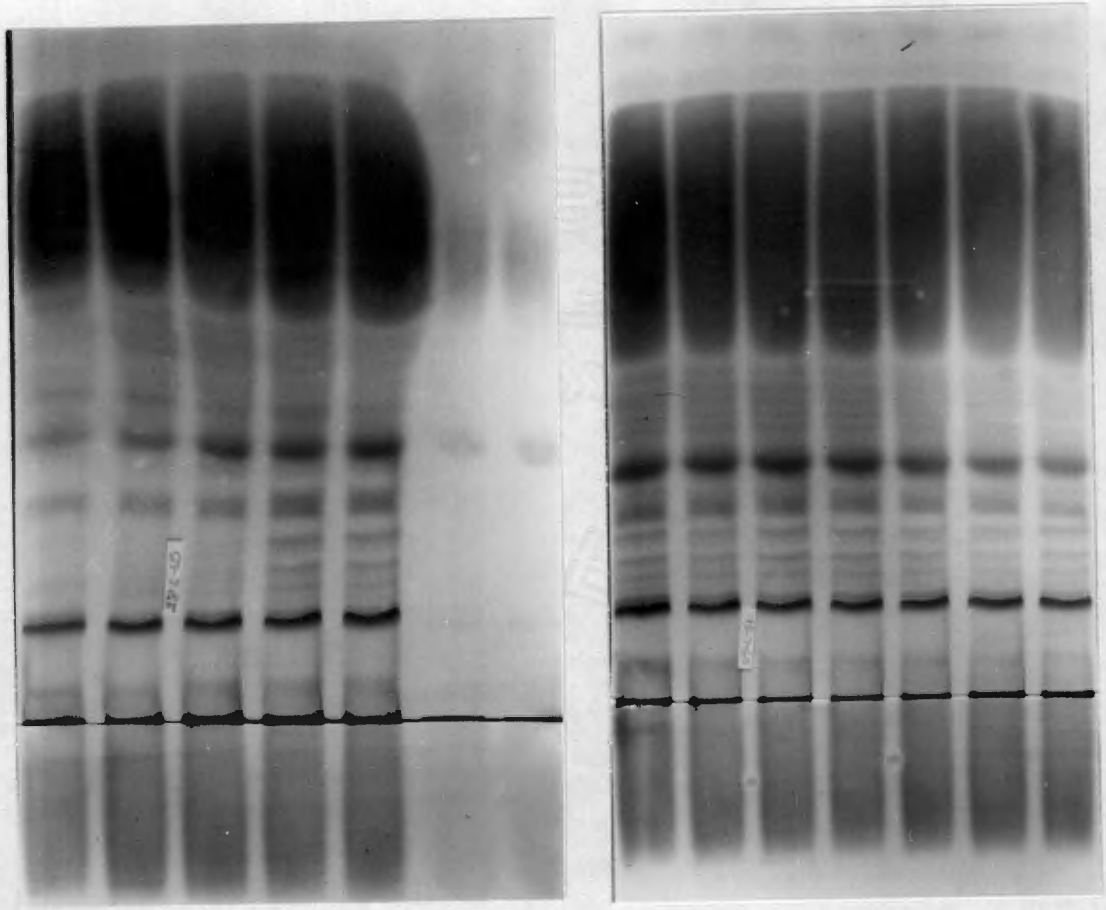
Serial daily changes are presented in fig. 15b. It is clear that by the 10th day after admission the electrophoretogram has practically returned to normal in that all the fractions

normally/...

FIG. 15 a, b.

Serial changes in the serum proteins of patient T.W., a moderate case of kwashiorkor.

a b



1 2 4 6 8 10 11 12 13 15 19 24

Days after admission.

normally expected are visible. Total serum protein concentrations increased from 4 g./100 ml. to 6.g./100 ml. during this period.

This patient demonstrates the order and rate at which changes occur in the serum proteins of a clinically moderately severe case of kwashiorkor responding rapidly to treatment.

VARIATIONS IN THE RATE AND ORDER AT WHICH SERUM PROTEINS RETURN TO NORMAL.

Haptoglobins:

It has already been noted that the concentration of haptoglobins in untreated cases of kwashiorkor is markedly decreased. In most patients who made an uneventful recovery after initiation of treatment, the concentration of these fractions increased rapidly and dramatically in parallel with the changes in albumin and β globulin C (transferrin). In some cases interesting variations on this theme were observed.

Lack of haptoglobin response:

In two patients the haptoglobins failed to return to normal concentrations long after an otherwise apparently normal pattern had been established. Both these patients, W.K. and S.A., (table IV) were severe cases of kwashiorkor clinically classified as of the marasmic type. An additional finding of great interest was the presence of a resistant anaemia associated with a megaloblastic bonemarrow. The electrophoretic patterns obtained on

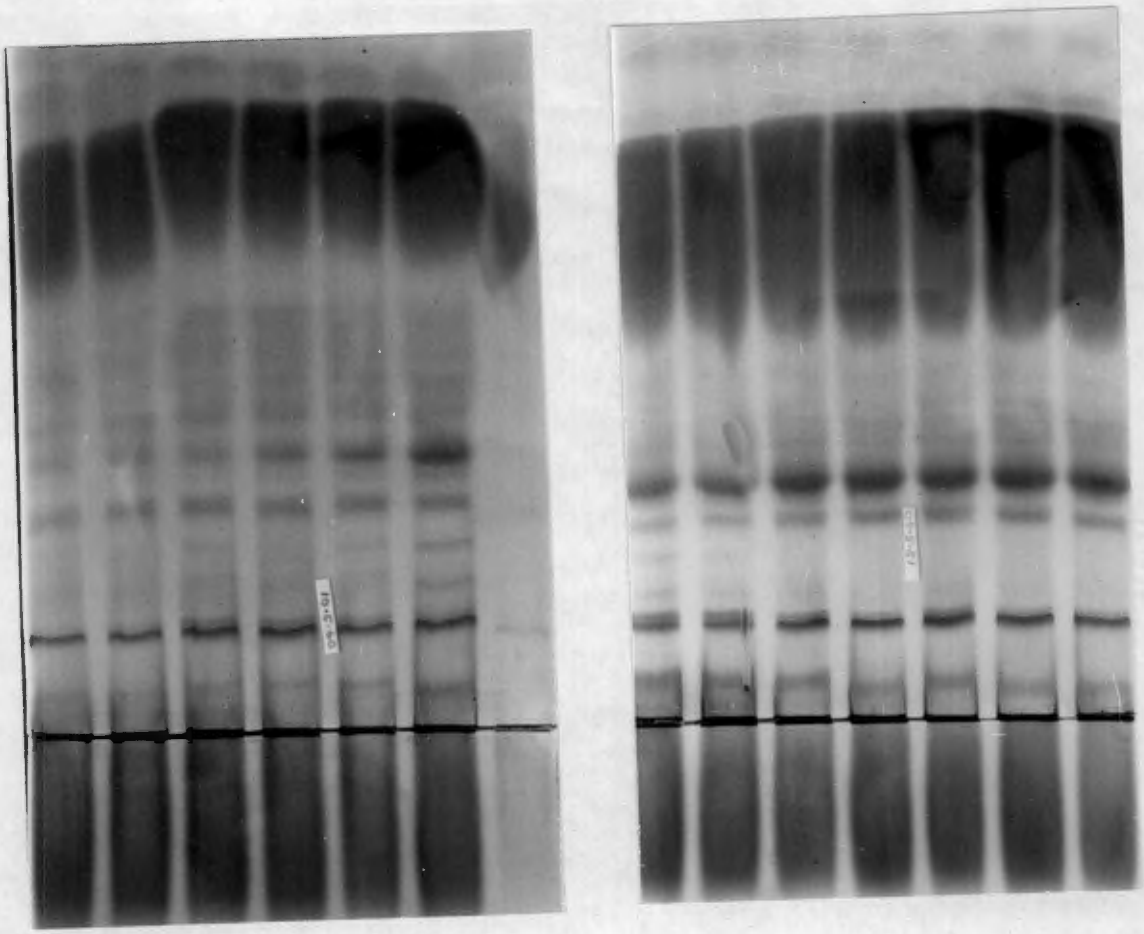
samples/...

FIG. 16 a, b.

Serial changes in the serum proteins of patient S.A., a severe case of kwashiorkor.

a

b



0 2 3 4 5 6 7 8 9 10 11 13 14

Days after admission.

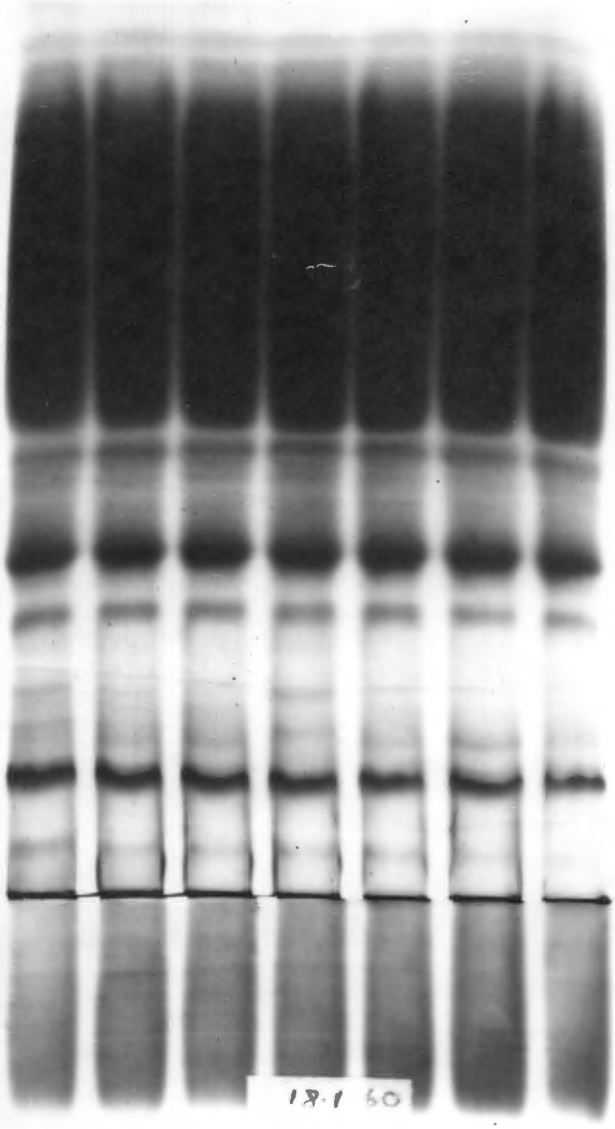
samples collected daily from S.A. for 13 days after admission to hospital are shown in fig. 16a, b.

The generally depleted pattern on the day of admission is evident with the exception of γ globulin which is present in high concentration. The last sample in fig. 16a represents oedema fluid from this patient. The dramatic improvement in the pattern even from the third day onwards is evident with albumin, β globulin C and α 2 B globulin A being the most prominent. It can also be seen that haptoglobin fractions become clearly visible on the 6th day but have disappeared completely by the eighth day after admission. These fractions did not return to normal concentrations for a period of two months as can be seen from the electrophoretic pattern obtained on a sample of serum collected two months after admission to hospital (fig. 14 sample 7). Interesting changes were also observed in the lipoprotein fraction marked T. There is a definite increase in concentration by the sixth day reaching a maximum by the seventh day with a subsequent gradual decrease in concentration. This increased concentration of B lipoprotein corresponds to the period of lipid mobilisation (phanerosis) from the liver described by Mathew and Dean [57] and is evident as visible lipaemia during this period. The total serum protein concentration in this patient increased from 4.15 g/100 ml. on the day of admission to 7.13 g./100 ml. on the fourteenth day after admission. Patient W.K. showed a very similar course of

events/...

FIG. 17

Serial starch gel electrophoresis diagrams of the serum proteins from patient W.K., three weeks after initiation of treatment.



events. Sample no. 1 in fig. 14 represents the pattern obtained three months after initiation of treatment in this patient. The pattern at this time is normal with haptoglobins present in the expected concentrations. It can be seen from fig. 17 which represents the daily pattern in W.K. during a period, three weeks after initiation of treatment that the haptoglobins at that time are hardly visible in an otherwise normal pattern.

SERIAL SERUM PROTEIN CHANGES IN A CASE COMPLICATED BY A STATE OF COLLAPSE AND CORTISONE THERAPY.

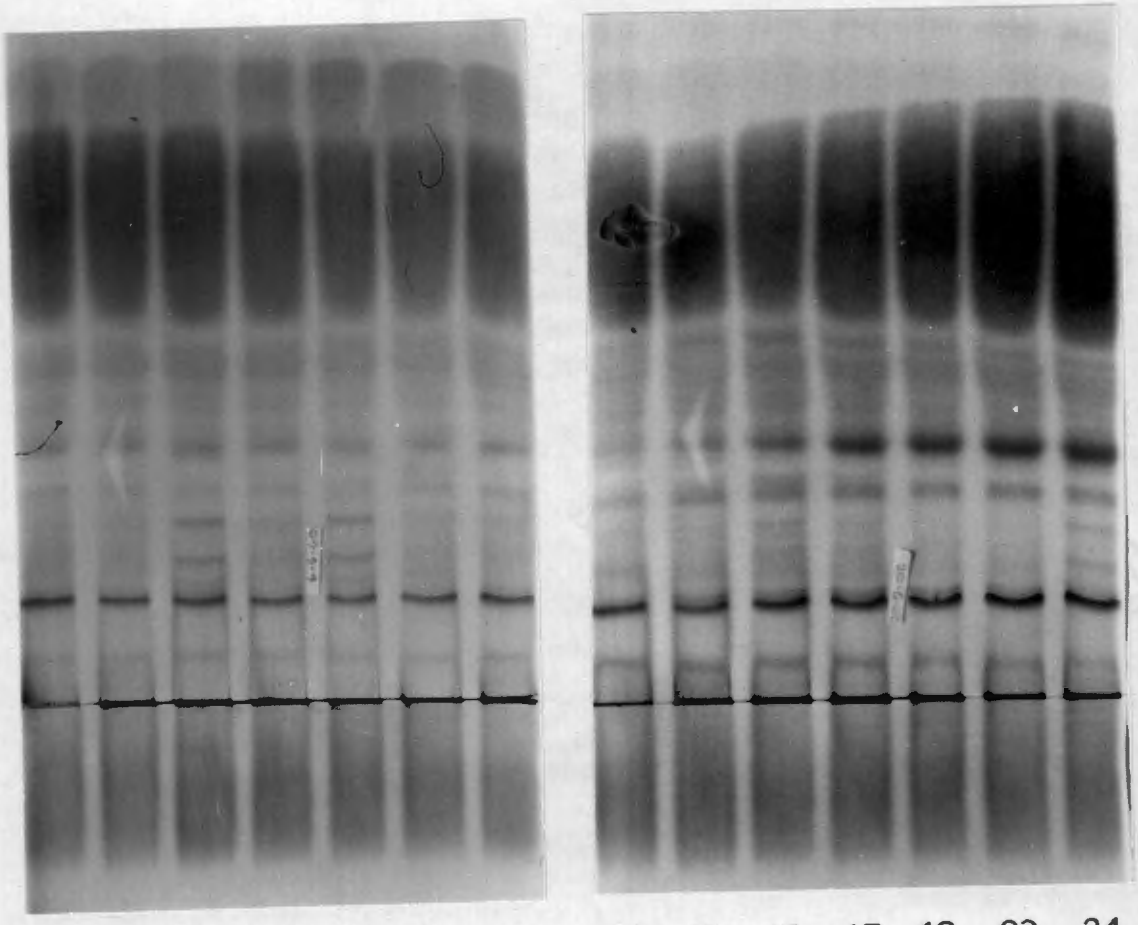
Fig. 18a, b, c demonstrate the serial changes in the serum proteins of patient M.B. who was judged to be only a moderately severe case of kwashiorkor on admission. This patients' recovery was complicated by the development of an unexplained state of collapse, 24 hours after admission, requiring hydrocortisone therapy and tube feeding. Cortisone treatment was continued until the 10th day after admission.

It can be seen from fig. 18a that the pattern on admission was moderately depleted with both haptoglobins and β globulin C clearly detectable. There is however a deterioration in the pattern during this 10 day period with fluctuating haptoglobin levels. From the 13th day onwards there is a dramatic increase in most fractions except for the haptoglobins which temporarily disappear (fig. 18b).

FIG. 18 a, b.

Serial changes in the serum proteins of patient M.B.

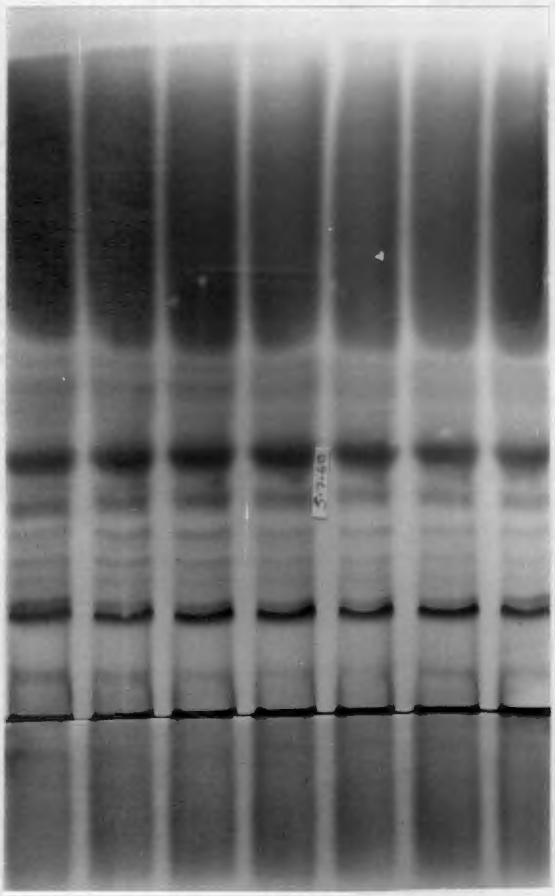
a b



Days after admission.

FIG. 18 c.

Serial changes in the serum proteins of patient M.B. (continued)



29 30 31 32 33 34 35

Days after admission.

Fig. 18c demonstrates the pattern after a further period of time. The striking feature again is the fluctuating levels of the haptoglobin zones in an otherwise fully recovered protein spectrum.

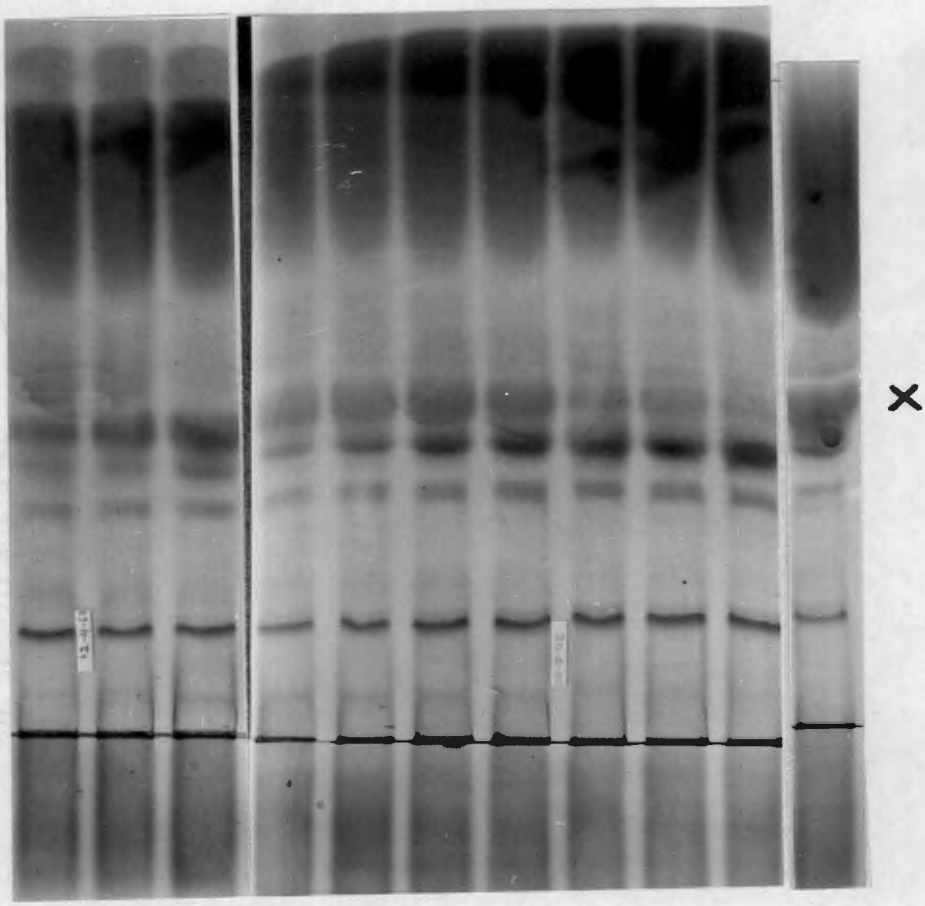
THE DEVELOPMENT OF UNUSUAL FRACTIONS DURING TREATMENT OF KWASHIORKOR.

Fig. 19 represents serial electrophoretogram on serum of patient A.N., (Table IV) a severe case of kwashiorkor developing after an attack of measles. This patient remained febrile throughout his stay in hospital and died 26 days after admission. Autopsy findings revealed a septicaemia. It can be seen that there is again a dramatic improvement in the pattern of this patient (genetic type Hp. 1-1) in spite of his febrile illness. A salient feature is the presence of a rather broad zone just ahead of β globulin C and marked X in the photograph, corresponding in position to the haptoglobin zone of this genetic type. This fraction increases during the first few days of treatment but subsequently decreases while β globulin C shows a concommittant increase. In the sample of serum obtained one day before death (25 days after admission) this unusual zone is again present in high concentration.

The/...

FIG. 19.

Serial changes in the serum proteins of patient A.N. who died of septicaemia.



Days after admission.

The effect of instituting a maize diet on the serum protein pattern in a case of treated kwashiorkor.

The effect of substituting the high protein milk diet with a diet consisting mostly of carbohydrate and maize protein was studied in one patient. (This patient (D.E.) represented a case of relapsed kwashiorkor with mental retardation and cerebral atrophy. After receiving the ordinary high protein milk diet for 25 days, the infant was given a diet consisting mostly of carbohydrate and maize with no milk. Fig. 20 shows the serum protein pattern after 25 days on the milk diet (first four samples) while samples 5-8 represent the pattern after approximately 40 days on a maize diet. The total protein concentration after 25 days was 7.14 g/100 ml. with albumin 4.45 g/100 ml. After a period of 40 days on the maize diet, the total protein concentration had decreased to 6.6 g/100 ml.

It can be seen that the haptoglobins and transferrin (β globulin C) zones are markedly reduced in concentration as judged by the intensity of staining of these fractions. Unfortunately albumin concentrations could not be determined during this second period and the quantitative reduction of albumin could therefore not be evaluated. In view of the fact that the total protein concentration was reduced by 0.5 g/100 ml. one can assume that the albumin level was also lower. However the globulins

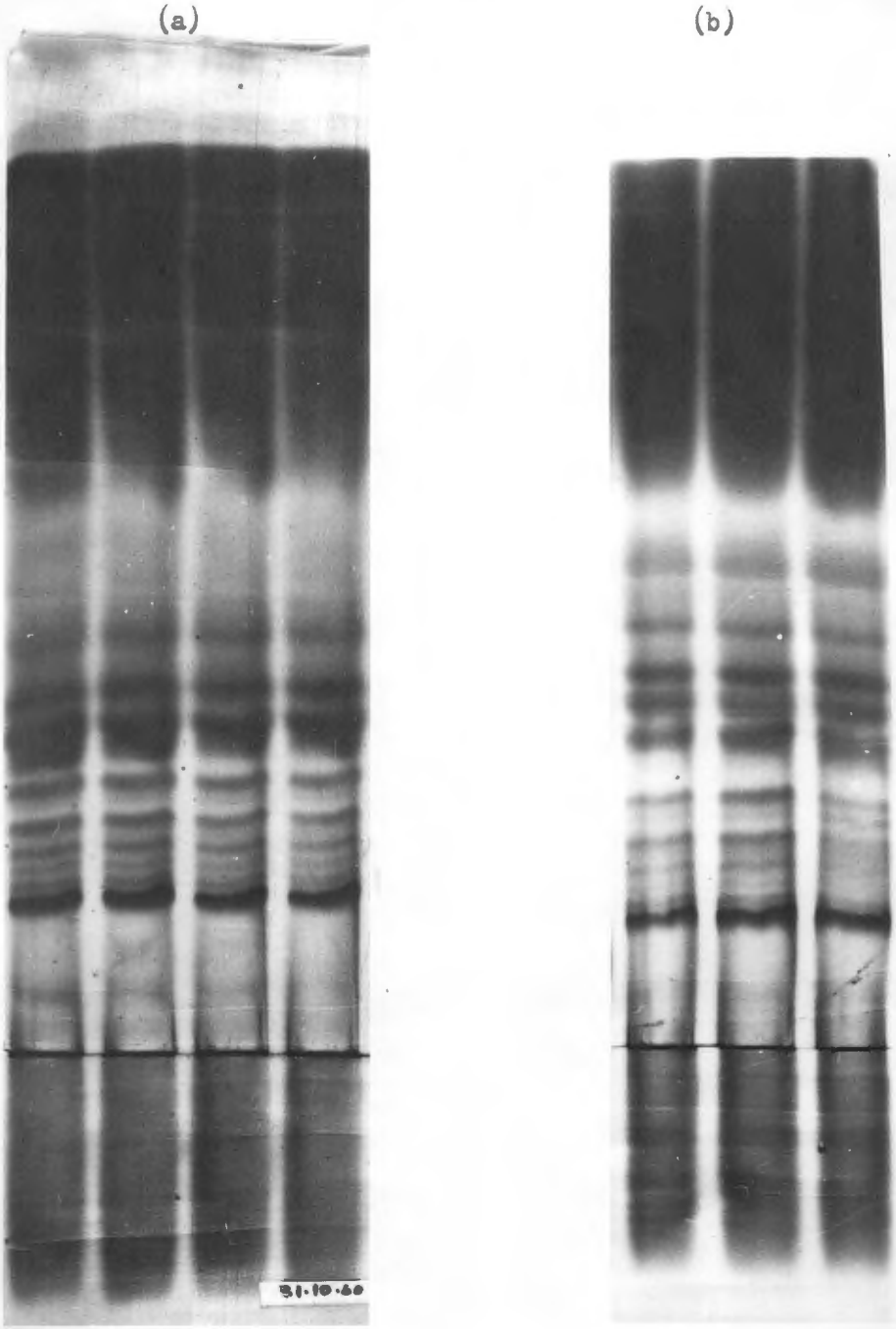
mentioned/...

FIG. 20.

Starch gel electrophoresis diagrams of the serum proteins from patient D.E.

a) After 25 days on a high protein diet.

b) After 40 days on a diet consisting of maize only.



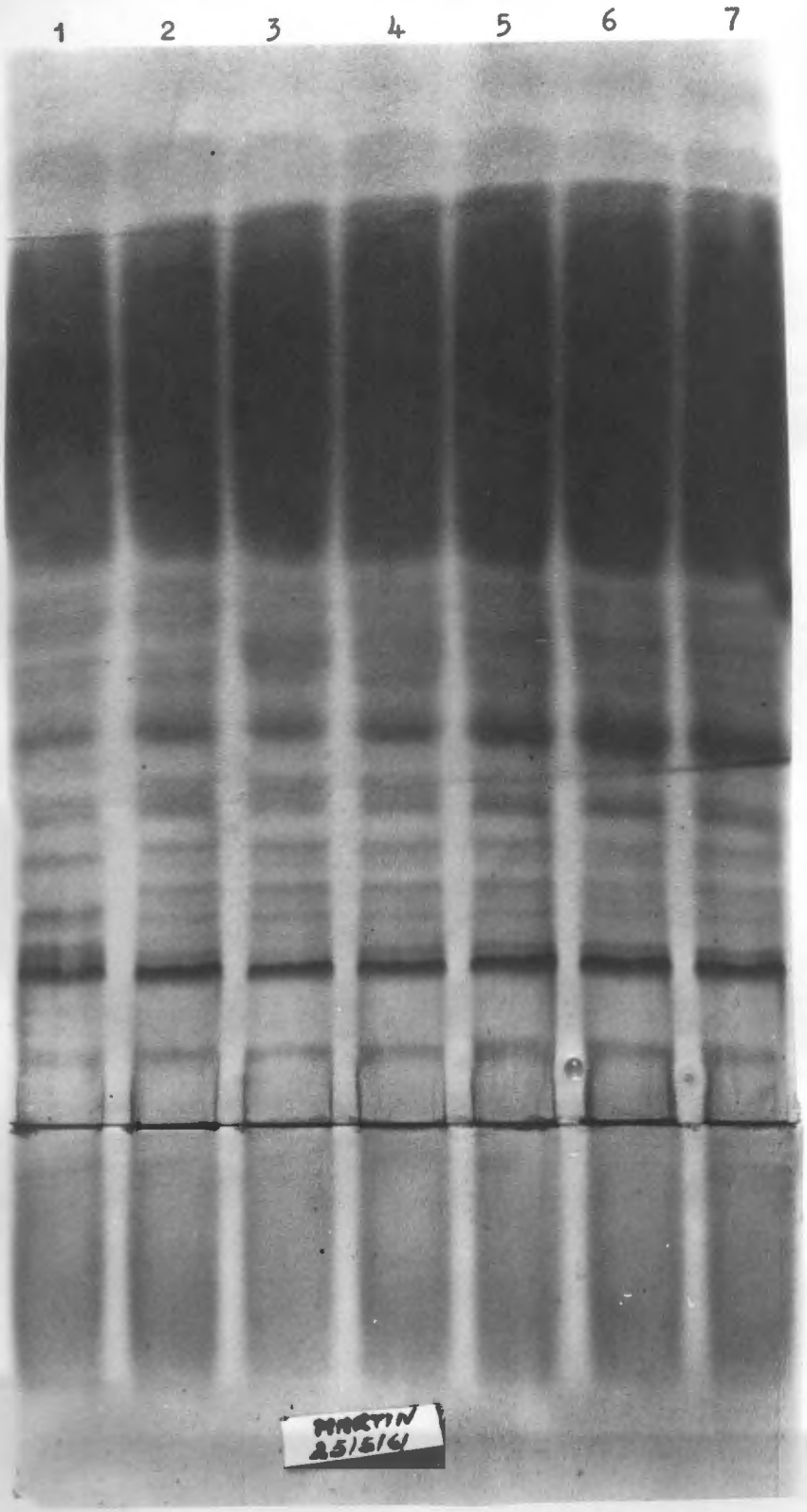
mentioned appeared to be relatively more effected during this time.

The effect of intravenous albumin and plasma transfusions on the electrophoretic pattern.

The effect of an intravenous infusion of albumin on the serum protein pattern in kwashiorkor was studied in three patients. Fig. 21 represents the serum protein changes in patient H.H., a mild case of kwashiorkor. 5 Grams of albumin was given intravenously at 10 a.m. between samples 2 and 3 which were collected at 9 a.m. and 2.30 p.m. respectively, on the same day. The only definite observation that can be made, is an increase in albumin concentration between the two specimens. Marked changes do occur from the third day onwards in the albumin and transferrin zones. The value of these experiments is however limited by the fact that these patients inadvertently received milk as well during this period. No conclusions can therefore be drawn as to the effect of a single injection of albumin on the serum protein pattern as a whole as the contribution of dietary protein to the changes observed, is probably highly significant. This experiment was conducted in order to determine whether the infusion of an intact homogeneous protein could act as a readily available source of amino-acids for the synthesis of other serum proteins or whether the intact molecule would be retained in circulation and only provide amino-acids during the process of normal catabolism determined by the halflife of the molecules.

FIG. 21.

Starch gel electrophoresis diagrams of the serum proteins from patient M.M. who received I.V. albumin between samples 2 - 3.



CHAPTER V.

STARCH GEL ELECTROPHORETIC STUDIES ON KWASHIORKOR.

DISCUSSION AND CONCLUSIONS.

CHAPTER V.

DISCUSSION.

No attempt has been made during this study to evaluate the statistical incidence of different types of patterns in untreated kwashiorkor or to correllate these with the clinical severity of the case. As already stressed, these patients studied here form a rather selected group with a predominance of severe cases. Certain observations and conclusions can however be made.

The starch gel electrophoretic pattern in untreated kwashiorkor:

In general, the observations of earlier workers on the serum proteins in kwashiorkor have been confirmed. Albumin and B globulin C (transferrin) concentrations were uniformly and severely reduced. The finding of low serum iron binding capacity in cases of untreated kwashiorkor is supported by the present starch gel investigations. The present studies however revealed that the haptoglobin levels in untreated kwashiorkor were also markedly reduced or even absent on starch gel electrophoretograms. Certain fractions notably δ globulin the postalbumins and the α 2 macroglobulin (S α 2 fraction) appeared to be maintained even in severely ill patients. These fractions may therefore receive a certain priority during protein synthesis in the depleted state or are maintained by a much more slowed down catabolic rate. However

the/...

the lack of knowledge regarding the precise biological function of some of the finer fractions in the serum proteins preclude any speculations as to the significance of these observations. However it is possibly fair to say that the reduced albumin concentrations may be no more specific a finding than changes in transferrin or haptoglobin levels. As albumin accounts for more than 50% of the total serum proteins normally, it is quantitatively the most striking change.

It is evident that the exact pattern during the depleted state, which shows marked variation from patient to patient, does not serve as a prognostic guide in the individual case. The clinical and biochemical response in the two patients S.A. and W.K., (table IV) both severe cases of kwashiorkor of the marasmic type indicated that a grossly depleted serum pattern can be associated with a dramatic response to treatment. In another case M.B. judged to be of only moderate severity clinically and in whom the serum protein pattern was less depleted than in the above mentioned cases, the clinical and biochemical response was initially unsatisfactory. This patients' recovery was complicated by an unexplained state of collapse requiring steroid therapy.

The rate and order of recovery of the Serum proteins:

In spite of the decrease in digestive enzymes, the rate at which the serum protein concentrations is restored to normal

remains/...

remains phenomenal. Obvious changes can often be detected in the starch gel electrophoretograms as early as three days after initiation of treatment with the maximal changes occurring between the fifth and tenth days in uncomplicated cases.

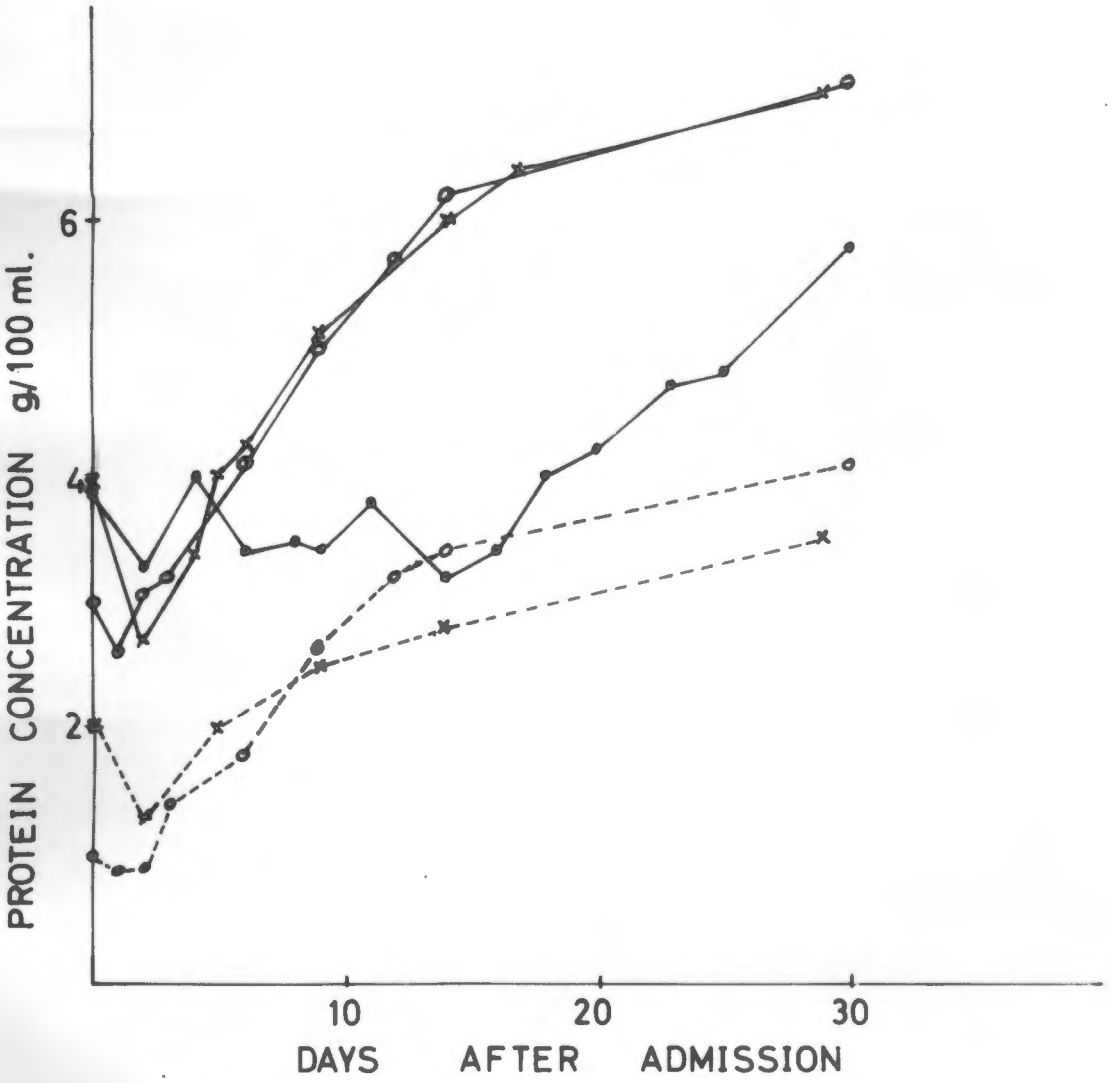
These observations tend to rule out decreases in enzyme concentrations as rate limiting factors during protein synthesis in this condition. The possibility that deficiencies of certain vital cofactor may be unmasked during this state of accelerated metabolism cannot be excluded and indeed seems almost certain in the case of haemoglobin formation.

In fig. 22 the total protein and albumin concentrations of three patients at different intervals after treatment are shown. There is usually a slight drop in the protein levels on the second and third day after admission which can probably be explained on the basis of rehydration of the intravascular compartment during this period. From the third day onwards both the total protein and albumin values increase dramatically. It is evident that the increase in albumin levels seems to lag behind the total protein concentration after the initial period of rapid response. In patient M.B. the total protein concentration shows fluctuating levels during this initial period when the state of peripheral circulatory collapse persisted. It could be suggested that during this period albumin may serve as a source of labile protein utilised for body protein synthesis. Some support for

this/...

FIG. 22.

CHANGES IN THE SERUM PROTEIN CONCENTRATIONS OF PATIENTS WITH KWASHIORKOR DURING TREATMENT.



PATIENT: W.K. ○ ○ ○
G.J. × × ×
M.B. • • •

TOTAL PROTEIN. ————

ALBUMIN. - - - - - -

this hypothesis comes from the evidence of increased turnover rates of albumin during this stage in kwashiorkor [73] and in experimental animals [95]. It is also evident that the restoration of the total serum protein and albumin concentrations to normal values is not an index of total body protein repletion. In the majority of the present patients, serum protein concentrations revealed normal levels 20-30 days after initiation of treatment whereas total body protein repletion takes approximately three months [73]. Similar observations were made by Senecal in French West Africa [49].

The pattern of Serum protein repletion during the treatment of kwashiorkor:

It is evident from the results presented that the order in which individual serum proteins are restored to normal concentrations varies from case to case. In uncomplicated cases increases in albumin, β globulin C and the haptoglobins seem to parallel one another. However in a number of patients interesting variations were noted. These affected predominantly the haptoglobin fractions.

The term haptoglobin was introduced by Polonovski and Jayle [127] in describing an α 2 globulin fraction which reacted stoichimetrically with haemoglobin to form a stable complex. These proteins are today regarded as specific haemoglobin carriers and when haemoglobin is injected intravenously it combines

immediately with available haptoglobin after which the complex as a whole is eliminated at a constant rate of 0.3-0.8 g/hour from the blood stream. The available evidence suggests that the haptoglobins consumed during this process cannot be reutilised as the haptoglobin concentration does not recover its original level before 5-7 days after an injection of haemoglobin [128]. Reduced haptoglobin levels or even the absence of demonstrable haptoglobins is an extremely sensitive index of increased haemoglobin catabolism not only as a result of intravascular haemolysis but also when red cells are destroyed extravascularly as in hereditary spherocytosis [129]. According to Nyman et al [130] a decreased haptoglobin level above all suggests increased haptoglobin consumption due to intra or extravascular haemolysis or even socalled increased intramedullary haemoglobin turnover (ineffective erythropoiesis). Subnormal values are rarely seen in chronic liver disease and in some miscelaneous conditions such as infectious mononucleosis. However in many of such cases there was evidence of increased haemoglobin turnover as evidenced by the increased serum carbon monoxide levels derived from the α methene bridge of the porphyrin nucleus when the ring is opened.

The observation of markedly reduced or absent haptoglobin concentrations in the patients with kwashiorkor raised interesting possibilities. The low or absent haptoglobins in the untreated cases may possibly be explained on the basis of a general depression

in serum protein synthesis during the depleted state but no information on this point is available. However the failure of the haptoglobin concentrations to return to normal levels or the persistent absence of these fractions in certain cases even after prolonged treatment certainly suggests a different mechanism as other evidence of severe chronic liver dysfunction is lacking in these cases. The adequate synthetic mechanisms for producing albumin and transferrin, both proteins known to be produced by the liver strengthen this contention. The high incidence of megaloblastic anaemia in kwashiorkor and the association between persistent anhaptoglobulinaemia in the two cases of resistant anaemia with megaloblastosis (W.K. and S.A.) in the present series of cases strongly suggest a haemolytic mechanism in some of these patients. The socalled "crisis" described by Walt [100] during which the haemoglobin may drop by 3-4 g./100 ml. within 24 hours further supports this hypothesis. Temporary fluctuations in haptoglobin levels were observed in several additional patients in the present series as exemplified by case M.B. fig. 18b. An interesting observation is the fact that conventional signs of haemolysis such as increased reticulocyte counts were absent in the cases where these were looked for. This however does not exclude increased haemoglobin catabolism of the type described as "defective erythropoiesis" by Nyman et al[130]. More sensitive indices such as blood carbon monoxide levels may have to be used

to/...

to demonstrate this degree of haemolysis.

The observation that the megaloblastic response is often evoked by the initiation of treatment and apparent cure may be the result of a conditioned deficiency of folic acid or associated cofactors during this stage of active nucleic acid and protein synthesis. The importance of folic acid not only in haemoglobin synthesis but in intermediate metabolism generally during the transfer of methyl groups suggests that experiments designed to investigate folic acid metabolism in this state could yield vitally important information.

The demonstration of a zone of unusual intensity which was present in case A.N. fig. 19 is of some interest. This fraction appeared in the position of the haptoglobins of genetic type Hp.1-1. Although the identity of this fraction could not be identified with absolute certainty it behaved as a haptoglobin since when haemoglobin was added to a sample of serum from this patient it travelled to exactly the same position.

The results of Nosslin and Nyman [129] suggest that the presence of associated tissue injury or inflammatory changes even in patients with increased haemoglobin catabolism restores the haptoglobin levels to normal almost immediately. Patient A.N. was febrile throughout his illness and died of septicaemia which could be the basis for this increased haptoglobin concentration. This is a rather unusual state of affairs as it appears as if tissue

injury is a more effective stimulant to haptoglobin synthesis than increased haemoglobin catabolism unless the binding sites on haptoglobin for haemoglobin are affected by the disease process preventing the haemoglobin-haptoglobin complex from forming and so decreasing the consumption of the latter.

This patient A.N. also demonstrates that in spite of a severe infection a dramatic improvement in the serum protein concentrations occurred. During the initial 12 day period the total serum protein concentration increased from 3.7 g./100 ml. to 5.8 g./100 ml.

The changes occurring in the serum proteins of a patient on a maize diet.

The order of serum protein depletion appears to be very similar to the order of repletion during treatment of kwashiorkor. It is evident from the electrophoretogram of patient D.E. (fig. 20) in whom a maize diet was instituted after the serum protein pattern had been restored to normal that the decreases in concentration effect mostly the transferrin, haptoglobin and α_2 B globulin A fractions with possibly the albumin which is more difficult to assess.

Protains of the oedema fluid:

The total protein concentration of the oedema fluid in those patients where this was examined varied between 0.45-0.5

g./100 ml./...

g./100 ml. The electrophoretic studies indicated that the main fractions present were albumin, transferrin and γ globulin. Loss of weight associated with the disappearance of oedema fluid in one child indicated that the oedema fluid weighed at least 5 lbs; the total protein contained in the oedema fluid must therefore have been at least 10-12 g. The return of even part of this protein to the intravascular compartment could have an appreciable effect on the composition of the serum proteins. The disappearance of oedema fluid often coincides with the period of maximal changes in the protein pattern, towards the end of the first week after initiation of treatment. It is not suggested that this mechanism entirely accounts for the dramatic early changes but it must be considered as a possibly significant, additional mechanism.

CONCLUSIONS.

The results of the present investigation suggest that the changes in the serum proteins of patients suffering from kwashiorkor, untreated and during treatment are more complex than generally appreciated. The concentrations of albumin and most of the globulin fractions with the exception of the post albumins, S α 2 macroglobulins and γ globulins are markedly reduced in the acute stage of kwashiorkor. The pattern varies however from patient to patient and does not always correlate with the clinical severity of the disease.

Infection per se does not necessarily affect the rate at which the serum protein concentration increases but may effect the pattern of repletion.

The haptoglobins show striking changes in this condition. The severly reduced values before treatment and persistent absence even after long periods on an adequate diet suggest a haemolytic mechanism for the anaemia in some cases of kwashiorkor. The effect of early supplementation of the diet with folic acid or related co-factors such as ascorbic acid on the haptoglobin levels in kwashiorkor should therefore yield interesting information.

The return of some of the proteins in the oedema fluid to the blood stream during the period of rapid loss of oedema fluid could account for some of the dramatic changes in the serum protein spectrum during this period.

Finally it is evident that digestion and absorption of protein and the general mechanisms for protein synthesis remain intact and efficient even in the severely depleted child. However the excessive demand for energy producing mechanisms during this state of tremendous metabolic activity may induce deficiencies of vital co-factors which may effect cellular metabolism and transport mechanisms adversely. Such acutely induced deficiencies may even be responsible for some of the cases of unexpected death which occur during treatment.

PART B.

THE AMINO-ACID COMPOSITION OF SERUM ALBUMIN IN KWASHIORKOR.

CHAPTER VI.

INTRODUCTION.

It is evident from the semi-quantitative studies on the serum proteins in kwashiorkor described in part A that the changes which occur are complex but not entirely specific, in that albumin and the majority of globulins show markedly decreased concentrations in the untreated cases. It was pointed out that certain fractions notably the postalbumins, $\alpha 2$ macroglobulin and γ globulin were generally less effected. The studies of Holt et al[84] on the plasma aminogram in kwashiorkor demonstrated a uniform pattern markedly different from the normal, irrespective of the country of origin. These changes effect certain amino-acids more than others, especially the essential amino-acids, but also some of the non-essential amino-acids and probably reflect an alteration in the composition of the available amino-acid pool in general, which means that the composition of the substrate pool for general metabolism and protein synthesis is altered. The order of reduction of the total available amino-acid pool cannot be readily assessed as all the amino-acids are actively metabolised, which makes interpretation of radio-active studies extremely complex.

It seems logical, therefore, that certain mechanisms must be put into operation by the depleted body to conserve nitrogen or utilise available nitrogen more effectively. Certain possible

mechanisms/...

mechanisms come to mind:-

1. Decrease in oxidative deamination of amino-acids and urea production.

All the available reports show that the urinary urea levels and total nitrogen excretion are markedly diminished on low protein diets [131, 132, 133, 134]. In the studies of Edozien [134], a group of children on a high protein diet excreted 79.5% of their total urinary nitrogen in the form of urea, whereas the comparative figures for normals on a low protein diet and kwashiorkor were 54.9% and 41.2% respectively. Total nitrogen excretion in normals on a high protein diet was 8.0 g./litre while in kwashiorkor only 3.8 g./litre was excreted. In adults similar observations were made [132]. The latter workers, however, demonstrated that the urea excretion was lower on low protein - high calorie diets than on diets deficient in both calories and protein. It was suggested that the high intake of carbohydrate reduces the requirements for energy derived from amino-acids by gluconeogenesis. In the present study, (Part A) the low blood urea levels were again observed. These observations suggest that losses of nitrogen may be lowered by a diminished rate of oxidative deamination and urea formation.

2. Decreased turnover rate of proteins.

Studies on the turnover rates of plasma proteins in experi-

mental animals [93, 94, 95] and on albumin in children with kwashiorkor [73] demonstrate clearly that the turnover rates are decreased and in Purves and Hansens' [73] studies the halflife of serum albumin was double that of normals. This, therefore, seems to be an additional mechanism of nitrogen conservation.

3. Utilisation of Reserve protein and re-utilisation of amino-acids liberated during tissue catabolism.

Although there is no true store of nitrogen and protein in the body comparable with glycogen and depot fat, it is evident that, in the protein depleted state, amino-acids liberated during catabolism of protein from certain organs must be utilised for synthesis of vital proteins such as enzymes when there is a lack of dietary protein. The experimental observations of Sprinson and Rittenberg [135] and Oleesen et al [136] suggest that in man about one third of the amino-acids in the body pool is supplied by tissue catabolism. If there is a dietary deficiency of amino-acids, the relative contribution from tissue catabolism may be significantly higher. Should the fractional rate of catabolism remain the same, it is evident that a net loss of protein nitrogen must occur from certain organs. Muscle, being the largest protein-containing organ in the body, probably bears the brunt of this protein-losing state in order to supply amino-acids for utilisation in the synthesis of vital proteins with rapid turnover rates such as enzymes. This is

borne/...

borne out clinically by the profound muscle wasting encountered in kwashiorkor. In order to achieve some sort of steady state, re-utilisation of nitrogen must therefore be more efficient, which is supported by the finding of decreased excretion of urea and total nitrogen in the urine. Levenson and Watkin [98] also concluded from their studies on the nitrogen which is lost after burns, that the integrity of active vital organs is maintained at the expense of less vital ones such as skeletal muscle.

4. Increased formation of amino-acids through transaminase reactions.

Although there is no experimental evidence to support this hypothesis, it is theoretically possible that carbohydrate may be a limited source of certain amino-acids in the depleted state, through transamination reactions. This would obviously depend on the availability of the amino-acid donating the amino-group in the reaction. With the possible exception of lysine and threonine, all amino-acids, even the essential ones, take part in these reactions. For instance in the rat, after feeding of ammonium salts, glycine, L or D Leucine, or L tyrosine labelled with isotopic nitrogen (N^{15}), almost all of the amino-acids subsequently isolated from the proteins of the tissues contained some N^{15} [137]. It would, therefore, be of fundamental importance to study the rate of transaminase reactions in the depleted state as compared with the normal in order

to/...

to determine whether specific amino-acids derived in this way may contribute significantly to the general pool.

5. Replacement of essential amino-acids by non essential amino-acids in certain proteins.

It is, theoretically, possible that a certain amino-acid within a protein molecule may be replaced by another of equal charge, without significantly effecting the net charge on the protein molecule. If this could, indeed, happen in the protein depleted state, the animal may be equipped with a potent tool for conserving essential amino-acids by replacing them with non essential amino-acids, provided that the biological function of the particular protein is not interfered with. From the studies on adrenocorticotrophic hormone by Hoffman et al [138] and Lee et al[139], it is evident that only the first twenty three amino-acids in the straight chain polypeptide of thirty nine amino-acids are required for full activity of this hormone. The remaining thirteen amino-acids vary according to the animal source, without affecting the potency of the hormone. There appears, therefore, to be no fundamental objection to the hypothesis of interchangeability of amino-acids in a protein molecule, provided that the particular animal possesses the required synthetic mechanisms for affecting such changes, or that these can be induced for instance by a change

in/...

in the substrate composition. Some support for this argument comes from the persistence of the gene for production of foetal haemoglobin in states where normal adult haemoglobin (A1) is not formed. The synthetic mechanism for production of foetal haemoglobin is normally suppressed shortly after birth. Interesting observations have also been made with respect to albumin, which, up till the nineteenth week of foetal life, appears to consist of two electrophoretic components [140, 141]. This condition of bisalbuminaemia has now been described in adults by several authors[142, 143, 144]. The "abnormal" albumin showed no demonstrable antigenic differences and coupled specifically with n-tolyl-alpha-napthylamine-8-sulphonic acid. To our knowledge, no results on the amino-acid composition of the two fractions have been published. These considerations stimulated our interest in the amino-acid composition of certain body proteins, notably albumin which accounts for the bulk of the readily accessible labile serum protein pool. We were particularly interested to find out whether the body could affect changes in the amino-acid composition of a protein such as albumin when the availability of essential amino-acids was extremely low and the level of amino-acid substrates as a whole was markedly diminished. However, some theoretical background to the specificity of protein biosynthesis and the microheterogeneity of protein structure is required before this argument can be further developed.

OUTLINE OF PROTEIN SYNTHESIS.

The active site of protein synthesis in the cell resides in the ribosomes which are dense spherical particles attached to a network of membranes, tubules and saccules (endoplasmic reticulum) and contain 80% of the cells' ribonucleic acid (R.N.A.). The desoxyribonucleic acid (D.N.A.) of the cell nucleus contains the genetic code for protein synthesis and transfers its specific genetic information to the protein synthesising mechanism in the cytoplasm. Both R.N.A. and D.N.A. consist of long chains of nucleotides containing large numbers of purine and pyrimidine bases. The differences in sequential order of these nitrogenous bases determine the arrangement of the amino-acids in the peptide chains of different proteins.

The genetic code is transferred from D.N.A. to R.N.A. by the formation of a complimentary R.N.A. strand for each hybrid D.N.A. helix produced in the nucleus. This R.N.A. molecule, designated messenger R.N.A., migrates into the cytoplasm via the endoplasmic reticulim and attaches itself to the ribosomal complex of this structure, where it acts as a template for the synthesis of a specific protein. For the incorporation of each amino-acid into the peptide chain, a specific configuration of the purine and pyrimidine bases in the messenger R.N.A. strand is present. This configuration is referred to as the code word for that particular

amino-acid. It is now evident that these code words consist of a triplet of three bases. Therefore, to direct the specific sequential incorporation of amino-acids into a peptide chain, which usually consists of twenty different amino-acids, a minimum of at least twenty code words is required. As there are four of these bases - adenine, guanine, cytosine and uracil, sixty four possible code words may exist. Work on suitable cell free systems has led to the assignment of triplet code words for twenty amino-acids [145]. Two of the bases in each triplet carry most of the information. Although the code is quite specific in that only one amino-acid will be incorporated by a particular code, there is more than one code word for many amino-acids. However, to date, no overlapping of code words for different amino-acids has been shown.

The first stage of protein synthesis requires the activation of amino-acids by a specific enzyme to form the adenosine-monophosphate (AMP) - amino-acid compound. These activated amino-acids are attached to short-chain R.N.A. molecules (transfer R.N.A.) which transfer the activated amino-acids to the messenger R.N.A. where the specific sequential incorporation of amino-acids into the peptide chain is determined by the order of the code words in the R.N.A. strand.

THE SPECIFITY OF PROTEIN BIOSYNTHESIS.

The available evidence then suggests that the process

of protein biosynthesis is highly specific and that, not only the amino-acid composition, but also the sequential arrangement are fixed parameters for a particular protein species. However, this view has not been excepted entirely without question[146, 147, 148]. Tristram [148] suggested that although certain groups in the protein must be correctly spaced and orientated for function, the remaining residues may not be very important and could depend on environmental circumstances. This has certainly been demonstrated in the case of adrenocorticotrophic hormone [138, 139].

The whole problem related to the specificity of protein biosynthesis was reviewed recently by Vaughan and Steinberg [149].

SEQUENTIAL PURITY OF PROTEINS AND PEPTIDES.

The available data on the complete or partial amino-acid sequences of proteins were collected by Vaughan and Williams[149] (p. 121).

In not a single case could deviations from the unique amino-acid sequence for a particular protein be demonstrated. Even the positions of divalent bonds such a cystine did not vary in the proteins where this was examined. There is, therefore, no evidence, as yet, to suggest that variations in the primary structure of a particular protein occur under normal circumstances.

This conclusion, of course, is only valid if the method used in the preparation of the protein is non-selective and if

one/...

one can assume that the protein sample finally isolated is representative of the population synthesised by the tissues.

EVIDENCE FOR THE MICROHETEROGENEITY OF PROTEINS.

Colvin et al [150] introduced the term microheterogeneity to describe the statistical distribution of the structural properties of a particular protein. These authors believed that a unique structure for a particular protein was unlikely, and based their assumption mainly on heterogeneity demonstrated by physico chemical procedures.

Differences in the physico-chemical properties of a molecule, however, do not necessarily imply differences in the primary structure of the molecule, but may be determined by several factors.

During isolation and purification certain changes may occur especially as a result of deamidation of amide bonds which are known to be labile. This has been demonstrated in the case of corticotrophin [151].

Configurational isomerism may be induced with resultant changes in the secondary structure of the protein by certain isolation procedures. The apparent microheterogeneity of albumin is almost certainly due to isomerism with the formation of dimers, and even polymers as demonstrated by Sogani and Foster [152] when albumin is subjected to increased hydrogen ion concentrations.

Apparent/...

Apparent inhomogeneity of a protein may also be produced by the binding of different ions. It is well known that proteins in general, and albumin particularly, have a great affinity for small ions, especially anions. Differences in the number of ions bound at a particular time may, therefore, produce apparent heterogeneity. The observation by Hughes [153] and other workers that only two thirds of the albumin population possess a free sulphydryl group, of course, suggests true heterogeneity, but again this may only reflect a difference in secondary structure.

DIFFERENT MOLECULAR SPECIES OF A PROTEIN OCCURRING IN THE SAME INDIVIDUAL.

There is definite evidence that more than one molecular species of protein may exist in a particular individual. The example of bisalbuminaemia in adults [142, 143, 144] has already been mentioned. Haemoglobin and blood group substances are additional proteins where this principle applies. However, it is almost certain that, under these circumstances, the synthesis of these proteins are directed by more than one gene.

ATTEMPTS AT INDUCING ALTERATIONS IN THE AMINO-ACID COMPOSITION OF PROTEINS BY CHANGING THE COMPOSITION OF THE AMINO-ACID POOL AVAILABLE FOR PROTEIN SYNTHESIS.

Several attempts to test this hypothesis have been made in the past. Czonka [154] claimed to have produced such changes in

eggwhite/...

eggwhite protein. Rao and Wadhwani [155] made similar observations on the total cellular protein of Mycobacterium tuberculosis, while Cohen [156] claimed that the valine content of the proteins formed by a valine-sensitive mutant of E. coli increased when the organisms were grown in a medium containing an excess of valine. However, none of these experimental conclusions are valid as no attempt was made to isolate a specific protein from the organism. Changes in the amino-acid composition so demonstrated, almost certainly reflect an alteration in the relative amounts of different proteins formed under these conditions.

There appears, therefore, to be no concrete evidence that the apparent microheterogeneity of certain proteins under normal conditions is due to differences in primary structure of the molecules. To our knowledge, the only report dealing specifically with this problem is that of Keller and Block [157]. These workers examined the amino-acid composition of bovine serum mercaptalbumin separated into three fractions by chromatography on DEAE cellulose, and claimed that the cystine content of one fraction was significantly different from the other fractions. The experimental conditions of hydrolysis and amino-acid analysis were not discussed by these workers nor was any attempt made to measure sulphydryl groups. The fact that the percentage nitrogen of the albumin preparations was less than 14% renders the significance of these

observations rather doubtful. In the experiments where attempts were made to induce such changes, the results were rendered meaningless through failure to isolate pure proteins from the test organism.

It was therefore decided to investigate the amino-acid composition of serum albumin in kwashiorkor as compared with that of normal persons. Although this condition is not an ideal example of pure protein depletion, the complexity of the metabolic strain imposed upon the biosynthetic mechanisms may be sufficient to induce or unmask pathways for altering the amino-acid composition of a particular protein such as serum albumin should such occult pathways exist.

With these considerations in mind, it is essential to define certain problems related to these particular investigations before discussing the experimental methods adopted.

1. Size of the blood samples available.

The blood samples that can be safely taken from a patient with kwashiorkor are small, and, as aliquots from the specimen collected on the first day of admission were required for blood culture and other biochemical investigations, no more than 5 ml. of clotted blood were available to us in most cases. The serum protein concentrations hardly ever exceeded 4 g./100 ml. of which only approximately half was albumin, the total albumin in the starting material ranged between 40-70 mg. only. It was, therefore,

essential that the method employed should produce high yields and be suitable as a micro-method.

2. Selectivity of the method.

From the theoretical considerations outlined in the previous chapter, it is evident that the preparative method should be non selective and yield a final product which is representative of the entire molecular species of albumin. No changes are likely to be demonstrable if only a fraction of the total albumin is finally obtained as molecules in which differences could be found will be excluded.

3. Effect of the isolation procedure on the native state of the protein.

As outlined before, certain changes especially in the amide content of proteins, may be produced by drastic isolation and purification procedures. Changes in the secondary structure of the protein molecule should not effect the amino acid composition but may influence the hydrolytic process as will be discussed. It is, therefore, essential that manipulations during the preparation of the albumin sample should not be unduly drastic, so as to avoid splitting off fragments from the molecule.

4. Purity of the sample.

Before the structure of any substance can be determined, the preparation to be analysed must be obtained in pure form.

Whereas/...

Whereas this objective can usually be achieved in inorganic chemistry, the same does not apply to macromolecules such as proteins. In the present study, this aim almost certainly could not be realised without employing highly selective procedures producing low yields, which would invalidate any results obtained. In the present investigation, a satisfactory preparation was defined as one which was homogeneous on paper electrophoresis in veronal buffer of ionic strength 0.075 and pH 8.6. Although the albumin component on the paper electrophoretogram contains some α_1 globulin this is quantitatively insignificant [112] and should not effect the validity of the results obtained.

These criteria were considered in the development of methods for the preparation of albumin described in the following section.

CHAPTER VII.

INVESTIGATION OF METHODS FOR THE PREPARATION OF ELECTROPHORETICALLY HOMOGENEOUS ALBUMIN.

exchangers have a high capacity and will separate the plasma proteins into a large number of fractions. The experience of most early workers tended to suggest that homogeneous preparations could not be obtained without some preliminary purification of a complex mixture such as serum or plasma [164, 165, 166]. However, Cohen and Gordon [167] claimed that albumin could be prepared in a sufficiently pure form for iodination to be used in turnover studies in rats. These workers used carboxymethyl cellulose and employed a salt gradient at constant pH for development of the chromatogram. It was, therefore, decided to investigate this procedure, or a modification thereof, as a method for the preparation of electrophoretically homogeneous albumin.

A.

THE PREPARATION OF HUMAN SERUM ALBUMIN BY CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE.

In the procedure of Cohen and Gordon [167] the serum and resin were equilibrated with acetate buffer (0.05M : pH 5.0) and the adsorbed protein eluted by establishing a gradient between the above starting buffer and the limiting buffer (Acetate, 0.4M : pH 5.1), using a constant volume mixing chamber in the gradient device.

METHODOLOGY.

Reagents:

Carboxymethyl cellulose was obtained from Brown Co., U.S.A. By using test sieves, the crude resin was fractionated and the fraction between 150-200 mesh (dry form) was used in all subsequent experiments. A suitable aliquot of the selected fraction was then transferred to a beaker containing H_2O and after proper soaking the non-sedimenting particles were removed by decantation. The resin was then washed on a Buchner funnel with approximately one hundred times its volume of a solution containing NaOH 0.5 M and NaCl 0.5M. This was followed by washing with distilled H_2O until the filtrate was neutral to litmus paper. The resin was then equilibrated with starting buffer until the effluent had the desired pH.

Chromatographic Columns:

The chromatographic columns were made of pyrex glass tubes 1.2 cm. internal diameter and measuring 20 cm. long between the sintered glass disc (porosity grade 2) at the bottom and a B14 groundglass joint at the top. The columns were fitted with ball and socket joints at the bottom for convenient connection of the effluent stream to an automatic recording instrument (L.K.B. Uvicord).

Pouring of the Columns:

Before use the glass columns were cleaned by soaking in a saturated solution of KOH in ethanol, followed by exhaustive rinsing in distilled H_2O. The resin, after proper equilibration, was poured into the columns as a slurry in three sections using an extension tube attached to the top of the column. The resin bed was firmly packed by applying a pressure of 8 lb. per square inch from a nitrogen cylinder until constant height was reached. The resin column was usually between 15 and 18 cm. in length.

Gradient Device:

The apparatus used for producing a gradient is shown in fig. 23. It consisted of a separatory funnel of 500 ml. capacity which contained the limit buffer connected to the mixing chamber, filled with starting buffer, by means of a length of polythene

tubing/...

FIG. 23.

EXPERIMENTAL ARRANGEMENT OF APPARATUS FOR PRODUCING GRADIENT.

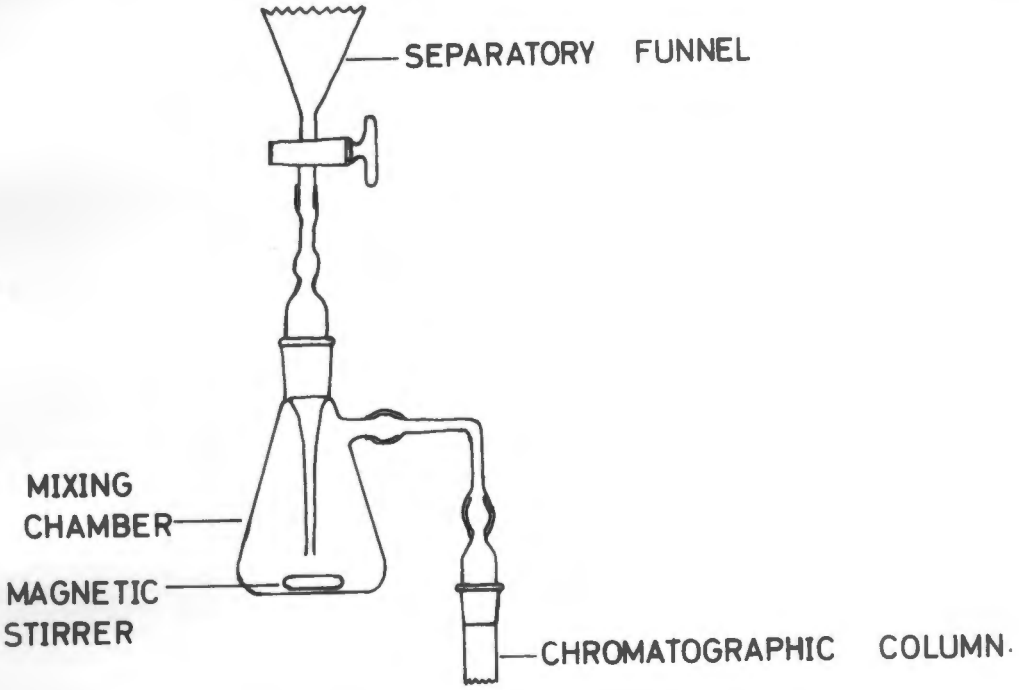
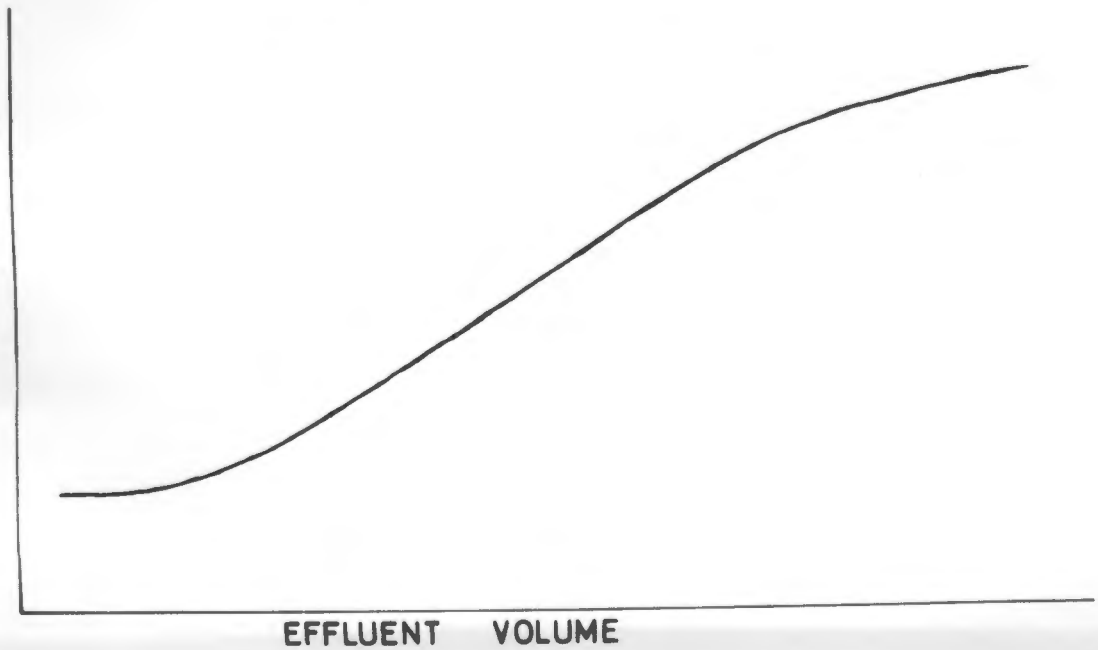


FIG 24

SLOPE OF GRADIENT PRODUCED BY ASSEMBLY SHOWN ABOVE.



tubing and fitted with a suitable ground glass connection. The effluent from the mixing chamber was fed into the chromatographic column using a length of polythene tubing of narrow bore to prevent excessive mixing of the effluent stream. The mixing chamber contained a magnetic stirrer. The gradient produced by this arrangement is demonstrated in fig. 24. As the slope of the gradient will be affected by the volume of the mixing chamber as well as the difference in molarity and hydrogen ion concentration between the starting and limit buffers, these parameters had to be investigated for each set of experiments.

Buffers:

In the pH range 4.25 - 5.25 Acetate buffers of different molarity and hydrogen ion concentrations were used. The composition of the buffers will be specified for each experiment.

GENERAL CHROMATOGRAPHIC PROCEDURE.

The serum to be fractionated was dialysed against the starting buffer at 4° C for 48 hours, changing the buffer at approximately 12 hour intervals. The precipitate, which formed during dialysis, was removed by centrifugation and an aliquot of the dialysed serum, containing approximately 120 mg. of protein, was applied to the column previously equilibrated with the appropriate starting buffer. The sample was allowed to enter the resin under gravity flow and washed in with two aliquots of approximately 2 ml. starting buffer. The space on top of the resin surface was then filled with starting buffer and the column connected to the mixing chamber, also containing starting buffer. The feeding chamber was filled with limit buffer and elution commenced. The

effluent/.....

effluent was collected in 5 ml. fractions by means of an automatic fraction cutter and the absorption of each fraction measured at 280 m μ in a Beckman Model D.U. Spectrophotometer. The absorption at 280 m μ was plotted against the effluent volume whereby a series of peaks were obtained. During the latter part of the present study the absorption of the effluent stream was automatically recorded at 262 m μ in a L.K.B. Uvicord instrument which then became available to us.

The fractions in each peak were pooled and concentrated by pervaporation with intermittent dialysis against distilled H_2O. After determination of the protein concentration on the pooled fractions by the biuret procedure [123], these were examined by paper electrophoresis in a barbiturate buffer of ionic strength 0.075 and pH 8.6

EXPERIMENT I.

Experimental Data:

Buffers:

Starting buffer	:	Sodium Acetate - Acetic acid (0.05M : pH 5.0)
Limit buffer	:	Sodium Acetate - Acetic acid (0.4M : pH 5.1)

Mixing chamber: 200 ml. capacity.

Sample: Normal human adult.

Serum. Total protein 7.8 g./100 ml.
Albumin 5.0 g./100 ml.

FIG 25a EXPERIMENT 1

FRACTIONATION OF SERUM PROTEINS ON C.M.C. GRADIENT, ACETATE, (0·05M : pH 5·0 - 0·4M : pH 5·2).

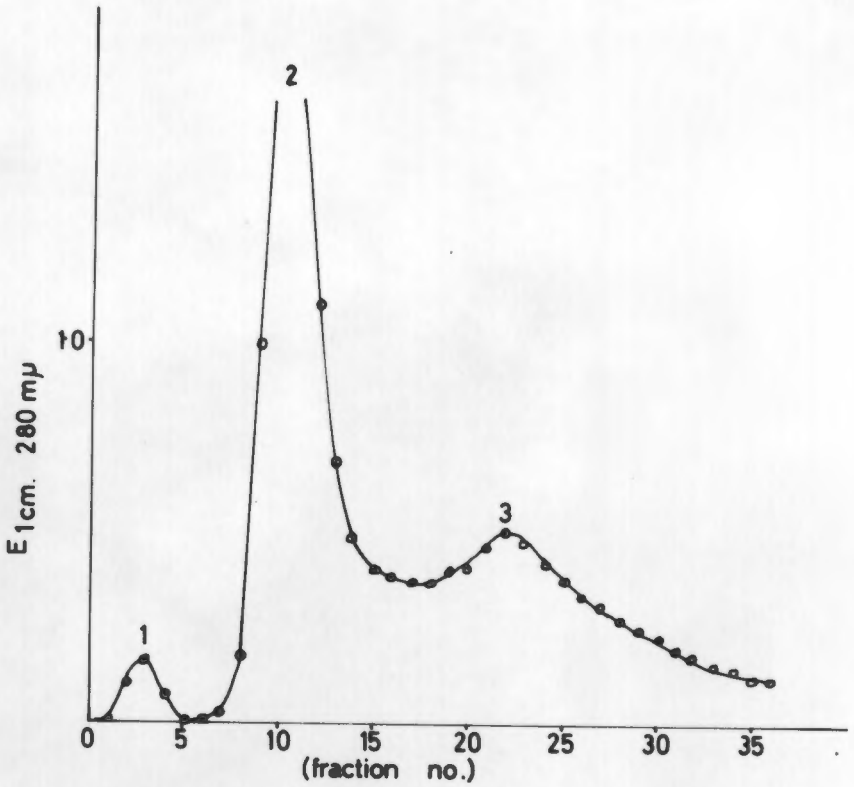
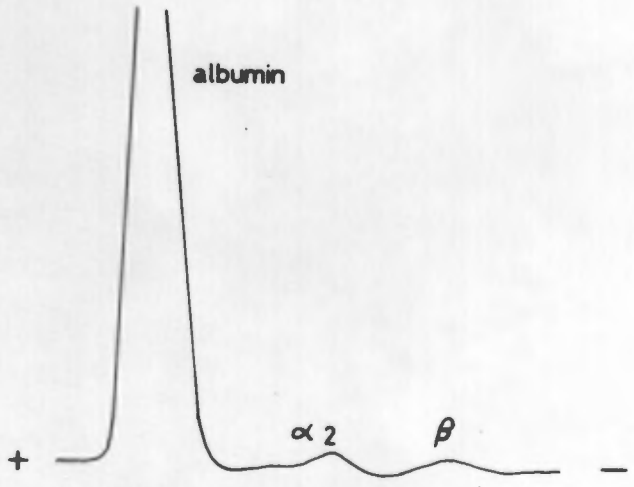


FIG 25b PAPER ELECTROPHORESIS PATTERN OF PEAK 2 (ALBUMIN), ABOVE.



RESULTS:

The effluent diagram obtained by plotting the absorption at 280 m u against effluent volume is presented in fig. 25a. Fig. 25b shows the electrophoretogram of the protein in the peak labelled 2 in fig. 3a. It is evident that, although most of the material migrated as albumin, significant contamination with α 2 and α 1 globulins was still present. Further experiments were conducted, varying the molarity of the limit buffer between 0.150 and 0.4 M while keeping the volume of the mixing chamber constant. Resolution was not improved by decreasing the molarity of the limit buffer but trailing of the effluent peaks became more marked.

EXPERIMENT II.

PRELIMINARY FRACTIONATION WITH $(NH_4)_2SO_4$.

In order to remove the bulk of the unwanted globulin fractions before chromatography, serum was treated with half saturated $(NH_4)_2SO_4$ and the albumin remaining in solution subjected to chromatography.

Procedure:

To fresh normal serum at room temperature was added saturated $(NH_4)_2SO_4$ (sp. gr. 1.245) to give a final concentration of 50% saturation using the calculation $X = \frac{CV}{(100-C)}$ where X is the volume of saturated $(NH_4)_2SO_4$ and V the volume of plasma.

It/...

FIG. 26a SERUM PROTEIN CHROMATOGRAPHY ON CMC FOLLOWING PRELIMINARY PURIFICATION WITH $(NH_4)_2SO_4$.

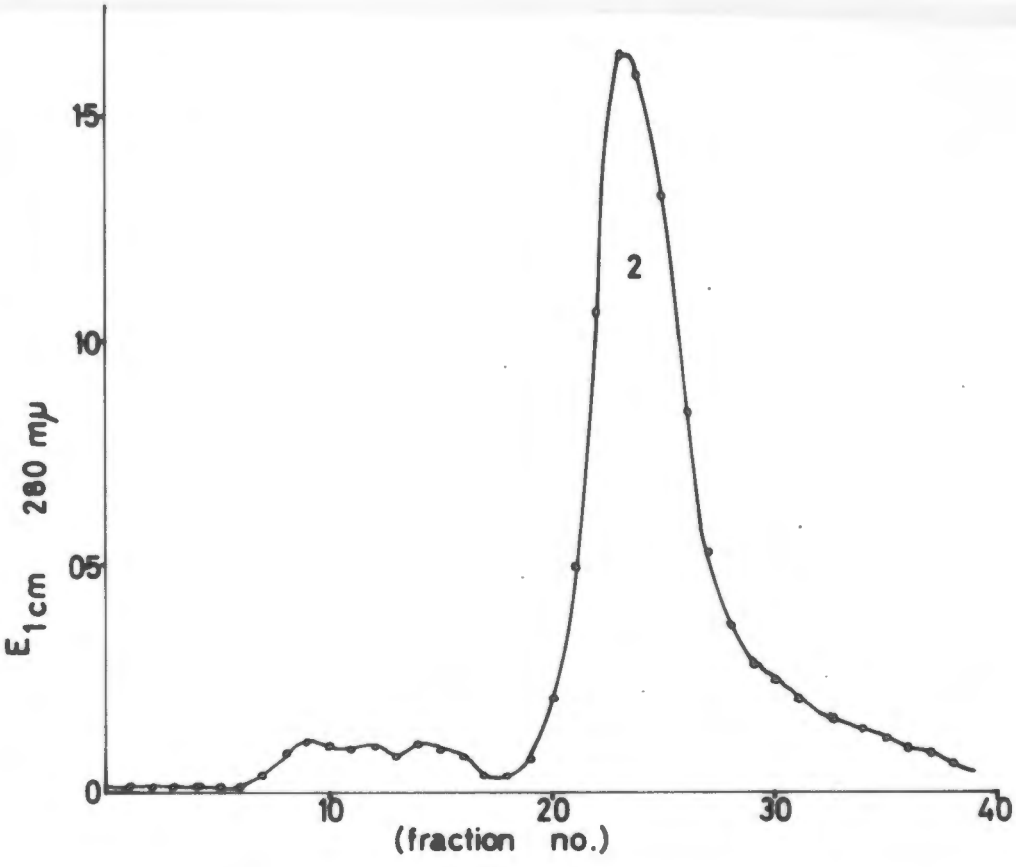
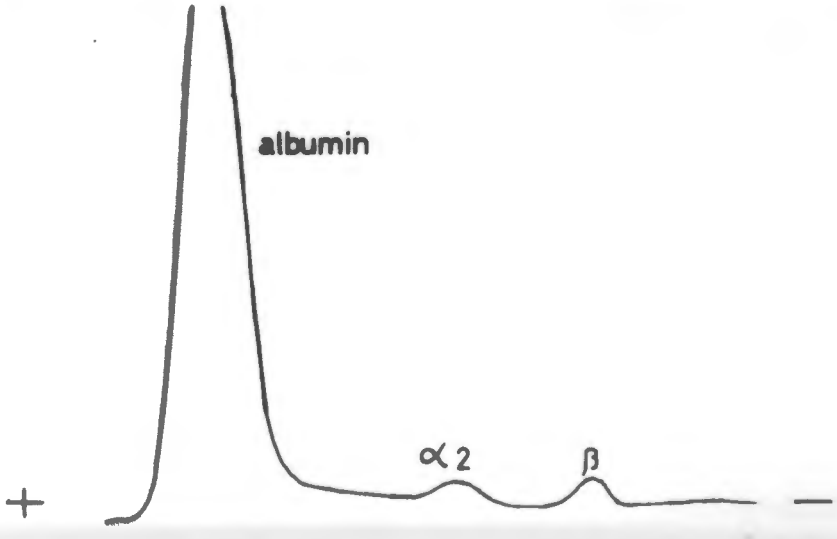


FIG. 26b PAPER ELECTROPHORESIS PATTERN OF PEAK 2 (ALBUMIN), ABOVE.



It was found advisable to check the pH of the $(NH_4)_2SO_4$ and adjust to neutrality when required as some preparations appeared to contain free acid.

The resultant precipitate was removed by centrifugation and the supernatant, containing albumin, dialysed against distilled H_2O until free of NH_4^+ ions. The salt free solution was then dialysed against starting buffer and subjected to chromatography as outlined in Experiment I.

RESULT:

The effluent diagram is shown in fig. 26a while fig. 26b represents the electrophoretogram of the concentrated pooled fractions 20 - 29. Although the effluent peak appeared more symmetrical, protein fractions having the mobility of α 2 and B globulins were still present on electrophoresis. This procedure had the additional disadvantage of requiring prolonged dialysis to remove the ammonium sulphate resulting in marked dilution of the sample. This method was, therefore, not investigated further.

EXPERIMENT III.

THE USE OF A COMBINED pH and SALT GRADIENT.

The results obtained in the previous experiments indicated that the chromatographic procedure used did not have the required resolving power for the preparation of homogeneous albumin as some globulin fractions persistently appeared in the albumin fraction. It was, therefore, essential to define more precisely the way in which the parameters of hydrogen ion concentration and

molarity affect the desorption of albumin from a column of carboxymethyl cellulose.

The conditions required for elution of a given molecule depend upon the number of bonds that can be formed between the absorbent and the adsorbed molecule. Both proteins and cellulose ion exchangers are polyelectrolytes and, therefore, capable of reacting at several points. A molecule having the same nett surface charge density as another (and hence a similar electrophoretic mobility) but exceeding it significantly in size, will require stronger eluting conditions because of the greater number of bonds that can be formed, provided intercharge distances are favourable. This principle was demonstrated for a series of homologous polynucleotides which emerged from a cellulose ion exchange column in the order of increasing size [168, 169]. For this reason a constant relationship between eluting conditions and electrophoretic mobility will not be maintained even at constant pH [170, 171]. It is also known that the concentration and type of anions present in the protein solution will effect the isoelectric points of individual proteins but not necessarily to the same extent [172]. For instance, the isoelectric point of albumin decreases progressively with increasing ionic strength [173].

With these facts in mind variations in the molarity of the starting buffer combined with a pH and salt gradient during elution of the proteins were investigated.

DETERMINATION OF THE SALT CONCENTRATION AT WHICH ALBUMIN IS ELUTED NEAR ITS ISOELECTRIC POINT.

Experimental conditions:

Buffers:

Starting buffer : Sodium Acetate - Acetic acid, 0.01M : pH 4.4
Limit buffer : Sodium Acetate - Acetic acid, 0.1M : pH 5.0

Procedure:

Chromatography was performed, as before, by equilibrating both resin and serum, with starting buffer. The gradient was established through a mixing chamber of 150 ml. capacity.

The pH and sodium concentration of each effluent fraction was determined in a Beckman model G pH meter and EEL flame photometer respectively.

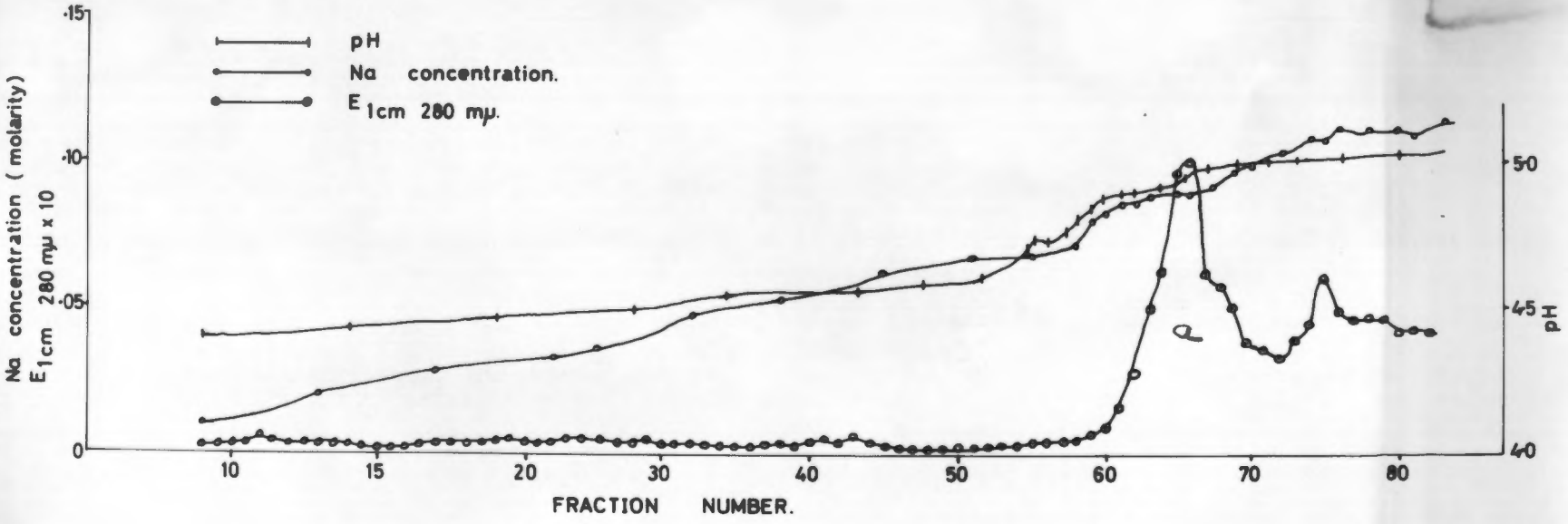
RESULT:

Fig. 27 shows the effluent diagram obtained by plotting absorption at 280 m μ; Na^+ concentration in meq./litre and pH against effluent fraction numbers. It is evident that the peak a, which consisted mostly of albumin on electrophoresis was eluted at an effluent pH of between 4.8 and 4.9 and sodium concentration of 80-90 meq./litre.

THE/.....

FIG. 27.

DETERMINATION OF THE SALT CONCENTRATION AT WHICH ALBUMIN IS ELUTED NEAR ITS ISOELECTRIC POINT.



THE PREPARATION OF SERUM ALBUMIN BY CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE USING A COMBINED PH AND SALT GRADIENT.

METHOD I.

In view of the fact that a significant number of globulins have iso-electric points below that of albumin, (4.8)[174] lowering the pH and increasing the salt concentration of the starting buffer may reduce the nett charge on these molecules sufficiently to prevent their adsorption on the resin or cause them to be eluted before albumin when a pH and salt gradient is used during the development of the chromatogram.

Experimental Conditions:

Gradient Buffers:

Starting buffer	:	Sodium Acetate - Acetic Acid. 0.08M : pH 4.25
Limit buffer	:	Sodium Acetate - Acetic Acid. 0.1M : pH 5.25

Elution: Gradient using a constant volume mixing chamber of 150 ml. capacity.

At fraction no. 59 the limit buffer was replaced by 0.5M NaCl. (fig. 28).

RESULTS:

The peaks obtained in the effluent diagram are shown in fig. 28 where the pooled fractions are numbered 1-4. Peak 4 was obtained by eluting all the retained fractions, following albumin, with a concentrated NaCl solution.

FIG. 28. CHROMATOGRAPHY OF SERUM PROTEINS ON C.M.C. USING A COMBINED pH AND SALT GRADIENT. METHOD I

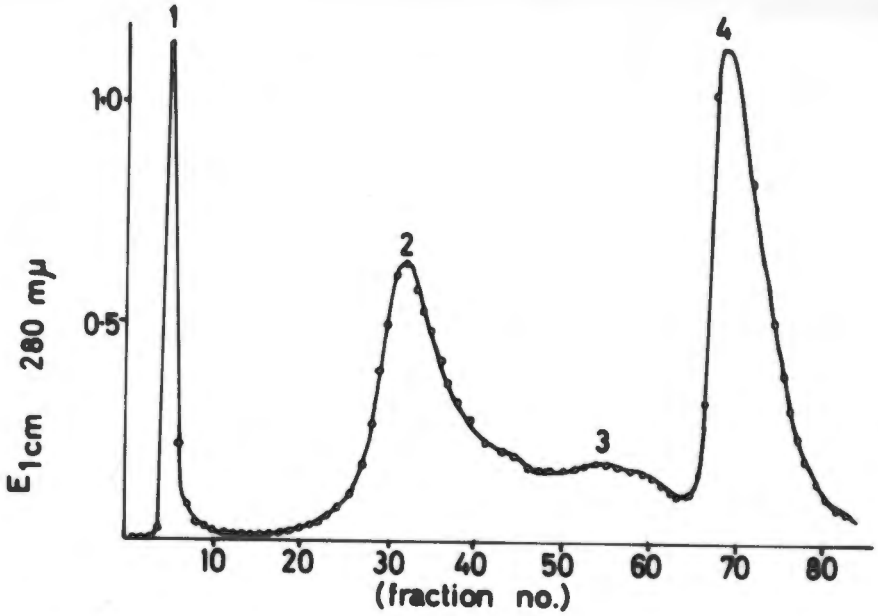
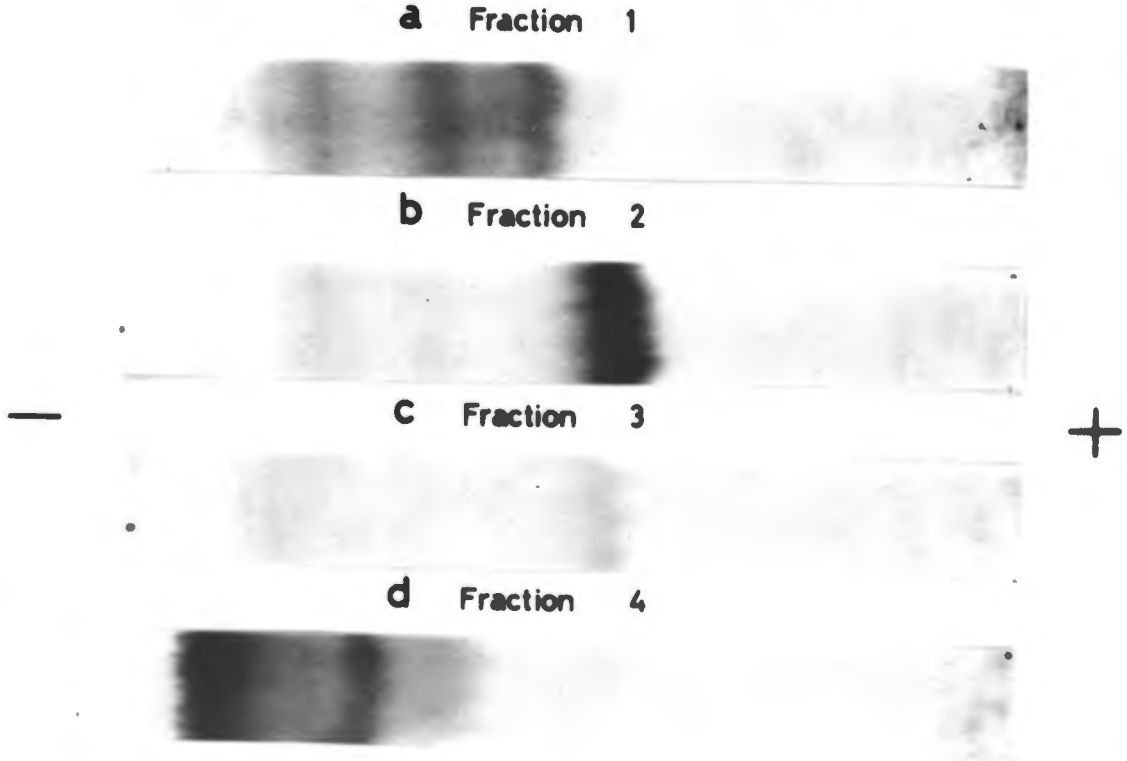


FIG. 29 a-d. PAPER ELECTROPHORETOGRAMS OF POOLED FRACTIONS 1-4 ABOVE.



Electrophoretograms of the pooled fractions are shown in fig. 29a, b, c, d. It is evident that pooled fraction no. 1 contained mostly α 1 and α 2 globulins; peak no. 2 the bulk of the albumin with some albumin in no. 3. Peak no. 4 contained β and γ globulins with some α 2 globulins.

Albumin prepared by this method was subjected to ultracentrifugation * and showed a single boundary (fig. 30) with a sedimentation constant $S_{20} = 4.44$ which agrees well with published figures [174].

* The ultracentrifugation studies were kindly performed by Dr. A. Polson of the U.C.T. and C.S.I.R. Virus Research unit, Cape Town.

COMMENT.

It is evident that by using this gradient system, a fraction of albumin could be obtained which appeared to be homogeneous on paper electrophoresis, and ultracentrifugation. By increasing the salt concentration and hydrogen ion concentration of the starting buffer the desired effect of preventing adsorption of some of the unwanted globulins was achieved. The main danger

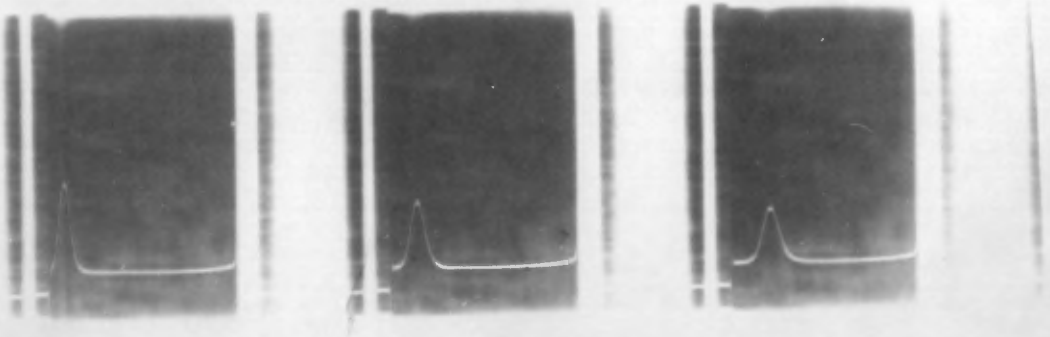
FIG. 30.

SEDIMENTATION DIAGRAM
ALBUMIN.

C.M.C. — ACETATE – pH 4·25—5·25

0·08 – 0·1 M.

GRADIENT ELUTION.



of this procedure is the possibility of isomerisation of albumin in this low pH range. This could lead to selective fractionation of the albumin and decrease the chances of demonstrating differences, in the amino-acid composition, between children with kwashiorkor and normal children. However, the electrophoretic and ultracentrifugation studies did not suggest this and are consistent with the observations by Callaghan and Martin [175] on the rotary dispersion behaviour of serum albumin which suggest that the native configuration of the molecule is retained in the pH range 4-10.

Recoveries of albumin from the original sample by this procedure were low and never exceeded 50-60%. This was a disadvantage as some degree of selective fractionation could therefore not be excluded.

In view of the simplicity and reproducibility of the method, it was decided to analyse some albumin samples prepared by this method from normal children and children with kwashiorkor and compare these results with those obtained on albumin prepared by different procedures, to be described in the following sections. This procedure will be referred to as Method I.

B.

PREPARATION OF HUMAN SERUM ALBUMIN BY CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE FOLLOWING PRELIMINARY FRACTIONATION WITH POLYETHYLENE GLYCOL.

METHOD II.

The albumin preparations obtained by the chromatographic procedure outlined in Section A, Method I, were not entirely satisfactory. As already pointed out, yields were low and in a few specimens prepared by this procedure contamination of α globulins still occurred.

As the contaminant fraction appeared to be the Sα 2 macroglobulin on starch gel electrophoresis, a fraction rich in carbohydrate, difficulties might be experienced as a result of humin formation during hydrolysis. Further experiments were, therefore, conducted in an attempt to find a more selective protein precipitant whereby unwanted globulins could be removed prior to chromatography.

POLYETHYLENE GLYCOL AS A PROTEIN PRECIPITANT.

Several watersoluble linear polymers such as polyethylene glycol, nonylphenol-ethoxylate, polyvinyl alcohol, dextran and polyvinyl pyrrolidone act as protein precipitants [176].

Polyethylene/...

Polyethylene glycol (p.e.g.) which is commercially available as polymers of different molecular weight ranging from 300-20,000 showed good selectivity as a protein precipitant and produced no detectable denaturation of the precipitated proteins even at room temperature. Polyethylene glycol (M.W. 6000) was used as a protein precipitant in the development of methods for the preparation of γ globulin and fibrinogen from human plasma. Homogeneous preparations could be obtained in high yield while the proteins retained full biological activity [176].

Earlier experiments established that hydrogen ion concentration was the most critical factor determining the selectivity of protein precipitation. For example, at pH 7.0, all the γ globulins and the majority of α and β globulins could be precipitated from human plasma by p.e.g. (M.W. 6000) at a concentration of 14% w/v in the reaction mixture. Lowering the pH to 5.9 ± 0.1 resulted in the precipitation of a significant proportion of the remaining globulins, leaving the albumin in solution. These established principles were therefore applied to the present problem, in an attempt to remove the contaminant fractions prior to chromatography on carboxymethyl cellulose.

METHOD.

Reagents:

1. Phosphate buffer (0.2M : pH 7.0).

2./...

2. P.e.g. MW. 6000 (Shell, S.A.).
A stock solution of polyethylene glycol 28% w/v in phosphate buffer (0.2 M : pH 7.0) was prepared. Solution can be facilitated by warming the buffer to approximately 50° C.

3. Acetic acid. 0.5 N.

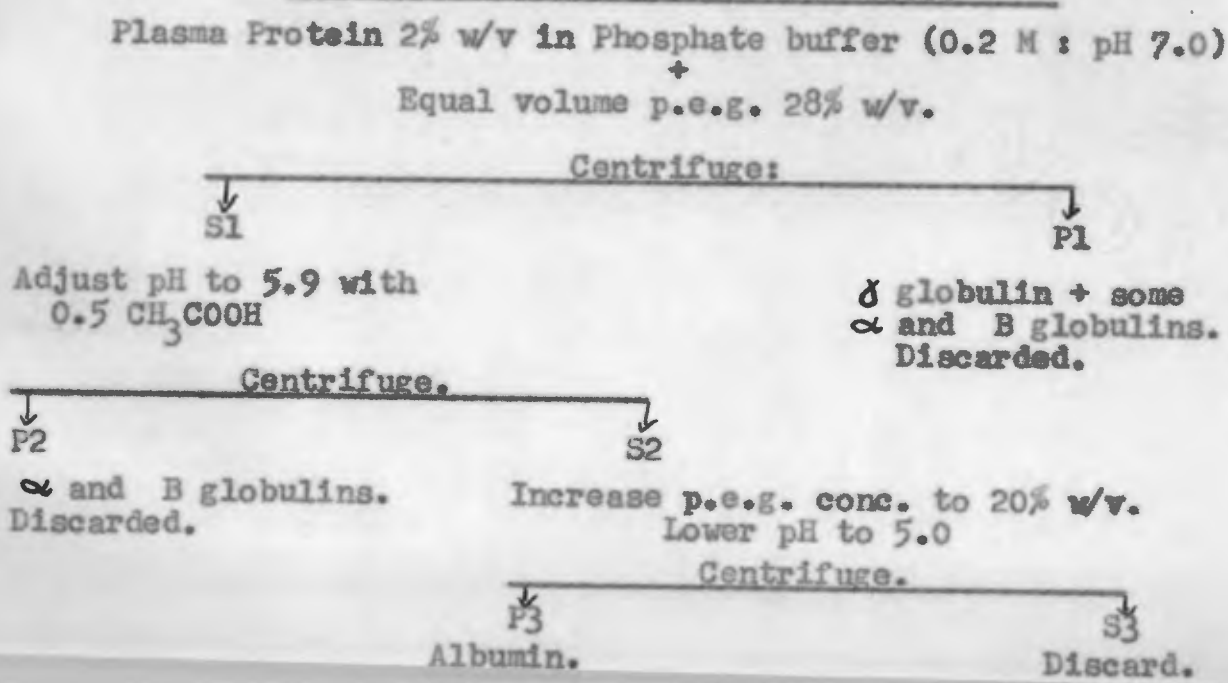
Method:

Normal hyman serum was diluted with phosphate buffer to a final concentration of 2% w/v protein. To the diluted protein solution was added an equal volume of 28% p.e.g. at room temperature (± 20°). The resultant precipitate P1 was removed by centrifugation at 6000 r.p.m. for 20 minutes.

The pH of the supernatant S1 was adjusted to 5.9 with 0.5 N acetic acid and the resulting precipitate P2 removed by centrifugation at 10,000 r.p.m. for 20 minutes. The p.e.g. concentration in the supernatant S2 was then increased to approximately 20% w/v and the pH adjusted to 5.0 with 0.5 N acetic acid, which resulted in the precipitation of the remaining protein P3. The supernatant S3 was discarded after centrifugation at 10,000 r.p.m. for 35 minutes. The precipitate dissolved readily in phosphate buffer (0.2 M : pH 7.0). The procedure is outlined in the flow diagram fig. 31.

Fig. 31.

Preliminary purification of Albumin with polyethylene glycol M.W. 6000.



Results:

Figs. 32a - c show the electrophoretic composition of the precipitated fractions dissolved in Phosphate buffer at pH 7.0 : 0.2M Electrophoresis was conducted at pH 8.6 in Veronal buffer of ionic strength 0.075.

It is evident that fraction P1 precipitated at pH 7.0 in p.e.g. 14% w/v consists of γ globulin with some β and α 2 globulins. Only a trace of albumin is present in this precipitate fig. 32a. Fig. 32b shows the composition of the precipitate P2 which formed by adjusting the pH of the supernatant fraction S1 to 5.9. β and α 2 globulins are selectively precipitated during this step.

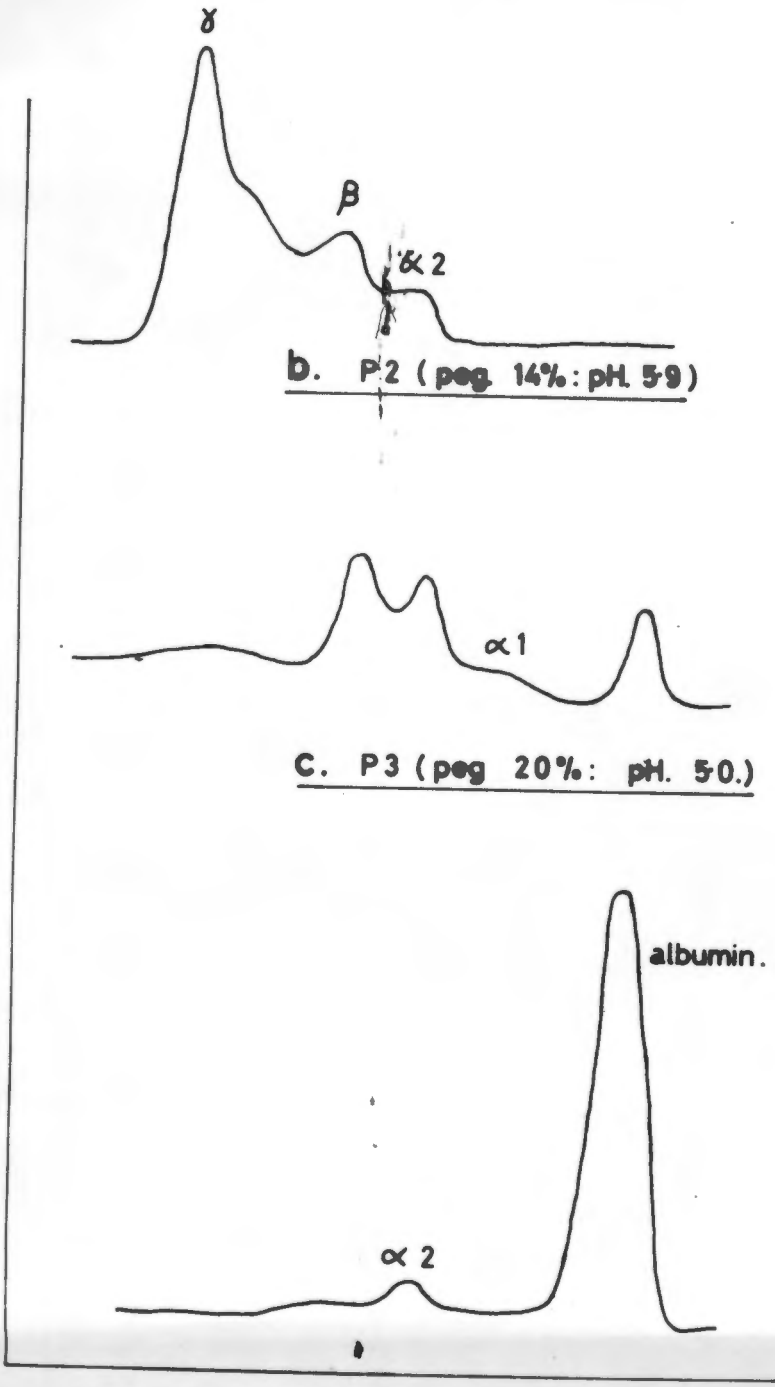
The composition of the final precipitate P3 is given in fig. 32C. Traces of α 2 globulin are still present but the bulk of the material is albumin.

FINAL PURIFICATION OF PRECIPITATE P3 BY CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE.

The partially purified albumin was subjected to chromatography on carboxymethyl cellulose after dialysis against starting buffer (Acetate 0.08 M : pH 4.25) for 48 hours. The limit buffer was Acetate (0.150 M : pH 5.25) and the volume of the mixing chamber 150 ml.

RESULT/...

FIG. 32 a-c. ELECTROPHORETIC COMPOSITION OF PRECIPITATES P1, P2 AND P3 OBTAINED DURING PRELIMARY FRACTIONATION OF SERUM PROTEINS WITH POLYETHYLENE GLYCOL.



RESULT:

Fig. 33 represents the effluent diagram obtained by continuous recording of the absorption at 262 m μ in an L.K.B. Uvicord instrument.

The first effluent peak A contained traces of α globulin and the remaining p.e.g. which behaved as an inert substance and passed straight through the column without being adsorbed.

The peak labelled B on the elution diagram contained the albumin and the last peak C traces of β and α 2 globulin.

It can be seen that the albumin peak B has a bifid configuration. The peak was divided in half by pooling fractions 1 and 2 as indicated in the diagram. These were subjected to paper electrophoresis and the results obtained are given in fig. 34a and b. Both fractions were homogeneous on electrophoresis with the expected mobility of albumin. The interesting finding was, however, that fraction 1 (34^a) travelled slightly further than fraction 2 (34^b) under identical electrophoretic conditions. This observation was confirmed in subsequent investigations.

Detection of polyethylene glycol in the final preparation:

The presence of 0.01% p.e.g. in a solution of protein could be detected by the formation of a white precipitate on

addition/...

FIG. 33. CHROMATOGRAPHY OF SERUM PROTEINS ON C.M.C. AFTER PRELIMINARY PURIFICATION BY PRECIPITATION WITH P.E.G.

Absorption of effluent recorded at 262 mμ in LKB UVICORD.

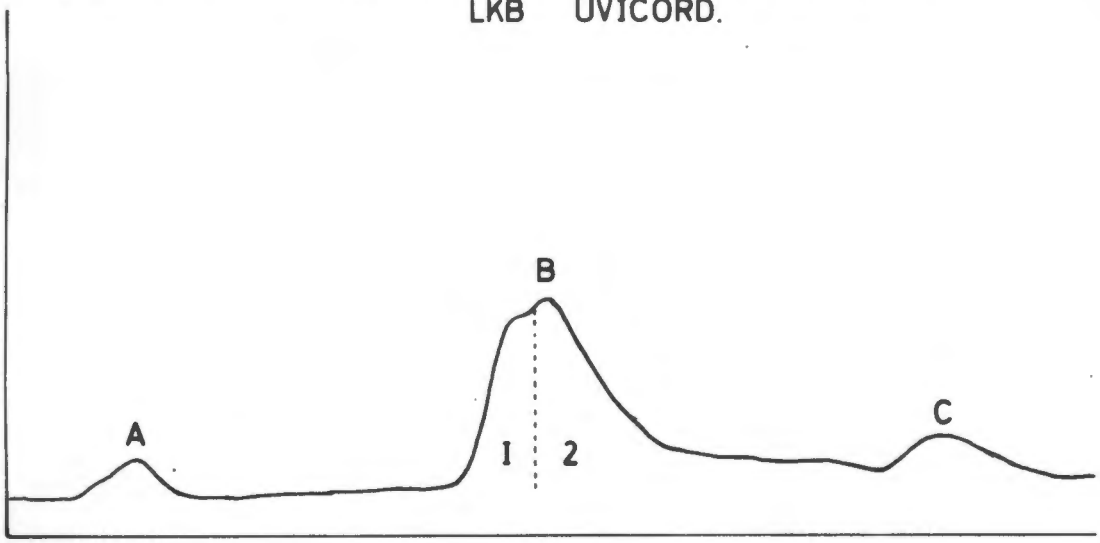


FIG. 34a. ELECTROPHORETOGRAM OF FRACTION 1.

FIG. 34b. ELECTROPHORETOGRAM OF FRACTION 2.

Site of application of sample.

CHAPTER VII.

INTRODUCTION.

The classical methods of protein fractionation and purification were reviewed in several recent publications[158, 159]. These include the wellknown precipitation procedures using organic solvents such as ethanol (Cohen) ether (Keckwick and MacKay) and acetone (Sumner). The use of neutral salts is another wellknown procedure and the sodium sulfate method of Howe [160] is still widely used in determining the albumin-globulin ratios in serum. Although method 6 of Cohen et al [161] or modifications thereof, is still regarded as a reference method for the preparation of various plasma protein fractions, this method did not appear suitable for the present project. In view of the number of different steps required in this procedure, recoveries could not be expected to be high when dealing with such small samples as were available. During the preparation of dog albumin by Allerton et al [162] using method 6 of Cohen only 1.6 g. of albumin was recovered from 440 ml. of plasma.

The introduction, by Petersen and Sobers [163], of the substituted cellulose ion exchange resins, provided a potent tool for the fractionation of protein mixtures. The most commonly used resins are the anion-exchangers, diethylamino ethyl cellulose (DEAE) and carboxymethyl cellulose (C.M.C.) a cation exchanger. These

exchangers/

addition of one drop of the material to 5 ml. of Nesslers' solution [176]. No p.e.g. could be detected in the samples prepared in this way indicating that residual material was effectively removed during the chromatographic procedure. The possibility that p.e.g. might be rendered undetectable by combination with protein was excluded by repeating the test after heat denaturation and extensive enzymatic digestion of the protein. The result was negative.

COMMENTS ON THE PROCEDURE.

(METHOD II.)

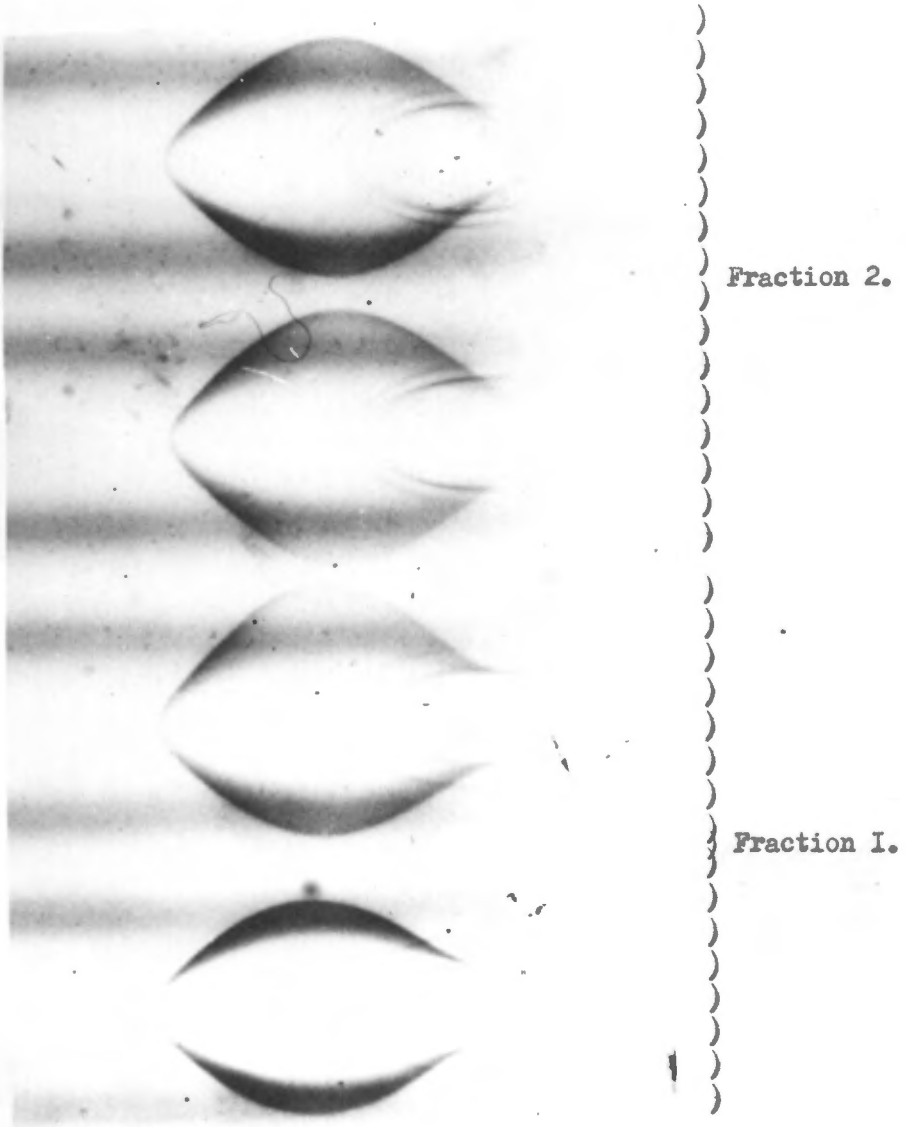
Although recoveries of albumin by this method were only 60-70% of the original the procedure had one important advantage in that the albumin could be separated into two components of different electrophoretic mobility. By hydrolysing and analysing these two components separately, differences in amino-acid composition were more likely to be detected. This practice was, therefore, adopted for samples prepared by this method from children with kwashiorkor and normals and will be referred to in the section on results.

It is evident from the results obtained on immuno electrophoresis (fig. 35) that the preparations obtained contained only faint fraces of material in the α 1 zone.

This procedure will be referred to as Method II (P.e.g. C.M.C.) in subsequent discussions.

FIG 35.

Immunoelectrophoresis diagrams of serum albumin prepared by Method II (P. e.g. C.M.C.).



C.

PREPARATION OF ALBUMIN BY PRECIPITATION WITH TRICHLORACETIC ACID.

METHOD III.

Method II based on preliminary fractionation with polyethylene glycol gave satisfactory preparations with regard to homogeneity but as already discussed yields were still low. It was decided to investigate additional methods for the preparation of albumin in order to obtain near quantitative recoveries of albumin from the samples of serum. This appeared to us to be an important consideration as preparations obtained in low yields could selectively exclude those molecular species of albumin in which demonstrable variations in amino-acid composition might be present.

Recently several workers observed that albumin was soluble in certain acidified organic solvents, whereas globulins were not. Delaville et al [177] appear to be the first workers to make this observation. At about the same time Levine [178] noticed that bovine albumin precipitates obtained in 5% w/v aqueous trichloracetic acid were soluble in acetone, methanol and ethanol but not in the higher alcohols, nor in benzene, dioxane, ethyl acetate, nitrobenzene, or carbon tetrachloride.

The implications of these observations were realised by

Schwert [179] who applied this principle to the preparation of albumin from serum and examined the physicochemical properties of the product so obtained.

Debro et al [180] described a method for measuring the albumin-globulin ratios in serum using the trichloracetric acid procedure and his results showed excellent agreement with those obtained by the classical moving boundary procedure suggesting that quantitative recoveries of albumin could be obtained by this procedure. Vallance Owen et al [181] modified the procedure used by Debro [180] somewhat and used this method to prepare albumin for biological studies. Michael [182] recently again investigated these observations and described the solubility of albumin in different combinations of acid and organic solvents. He also studied the physico-chemical and antigenic properties of the preparations.

From the results of these workers, it was evident that albumin homogeneous on ultracentrifugation and electrophoresis could be obtained by methods employing this principle. Furthermore, it appeared as if this somewhat revolutionary and harsh treatment of a complex protein molecule produced no untoward effects on the physico-chemical or antigenic properties of the preparation.

In the method of Michael [182] the globulins are precipitated from solution by the addition of acidified methanol. The albumin retained in solution is then precipitated by neutralisation of the solution and washed with ethanol and ether before drying over P_2O_5.

This procedure proved to be unsatisfactory in our experience as the dried protein failed to dissolve properly. The method described by Vallance Owen et al [181] in which the albumin and globulins were both precipitated in aqeous trichloracetic acid followed by dissolving the precipitated albumin in acidified ethanol, was finally adopted.

METHOD.

Reagents:

1. Trichloracetic acid, 10% w/v made up in deionised water.
2. Trichloracetic acid 5% w/v in deionised water.
3. Trichloracetic acid 1% w/v in 96% aqeous ethanol.

Procedure:

To serum at room temperature was added an equal volume of 10% trichloracetic acid. After thorough mixing, the solution was allowed to stand for 10 minutes followed by centrifugation at 2800 r.p.m. for 15 minutes. The supernatant was discarded and the precipitate washed with 5% trichloracetic acid. The washed precipitate was resuspended in 1% trichloracetic acid in 96% alcohol to dissolve the albumin. The undissolved globulins were removed by centrifugation and the supernatant, containing albumin, dialysed against several changes of dist. H_2O for 48 hours at 4° C to remove the trichloracetic acid and alcohol. A heavy precipitate formed during the first few hours but disappeared later, leaving only a faint precipitate after 48 hours. The solution was finally clarified by centrifugation and concentrated by pervaporation. Recoveries of albumin by this procedure varied between 80 and 90% in our experience.

Electrophoretic Properties of T.C.A. prepared albumin.

Paper electrophoresis at pH 8.6 revealed a single peak

with/...

FIG. 36. PAPER ELECTROPHORESIS DIAGRAM OF HUMAN SERUM ALBUMIN PREPARED BY THE T.C.A. METHOD.

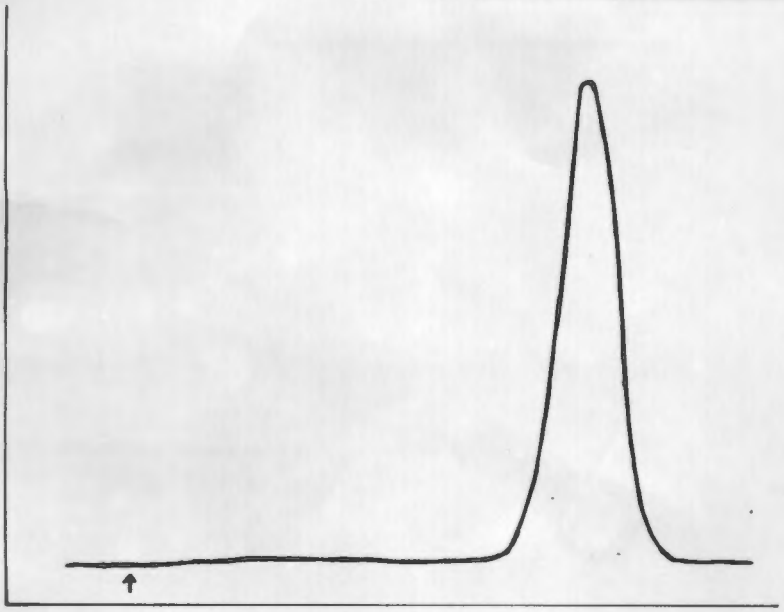
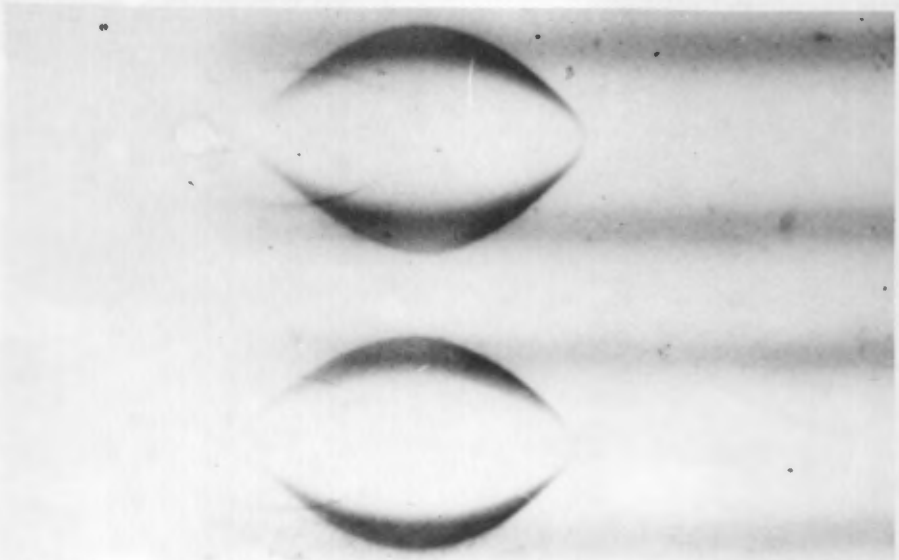


FIG. 37. IMMUNOELECTROPHORESIS DIAGRAM OF HUMAN SERUM ALBUMIN PREPARED BY THE TCA METHOD.



with the expected mobility of albumin. There was no evidence of denatured material remaining at the site of application of the sample (fig. 36).

On immuno electrophoresis two faint zones were detectable in the α 1 position but these appeared to be quantitatively insignificant (fig. 37).

COMMENT.

The elegance of the method and the high yields of albumin obtained in electrophoretically homogeneous form were important advantages. In addition this method can be used satisfactorily on small samples of serum and has no deleterious effect on the albumin so prepared. The observations of Rubin et al [183] who showed that small peptides were split off the albumin molecule at low pH values, were a source of concern. However neither Schwert [179], nor Michael [182] could demonstrate any alterations in the physicochemical or antigenic properties of albumin after treatment with trichloracetic acid.

CHAPTER VIII.

AMINO-ACID ANALYSIS OF PROTEIN HYDROLYSATES.

CHAPTER VIII.

INTRODUCTION.

The numerous and formidable difficulties attending amino-acid analysis of complex mixtures obtained during the hydrolysis of proteins, are attested to by the vast literature on the subject. Until the development of suitable chromatographic procedures by Moore and Stein the main methods used were:-

1. Chemical:

These depended on purely chemical procedures consisting either in isolating and weighing the individual amino-acids or causing them to react specifically with a compound to form a coloured product which could be measured photometrically.

The use of specific precipitants for the isolation of individual amino-acids were discussed by Stein and Moore [193].

2. Physico-Chemical:

The classical example in this category is the isotope dilution technique of Shemin and Foster [194]. This method of estimating a given amino-acid in a protein depends on adding to an hydrolysate of the protein a definite amount of a pure preparation of the amino-acid containing a known percentage of N^{15} and then to determine the proportion of labelled to unlabelled N in a pure specimen of the amino-acid isolated from the hydrolysate.

Although/...

Although accurate, the cost of labelled materials and the necessity for isolating each amino-acid in pure form prevents this method from being generally adopted as a routine procedure.

Microbiological:

Methods based on the requirements of certain bacteria for specific amino-acids have been successfully employed in amino-acid analysis and are reviewed by Snell [195]. This method is sensitive but requires vigorous control and experience as the growth of living organisms is subject to numerous extraneous influences which are not always predictable.

AMINO-ACID ANALYSIS BY AN ION EXCHANGE CHROMATOGRAPHIC PROCEDURE.

In most of the modern methods of amino-acid analysis, the amino-acids in a protein hydrolysate are first separated by a chromatographic procedure on columns containing a suitable ion exchange resin. The separated amino-acids in the effluent stream are then measured by a photometric procedure based on the blue colour formed in the reaction between α amino-acids and ninhydrin.

In this section on amino-acid analysis, the ninhydrin reaction will be described first, as the standardisation of the chromatographic procedure is based on this reaction.

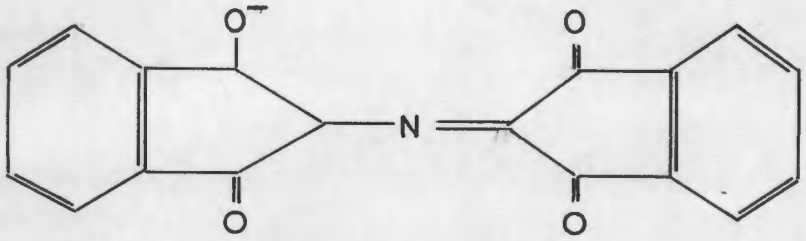
THE NINHYDRIN REACTION.

The blue coloured endproduct obtained in the reaction between amino-acids and ninhydrin (triketohydrindene hydrate) forms the basis for most of the colorimetric procedures used in the quantitative estimation of amino-acids today. Although the sensitivity of the reaction for qualitative work is well known, early workers had great difficulty in rendering the method quantitative [184, 185]. These difficulties appear to result primarily from the presence of dissolved oxygen in the reaction mixture and yields can be increased by performing the reaction in sealed tubes evacuated to 20 mm. [186]. By the addition of strong reducing agents the side reactions due to oxidation can be avoided. Ruheman [187] initially used hydrindantin, the reduced form of ninhydrin, but subsequently changed to stannous chloride. Ascorbic acid was found to be unsatisfactory as coloured condensation products were formed with the amino-acids [188]. Earlier work also established that the reaction is not specific for α amino-acids but coloured products are also formed with peptides, proteins, amino sugars or other substances possessing free amino groups [186].

NATURE OF THE REACTION:

Ruheman [187] and subsequently Moore and Stein [186] studied the reaction between α amino-acids and ninhydrin and isolated the

blue compound to which Ruheman [187] assigned the structure of diketohydrindylidene diketohydrindamine.

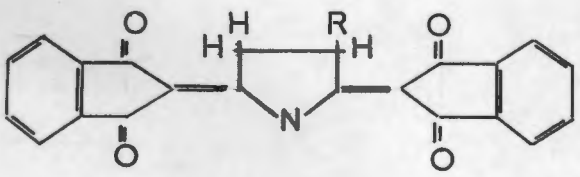


The reaction is thought to proceed as outlined in Fig. 38.

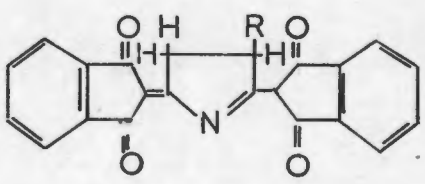
The absorption maximum of the isolated crystalline salt of this blue compound is at 570 m μ, the same as that of the reaction mixtures of ∝ amino-acids with ninhydrin [186].

The reaction between ninhydrin and hydroxyproline and with proline was investigated by Grassman and Von Arnim [189]. The chemical structure of the yellow coloured product formed by these two amino-acids was elucidated by these workers [189] who proposed structures 1 and 2.

1

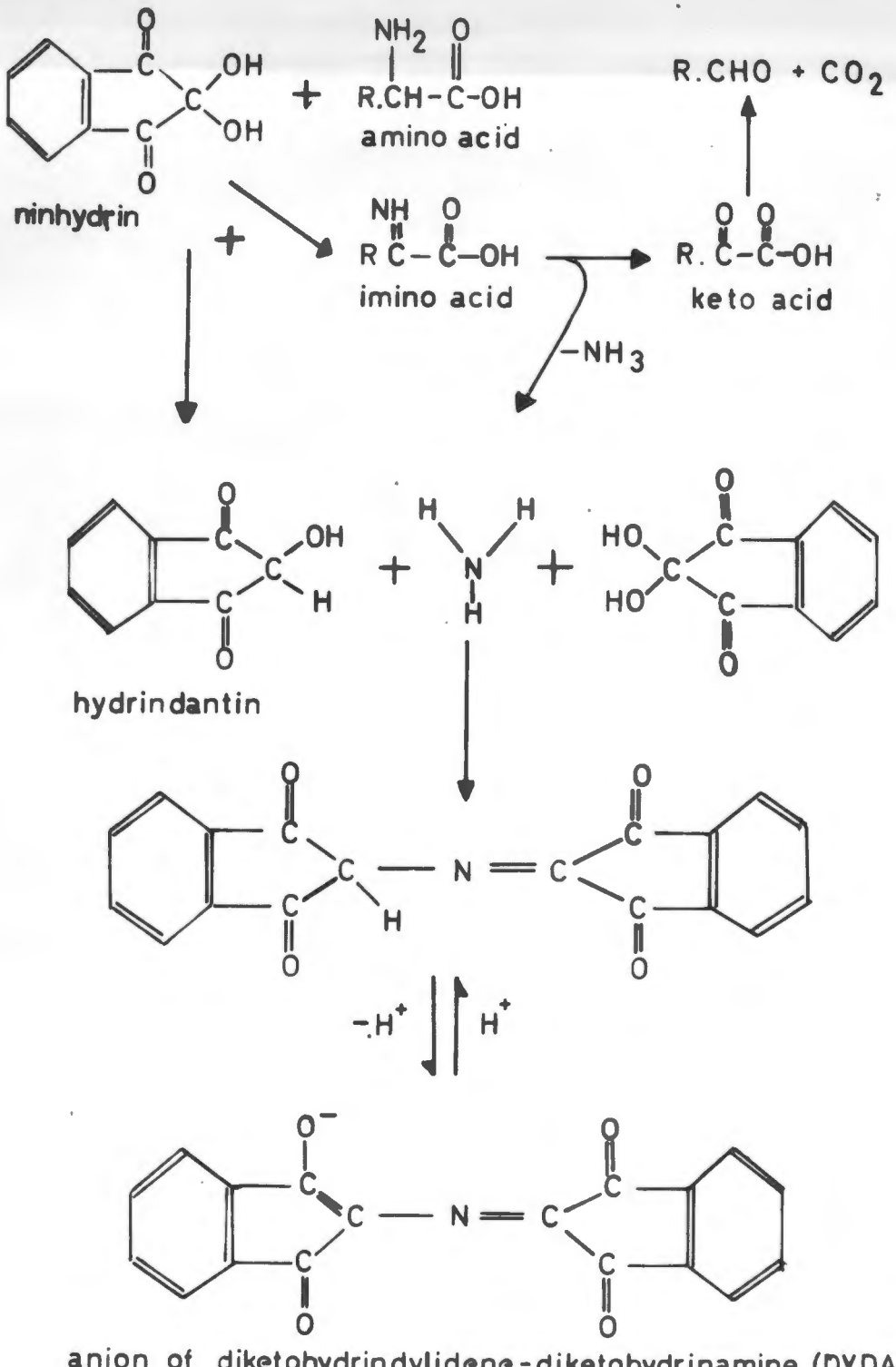


2



In/...

FIG 38 THE NINHYDRIN REACTION



In the photometric procedure described by Moore and Stein [186] stannous chloride was used as the reducing agent. These workers determined the colour yields from α amino-acids and other ninhydrin positive substances and found that the colour yields for all amino-acids were not the same. For example, when expressed on a molar basis relative to leucine colour yields from lysine were 12% higher and aspartic acid 12% lower. In the case of hydroxy-proline and proline, the yields of the yellow coloured products were only one-seventh and one-fourth respectively of those obtained with an equimolar solution of leucine. The absorption maximam of these products were found to be at 440 mμ.

Some other important observations were made.

REACTION WITH AMMONIA:

Ammonia gives no colour with ninhydrin unless hydrindantin is present in the reagent solution. Maximum colour yields for ammonia therefore depend on the presence of an adequate concentration of hydrindantin. During the oxidative deamination of amino-acids by ninhydrin, 1 equivalent of the reagent is reduced during the reaction to form hydrindantin. Ammonia itself cannot affect this reduction.

OPTIMUM pH OF THE REACTION:

Maximum colour yields for all amino-acids except tryptophan are obtained at pH 5.0. In the case of the latter the optimum was at pH 6.0.

In/...

In 1954, Moore and Stein [190] described a modified reagent replacing stannous chloride by an excess of hydrindantin in the reagent. Although the reagent was less stable, the troublesome precipitates encountered during the original procedure were avoided. The use of a strong reagent buffer eliminated pH adjustments of the effluent fractions prior to analysis. Colour yields were still below the theoretical obtained by performing the reaction in a predominantly organic solvent [191], but recoveries were reproducible. The ninhydrin procedure, adopted in the present study, was based on the modified reagent using hydrindantin instead of stannous chloride.

METHODOLOGY.

REAGENTS:

1. Ninhydrin:- British Drug Houses, A.R. Ninhydrin was not recrystallised before use.

2. Hydrindantin:- Prepared by reduction with ascorbic acid as described by Moore and Stein [190]. The prepared reagent was dried and stored over P_2O_5 in a desiccator kept in the dark.

3. Methyl Cellosolve: (2 Methoxy-Ethanol) B.D.H. Technical grade reagent. Each bottle was tested for peroxide content. It was soon discovered that the blank values obtained varied from batch to batch - Table VI.

TABLE/...

TABLE VI.

Blank values obtained on ammonia-free distilled water with different batches of methyl cellosolve.

B.D.H. technical grade Methyl cellosolve:	Blank Values Colorimeter Units.
Batch No. 635960	55
753450	401
696950	84
659810	65
468520	98
Merck's technical grade	
Batch No. 6160	79
May and Bakers Technical Grade.	
Batch No. 67006	81

Blank values remained high even after distillation under reduced pressure following treatment with cation exchangers, Na_2SO_4, $Na_2S_2O_5$ or CaO. The methyl cellosolve used was, therefore, selected by screening each batch for suitably low blanks.

Jacobs [192] recently recommended distillation of the solvent after addition of 10 ml. of a solution containing 50 g. $FeSO_4$ + H_2O in 100 ml. $2NH_2SO_4$.

4. Ethanol 50% v/v in dist. H_2O.
Used as a diluent for the coloured complex.

5. 4N. Sodium Acetate Buffer (pH 5.5).
To 2 Litres of dist. H_2O, add 2720 g. of NaO.AC.$3H_2O$ (A.R.) and stir on a steam bath until solution is complete. Cool to

room temperature, add 500 ml. of glacial acetic acid, and make up to volume of 5 litres after pH adjustment.

PREPARATION OF REAGENT:

20 gram of Ninhydrin and 3.0 gram of Hydrindantin dissolved in 750 ml. of methyl cellosolve in a dark reservoir from which the air has been displaced by nitrogen. Add 250 ml. of pH 5.5 acetate buffer and store under nitrogen. Sufficient reagent was prepared for 48 hours supply in most experiments.

COLORIMETRIC PROCEDURE.

As a general procedure colour development was carried out as follows:-

To 1 ml. of the standard or unknown solutions in a test tube was added 1 ml. of ninhydrin reagent using a burette. After shaking for 10 seconds, the tube was transferred to a boiling water bath and left for exactly fifteen minutes (stop watch). The reaction mixture was then diluted with 5 ml. of 50% aqeous ethanol. The contents were allowed to cool to room temperature and then shaken for half a minute to oxidise the excess hydrindantin before reading in a suitable photo-electric colorimeter. In our experiments a Klett-Summerson instrument was used. When analysing effluent samples from the chromatographic columns, analysis were performed in batches of 25 by using removable copper racks which fitted the waterbath exactly. With each alternate set of twenty-five effluent fractions, a standard amino-acid solution of known

composition was examined in duplicate as a check on the colour yield of the ninhydrin reagent. When yields were below the expected value the reagent was discarded and a fresh solution prepared.

EXPERIMENTAL.

a. Determination of absorption spectra of the ninhydrin complexes.

Fig. 39 shows the absorption spectra of different amino-acids, at varying concentrations, as determined in a Beckman Model DU Spectrophotometer. In addition to the main peak at 570 m μ there appears to be a second peak at approximately 400 m μ best demonstrated at higher concentrations of amino-acids. The sharp increase in absorption below 400 m μ is due to the reagent itself. The absorption peak of proline is poorly resolved and a maximum could not be accurately established.

b. Selection of Proper Filters for use in the photoelectric colorimeter:

A filter with maximum transmission at 570 m μ could not be obtained for the Klett Summerson instrument. As the coloured complex absorbs over a wide range with a maximum at 570 m μ and the curve is symmetrical, the suitability of a green filter with peak transmission at 540 m μ was investigated.

The ratios between the absorption of the coloured complex

at/...

FIG. 39

SPECTRAL ABSORPTION OF AMINO ACID — NINHYDRIN COLOUR COMPLEX.

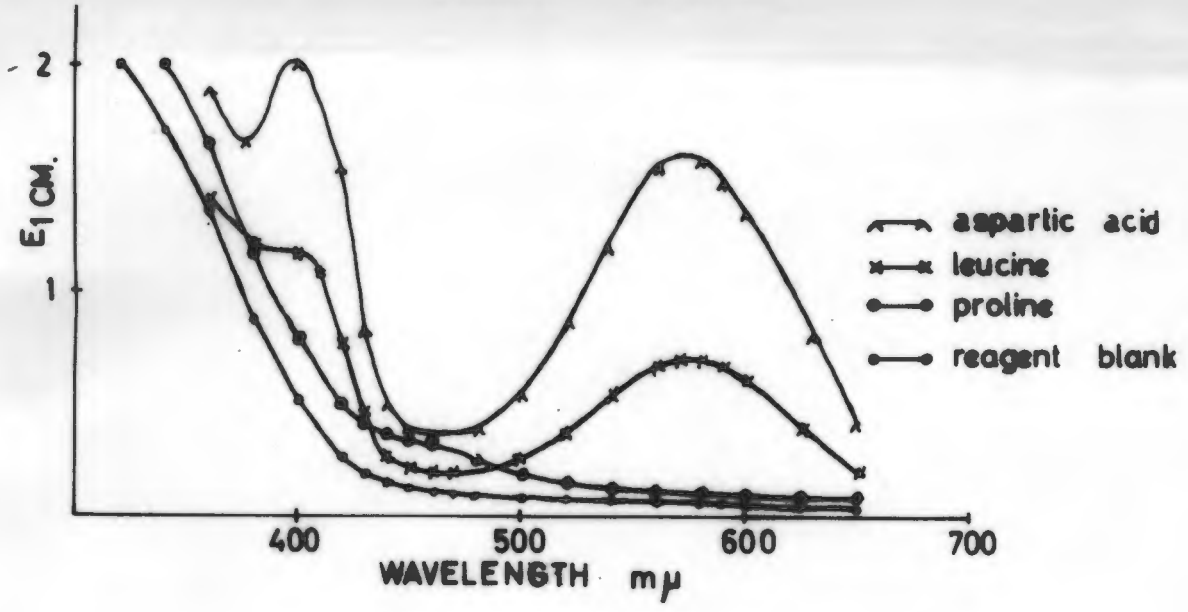
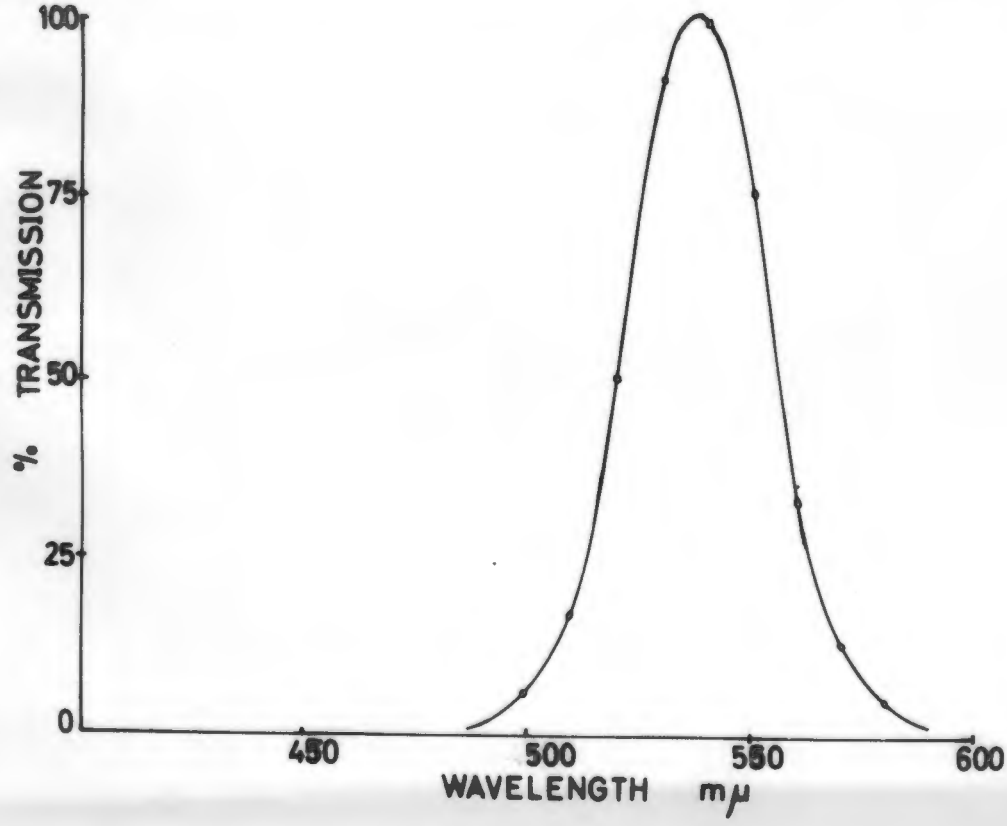


FIG 40

SPECTRAL TRANSMISSION OF GREEN COLORIMETER FILTER



at 540 m μ and 570 m μ was therefore determined in the Beckman DU Spectrophotometer. See table VII.

TABLE VII.

Ratios between absorption of different amino acid + ninhydrin complexes at 540 m μ and 570 m μ

Amino Acid.	Ratio. $\frac{540}{570}$ m μ
Aspartic Acid	0.795
Arginine	0.805
Phenyl alanine	0.813
Threonine	0.788
Leucine	0.798

It is evident that the ratios are remarkably similar for different amino acids and that the absorption at 540 m μ is about 80% of the maximum at 570 m μ. This would mean that by measuring the coloured complex at 540 m μ instead of 570 m μ the sensitivity of the method would be decreased by approximately 20%.

TRANSMISSION SPECTRUM OF THE GREEN FILTER.

In order to test the efficiency of the green filter as a

source of monochromatic light in the wavelength range 540 m μ, the transmission spectrum of the filter was determined in the Beckman model DU spectrophotometer.

It can be seen from fig. 40 that the transmission curve of the filter is symmetrical with a maximum at 535 m μ. As only light of wavelengths between 500 and 600 m μ is transmitted, unpredictable results due to nonspecific absorption should not occur. Although the sensitivity of the method would be somewhat reduced, there appeared to be no other theoretical objections provided that those amino acids occurring at lowest concentrations in the albumin molecule could be accurately measured on the quantities of protein usually analysed in the chromatographic procedure.

STANDARD CURVES OF INDIVIDUAL AMINO ACIDS.

Amino acids:-

All amino acids were Laboratory Grade Chemicals obtained from B.D.H. Before preparation of standard solutions, the chemicals were dried to constant weight over P_2O_5 in a vacuum desiccator.

Stock solutions were prepared in 0.8 N.HCl (ammonia-free) and contained between 5 and 10 micromoles of amino acid/ml. Dilutions of the stock preparations were made to give final concentrations in the range 0-0.4 μ moles per ml. Each dilution

FIG. 41

STANDARD CURVES FOR SOME AMINO ACIDS DETERMINED BY THE NINHYDRIN REACTION.
(reaction volume 7 ml)

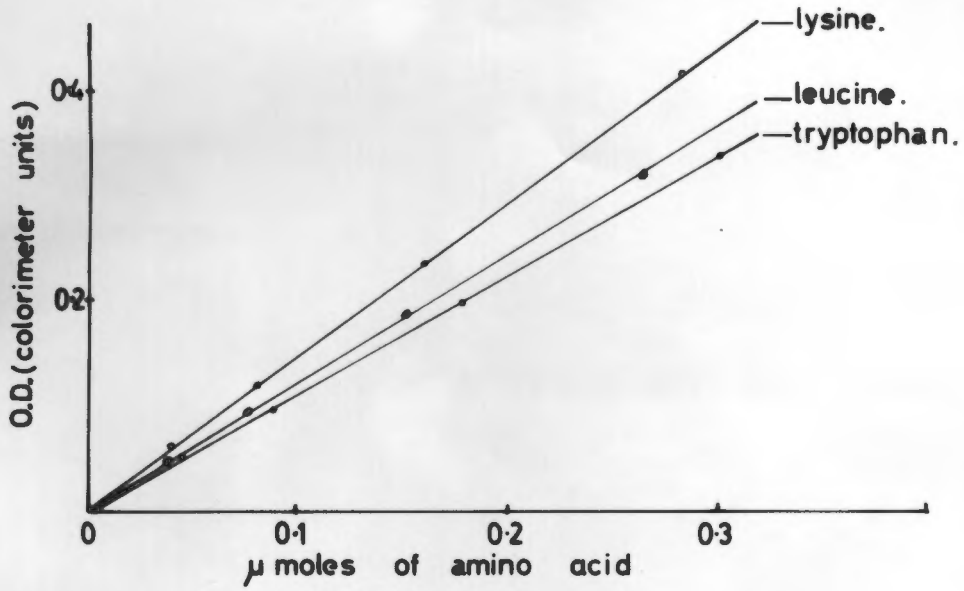
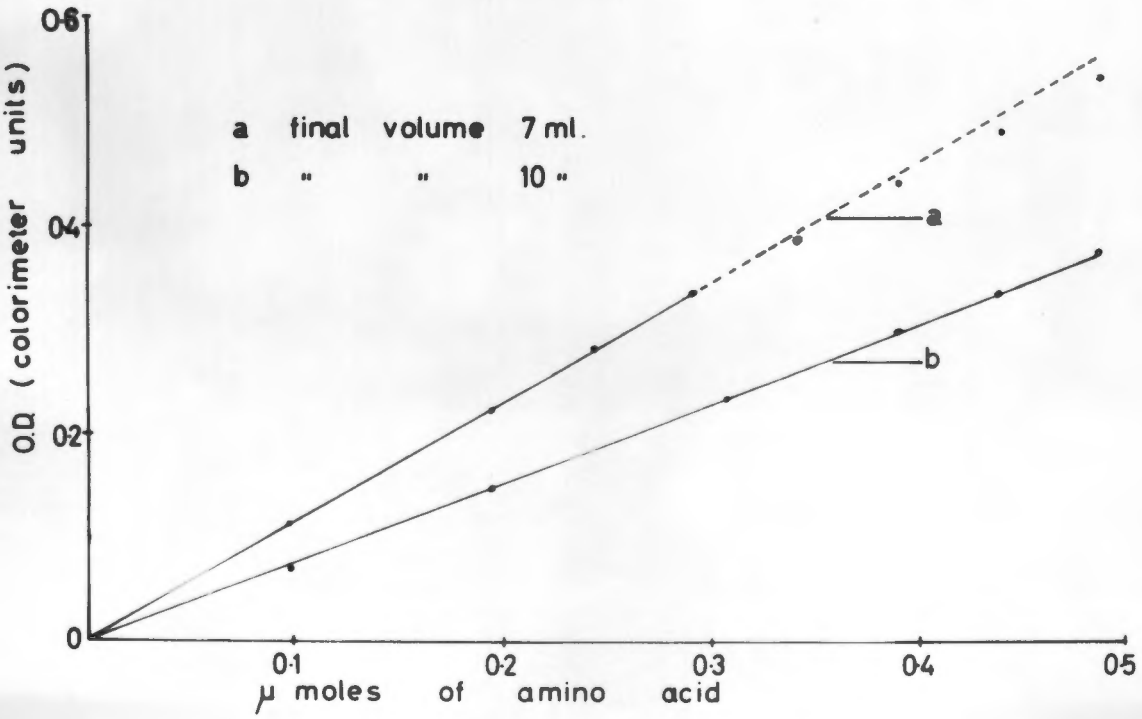


FIG. 42

STANDARD CURVES FOR GLYCINE AT DIFFERENT DILUTIONS OF THE REACTION MIXTURE.



was analysed in triplicate by the ninhydrin procedure described and the optical density measured in the Klett-Summerson colorimeter using the green filter (540 m μ). Appropriate solvent blanks were carried through with each experiment. The standard curves obtained on representative amino acids are given in fig. 41.

It is evident that Beer's law is obeyed for concentrations up to 0.35 μ moles in a final reaction mixture of 7 ml. At higher concentrations, there is a deviation from the straight line relationship but by diluting the reaction mixture to a final volume of 10 ml. the linearity of the curve is re-established (fig. 42). In all cases where the optical density exceeded 400 colorimeter units, further dilutions were made using appropriate correction factors when calculating the results.

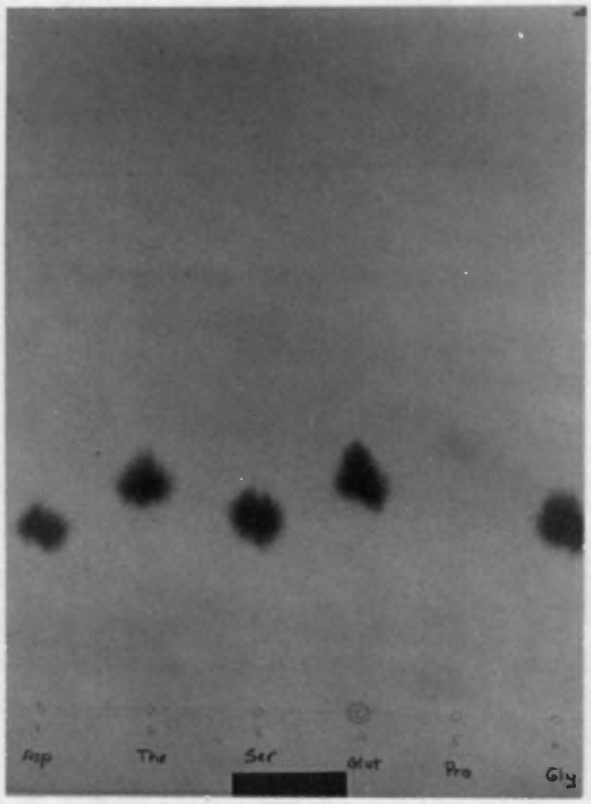
Colour Yields of Individual Amino acids.

From the slopes of the standard curves (fig. 41) it becomes evident that the colour yields for different amino acids are not the same.

The yields obtained from the different amino acids, on a molar basis relative to leucine, are given in table VIII and compared with the results obtained by Moore and Stein [190]. Good agreement is shown generally between the present results and those of the above authors. There are, however, significant differences between the two sets of figures in the case of proline, glutamic

FIG. 43.

Single dimensional paper chromatography of standard amino acids in Butanol - HAC - H_2O.



acid, lysine and phenyl alanine. All the amino acids were checked for purity by paper chromatography (see fig. 43) and in each case a single ninhydrin positive spot was obtained.

The nitrogen content of the amino acids determined by Kjeldahl estimation agreed with the theoretical in each case where discrepancies in colour yields were found. Although slight differences are to be expected in different laboratories, the reason for the significant differences encountered could not be explained.

TABLE/...

TABLE VIII.

Colour Yields of different amino acids on a molar basis relative to leucine.

	Colour Yield	
	Moore and Stein[190]	Present Author.
Aspartic Acid	0.94	0.96
Threonine	0.94	0.95
Serine	0.95	0.96
Glutamic acid	0.99	1.06
Proline	0.225	0.35
Glycine	0.95	0.96
Alanine	0.97	1.00
Half cystine	0.55	0.59
Valine	0.97	1.01
Methionine	1.02	1.02
Iso leucine	1.00	0.99
Leucine	1.00	1.00
Tyrosine	1.00	1.00
Phenylalanine	1.00	0.94
Lysine	1.10	1.16
Histidine	1.02	1.01
Ammonia	0.97	0.98
Arginine	1.01	1.02
Tryptophan	0.94	0.93

THE SEPARATION OF AMINO ACID MIXTURES BY COLUMN CHROMATOGRAPHY ON ION EXCHANGE RESINS.

The first attempts at quantitative analysis of the amino acids in protein hydrolysates were made by Stein and Moore [196] using starch columns. This procedure - a stimulating advance - lacked adequate resolving power and suffered from the additional disadvantage that traces of soluble material off the starch columns interfered with subsequent photometry.

In 1949 Partridge [197] introduced columns of Zeocarb 215 ion exchange resin for the fractionation of protein hydrolysates. In a subsequent report Moore and Stein [198] described the use of an eight per cent crosslinked sulphonated polystyrene resin (Dowex 50) but later changed to the 4% crosslinked resin (Dowex 50 x 4) which had the advantage of resolving small peptides as well [199]. The main disadvantage of the Dowex resins was the fact that the manufactured product varied from batch to batch, which created serious difficulties during standardisation of the procedure.

Markedly improved results were obtained with Amberlite (C.G.120) a finely pulverised sulphonated polystyrene resin (8% crosslinked) [200]. Hamilton [201] described an ingenious method for separating the crude resin into fractions of different particle size and demonstrated that resolution could be improved by using resins of uniformly small particle size. At the same time Spackman

et al [202] described an apparatus whereby colour development is automatically performed with continuous recording of the effluent stream. The method adopted in the present study was basically as described by Moore and Stein [200] using Amberlite C.G. 120 fractionated by Hamilton's procedure [201].

In this method the acidic and neutral amino acids are separated on a column 150 cm. in length while the basic amino acids are determined on a column 15 cm. in length.

METHODOLOGY.

Preparation of the Resin:

Amberlite C.G.120 was obtained from Malinkrodt Chemical Works, New York, U.S.A. One pound of the dry resin was suspended in 10 litres of H_2O in a large beaker and allowed to settle for 6 hours. Fine particles remaining in the supernatant were removed by decantation and this process was repeated a few times. The resin was then washed on a Buchner funnel with 2 litres of 4N.HCl followed by 500 ml. of H_2O or until the filtrate was neutral. The moist resin was suspended in 2 litres of 2 N.NaOH and heated on a steam bath for 1 hour. If the supernatant turned markedly yellow this step was repeated. The treated resin was finally washed with H_2O on a Buchner funnel until neutral and stored in 0.2 N. NaOH.

Fractionation of the Resin:

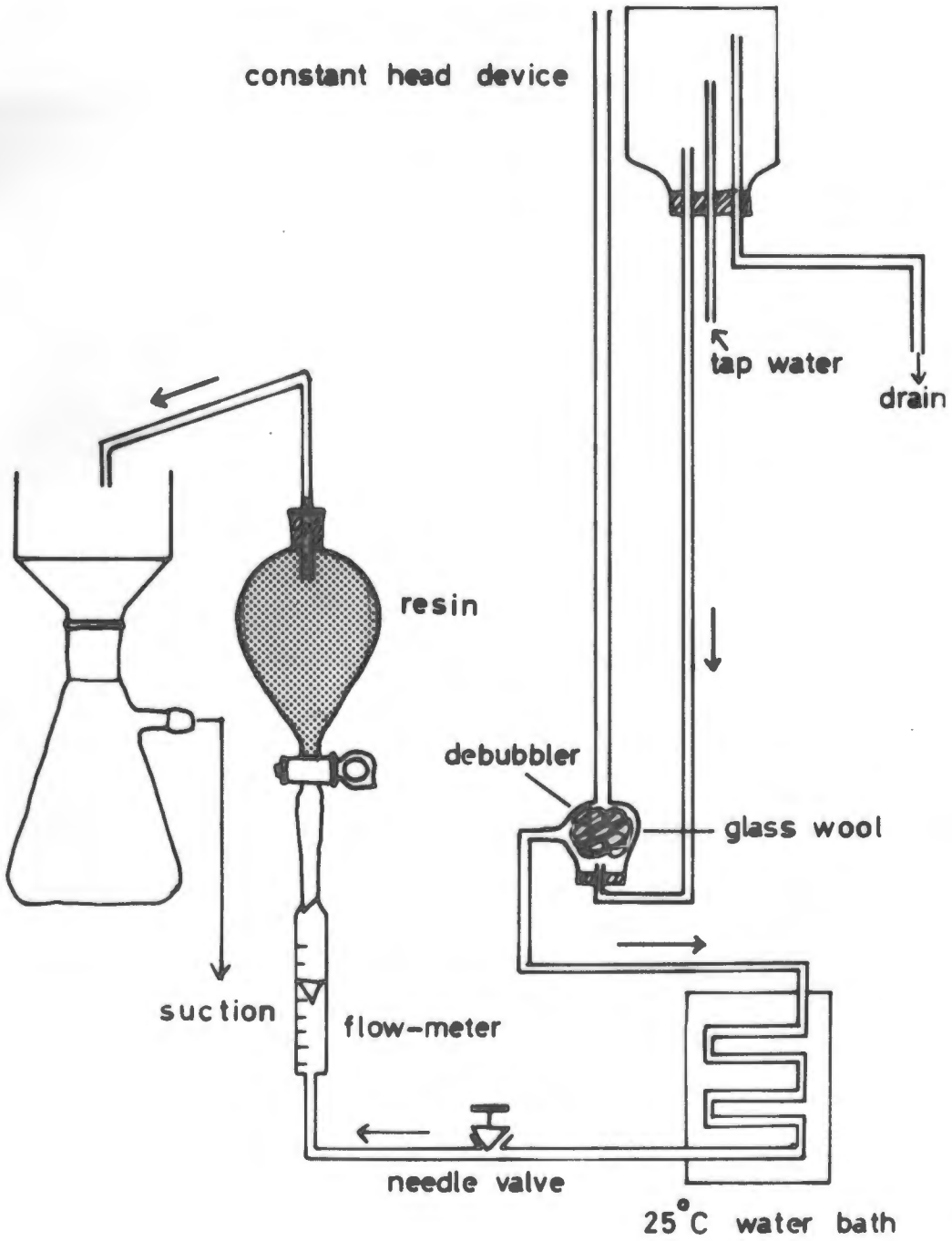
The resin was fractionated in an apparatus based on the design of Hamilton[201](fig.44).

Principle:

Deaerated water at constant temperature ($25^o \pm 2^o$) is passed upwards through a separatory funnel containing the crude resin to be fractionated. The flow rate can be adjusted by means of a needle valve connected to a flow meter. The effluent from the

top/.....

FIG 44 HAMILTON'S HYDRAULIC FRACTIONATOR FOR RESINS



top of the separating funnel is collected on a Buchner funnel to which slight suction is applied. By stepwise increase of the flowrate the resin can be fractionated as at a particular flow rate only particles of a certain dimension will remain suspended and be carried over with the effluent which is collected on the Buchner funnel.

In the design used in the present investigation, the temperature of the tap water was controlled by passing it through glass coils submerged in a thermostatically controlled water bath. For each alteration in flow rate the temperature of the waterbath may have to be re-adjusted to ensure that the contents of the separating funnel remain at the correct temperature.

The dimensions of the particles obtained at different flow rates are given in table IX based on the findings of Hamilton[201].

TABLE IX.

Dimensions of the particles in the fractions obtained at different flow rates.

Fraction.	Water flow ml./min. (2 litre funnel)	Particle Size.	Use.
A	50 ml.	25 μ	Not used.
B	50-110 ml.	25-30 μ	A.R. 15 & 50 cm. columns.
C	110-280	40 ± 7 μ	A.R. 150 cm. columns. F.C. 15 & 50 cm. columns.
D	280-580	56 ± 9 μ	F.C. 150 cms. columns.

A.R. Automatic recording apparatus.

F.C. Manual method by fraction collection.

As/.....

As tap water was used in the fractionation procedure the resin was treated again by washing with HCl and NaOH.

Preparation of the columns:

The Chromatograph tubes were made of pyrex glass 0.9 cm. in diameter with a sintered glass disc of porosity grade II fused into the tube 1.5 cm. from its lower end. The tube to hold the 150 cm. column measured 158 cm. from the sintered glass plate to a B19 ground glass joint fitted at the top while the total length of the glass tube to contain the 15 cm. column was 25 cm. Each column was fitted with a water jacket 2.5 cm. internal diameter, fused to the chromatograph tube at either end. The jackets were fitted with inlets and outlets which were connected to a thermostatically controlled water bath. The lower ends of the columns were not constricted but cut away at an angle of 45^{o} to prevent eddying in the effluent stream and resultant admixture of fractions.

Before use the columns were cleaned by soaking overnight in a saturated solution of KOH in aqeous ethanol 96% v/v followed by exhaustive washing with dist. H_2O.

Pouring of the columns:-

150 cm. column:

For this column, fraction C (See table IX) was used. The resin was equilibrated with citrate buffer (0.2 M, pH 4.25) without B.R.I.J. or thiodiglycol (see table X for preparation and composition of buffers). Approximately 100 ml. of settled resin is required for each 150 cm. column. This volume of resin was suspended in twice its volume of pH 4.25 buffer and divided into six sections of equal length.

The column was mounted on a frame attached to the bench and properly alligned in a vertical plane by means of a spirit level.

After resuspending the settled resin the first section is poured using a funnel and with the outlet of the column closed. The outlet is opened after allowing the resin to settle to a height of 2 cm. Pressure of 30 m.m. mercury is then applied to the column from a nitrogen cylinder, fitted with a reducing valve and

connected/...

connected to the column by a length of rubber tubing passing through a manometer. Pressure is maintained until this section of resin has completely settled. The next section is poured when approximately 2 cm. of supernatant buffer is left on the surface of the resin. In this way the column is poured in 6 sections with a final height of approximately 155 cm. to allow for subsequent settling of the resin. The last two sections are poured after fitting an extension tube to the top of the column.

15 cm. column:-

Moore and Stein [200] recommended the fraction of resin obtained at flow rates between 110-280 ml./min. (C) for the 15 cm. column operated by the manual method. In our experience, fraction B, recommended for the automatic procedure, gave better results as will be discussed later and this resin fraction was used. The column is poured in a single section through an extension attached to the chromatograph tube after equilibration of the resin with pH 5.28 buffer.

Preparation of Buffers:-

The composition of the citrate buffers used in the operation of the 150 and 15 cm. columns were as described by Moore and Stein [200] (See table X).

Buffers were stored at room temperature in polythene containers. Phenol proved to be an effective preservative as no contamination of buffers by microbial or fungal growths occurred even on prolonged storage.

TABLE/...

TABLE X.

Composition of the citrate buffers used in the operation of 150 and 15 cm. columns.

pH.	Na Concentration N.	Citric Acid. H_2O. -g.	NaOH. g.	HCl. (conc.) ml.	Final Volume Litres	Phenol. g.
3.25 ±0.01	0.2	420	165	213	20	20
4.25 ±0.02	0.2	420	165	94	20	20
5.28 ±0.02	0.35	491	288	136	20	20

Phenol was redistilled and added to the buffers after the final volume had been adjusted. Analytical grade reagents were used throughout.

The pH of the 3.25 buffer should be carefully adjusted and repeatedly checked during the first week after preparation. A Radiometer Model P4 instrument was used and the optimal pH for complete separation of alanine, cystine and valine was found to be 3.25 with the columns used.

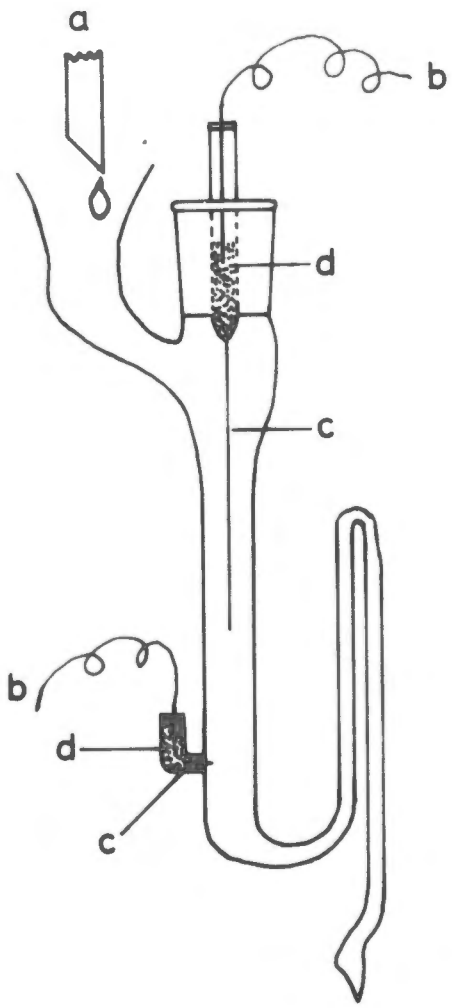
B.R.I.J. -35.

To permit faster flow rates without excessive pressure having to be applied to the column a detergent B.R.I.J. was added to the eluent as described by Moore and Stein [198]. The chemical was obtained from Pierce Chemical Company, Rockford, Illionois and prepared as a 33% w/v solution in dist. H_2O which was added to the buffers after boiling; 0.75 ml. of B.R.I.J. for each 100 ml. of buffer.

Thiodiglycol/...

FIG. 45

FRACTION COLLECTING SYPHON WITH CONDUCTIVITY ELECTRODES.



a Effluent from column.

b Leads to fraction collector circuit.

c Platinum electrodes.

d Mercury bridges.

Thiodiglycol:

To prevent losses of methionine through oxidation, thiodiglycol was added to the pH 3.25 and 4.25 buffers (1 ml. per 100 ml. of buffer) before use. This chemical was obtained from British Drug Houses Ltd. and redistilled under reduced pressure.

Fraction Collector:

The fraction collector used, was constructed in the Department and the revolving drum designed to hold 200, 5" x ⅝" pyrex test tubes. The motor driving the drum mechanism was activated through pulses from the syphon feeding into time relays in the circuit.

The principle of the syphon used is demonstrated in fig. 45 and was constructed in the department. Electrical contact was established by means of the two platinum wires when the electrolyte containing buffer reached the level of the upper wire. The syphons were constructed to deliver a volume of between 1.4 and 1.5 ml.

Fraction collector tubes:

Moore and Stein [196] recommended the use of properly matched photo-electric colorimeter tubes both in the fraction collector and for subsequent colour development and readings in the colorimeter. Although this is convenient and time saving, this can be expensive due to breakages and scratching of the colorimeter tubes.

5" x ⅝" pyrex tubes were found satisfactory in the present experiments. The colour reaction was performed in these tubes but readings were taken after transfer of the contents to a photo electric colorimeter tube.

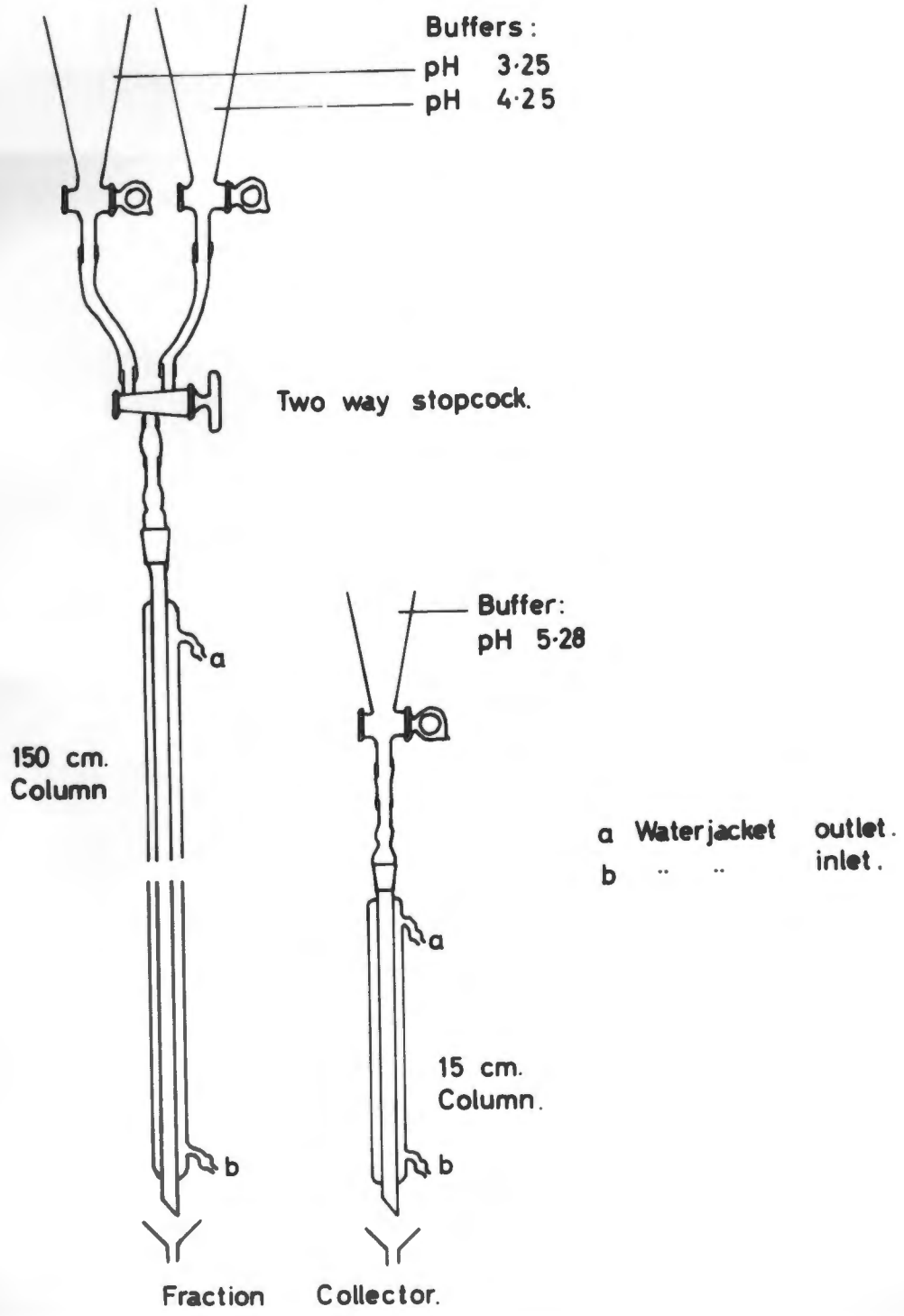
OPERATION OF THE COLUMNS.

Assembly of the Apparatus:

The apparatus was assembled and mounted over the fraction collector as shown in the diagram fig. 46. The buffers for immediate use were stored in 500 ml. separatory funnels under a

FIG. 46

EXPERIMENTAL ARRANGEMENT FOR MANUAL MOORE AND STEIN CHROMATOGRAPHIC PROCEDURE,



layer of liquid paraffin. In the case of the 150 cm. column, the funnels containing the pH 3.25 and 4.25 buffers respectively were connected to the column by lengths of polythene tubing through a two way stopcock as indicated in the diagram. The 15 cm. column was mounted next to the 150 cm. column. The waterjackets around the columns were connected to a thermostatically controlled water bath and water circulated through these by means of a small centrifugal pump.

OPERATION OF THE COLUMNS.

150 cm. Column:

Before use and after each run, the resin in the column was regenerated by allowing approximately 200 ml. of 0.2 N NaOH containing B.R.I.J. to run through under gravity. The NaOH solution was stored in a 500 ml. separatory funnel fitted with a soda-lime trap to prevent uptake of CO_2. The column was re-equilibrated for use by passing through it about 120 ml. of buffer at pH 3.25 either at room temperature or at 50° C under slight pressure.

Before each run, the separatory funnels were filled with the appropriate buffer brought to a boil just before addition to the reservoirs through long-stemmed funnels. B.R.I.J. and thiodiglycol were added after boiling. This procedure is required to deaerate the buffers, which prevents the formation of air bubbles in the resin bed when the buffers are heated to 50°. The water bath and pump for circulating water through the jackets around the columns were started approximately 1 hour before application of the sample and the temperature was maintained at 50° C

throughout/...

throughout the procedure.

Application of the Sample:

The layer of buffer on top of the resin was withdrawn with a pasteur pipette and the sample containing the equivalent of approximately 1 mg. of protein applied by means of a 1 ml. volumetric, mark to mark, pipette. The sample was allowed to run in under gravity and washed down with two aliquots of buffer of approximately 1 ml. each from the pH 3.25 reservoir. A pasteur pipette was convenient for this purpose. The residual space on top of the resin was then filled with pH 3.25 buffer and the reservoir containing pH 3.25 buffer connected to the column through ground glass B 19 connections. Pressure of 24 cm. of mercury was applied to the column from a nitrogen cylinder connected by a length of polythene tubing to the reservoir. At this pressure, flow rates of 15 ml. per hour could be obtained. All stopcocks were fitted with spring clips to prevent leakage when pressure was applied.

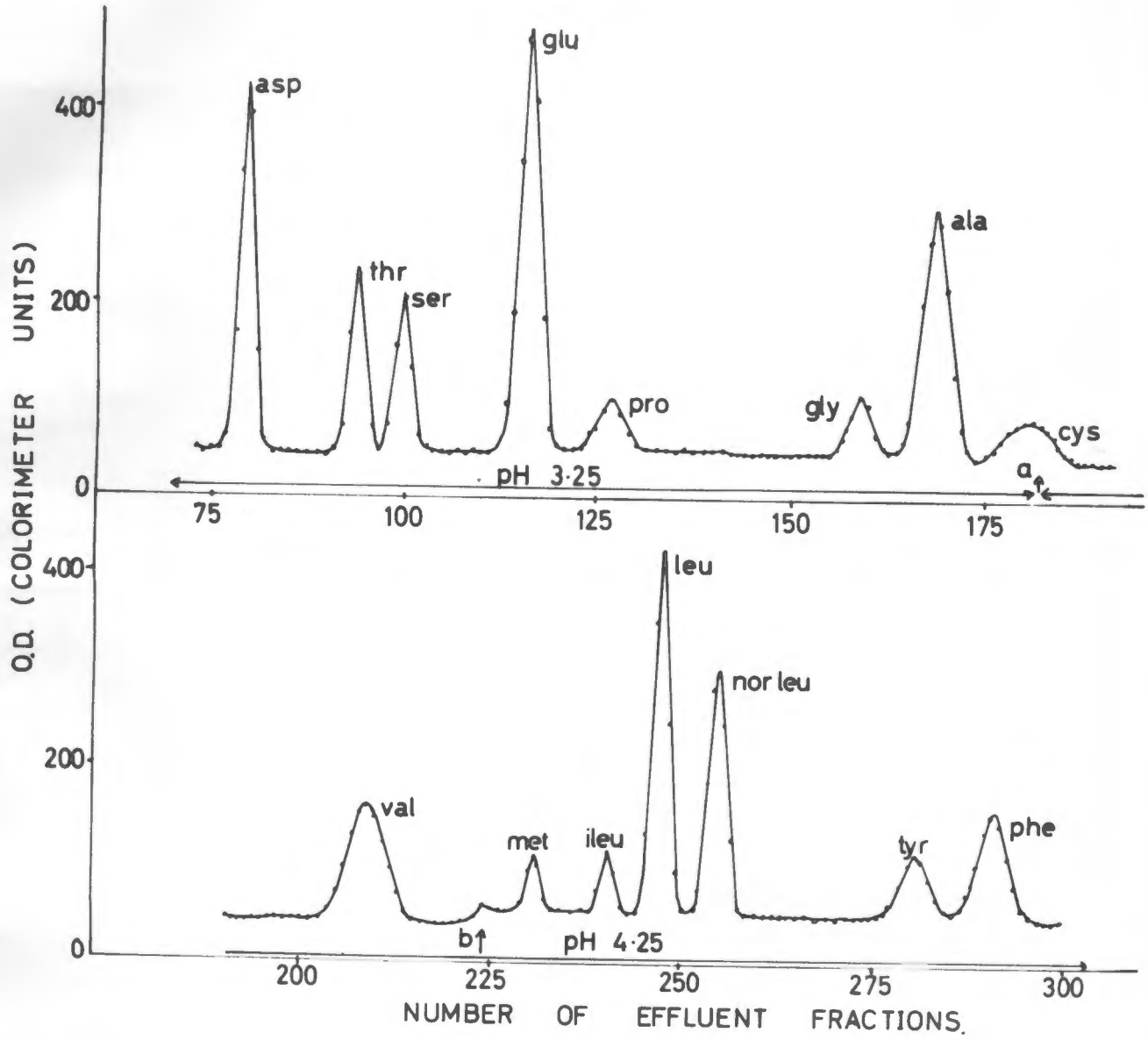
The effluent was collected in fractions of approximately 1.4 - 1.5 ml.

Moore and Stein[200] recommended that the eluent buffer of pH 4.25 should be introduced at a time designed to allow valine to emerge with the new buffer. This practice was initially adopted but the emergence of the new buffer usually produces a peak in the baseline which overlaps the valine peak, making accurate assessment

FIG. 47

SEPARATION OF ACIDIC AND NEUTRAL AMINO ACIDS IN A HYDROLYSATE OF ALBUMIN FROM PATIENT CON.

(150 CM. COLUMN)



a. buffer change 3·25—4·25 (pH)

b. emergence of pH 4·25 buffer

of the baseline for valine extremely difficult. The change over was therefore made slightly later, allowing valine to emerge with the pH 3.25 buffer, when the peak due to the pH 4.25 buffer front appears between valine and methionine. See fig. 47.

Operation of 15 cm. Column:

The basic amino acids were eluted with buffer of pH 5.28 at 50° C. The sample was applied in the same way and the column operated at a pressure of 20 cm. of mercury. As pointed out earlier the resin fraction B gave better results than C. With the coarser resin the arginine peak tended to be markedly assymetrical, with a broad base, whereas with the resin of smaller particle size a symmetrical and sharper peak could be obtained (fig. 48 a, b).

The basic column need not be regenerated with NaOH in between runs as no amino acids are retained on the column with the pH 5.28 buffer.

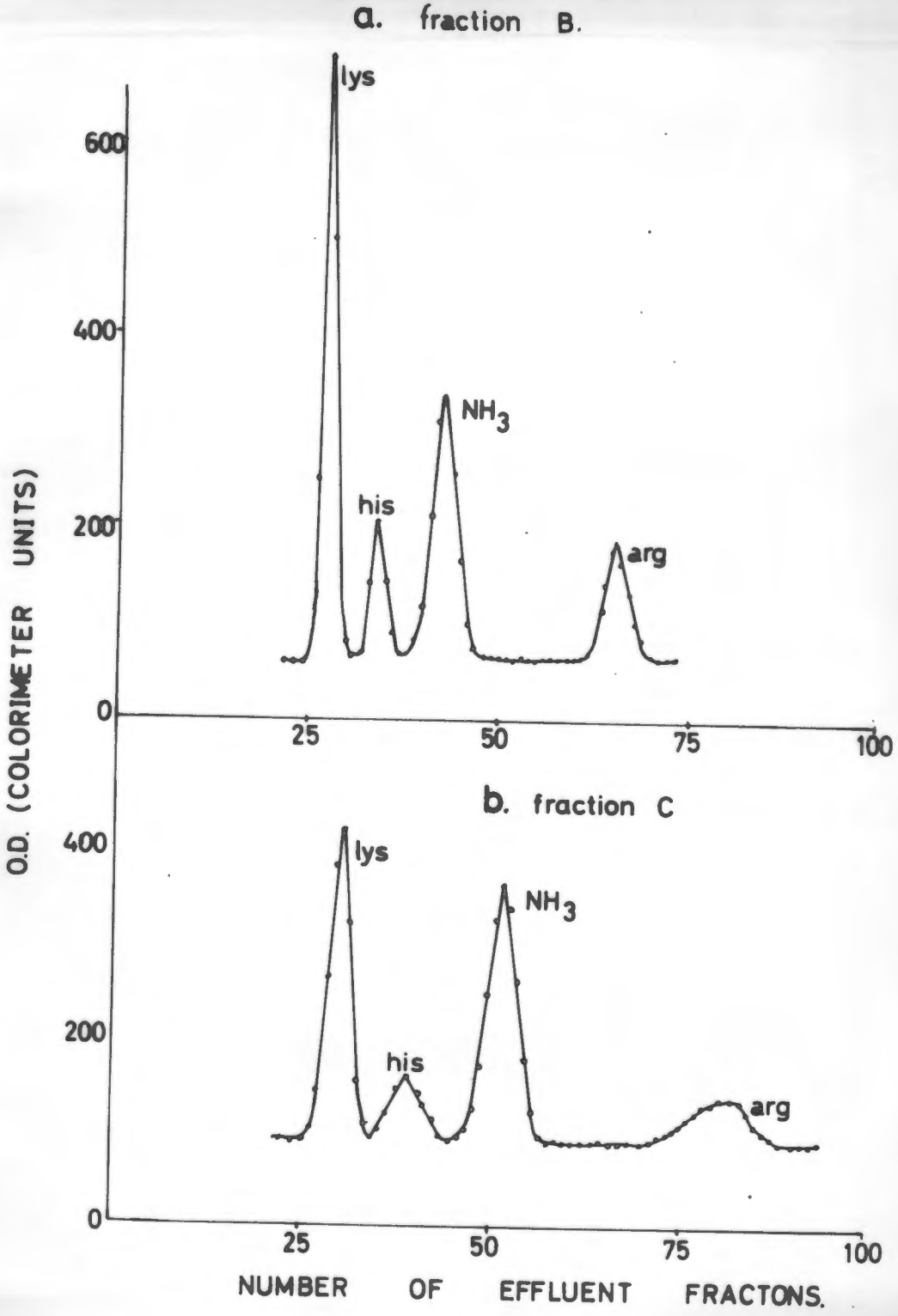
EXPERIMENTAL NOTES:

Resolving Power:

The resolving power of the 150 cm. column is demonstrated in fig. 47 which represents the effluent diagram obtained on a hydrolysate of albumin from a patient with kwashiorkor. It is

FIG. 48

ELUTION DIAGRAMS OF BASIC AMINO ACIDS WITH RESINS OF DIFFERENT PARTICLE SIZE.



evident that complete separation between different amino acids could be achieved, which eliminated the need for extrapolation of peaks for purposes of calculation. The two arrows on the effluent diagram indicate the point of change over from pH 3.25 - 4.25 buffers and the emergence of the pH 4.25 buffer front. The peak produced by the pH 4.25 buffer front does not overlap any of the amino acid peaks and settles to a new steady baseline before methionine is eluted. The blanks for the peaks up to valine were taken as the average of the baseline before and after each peak while blanks for the amino acid peaks emerging after valine were obtained from the baseline values following leucine. Average blank values obtained ranged between 38 and 45 colorimeter units in most experiments. In the case of the 15 cm. column, blanks for lysine, histidine and ammonia were taken from the baseline after ammonia. For arginine the average value before and after the arginine peak was used. This is necessary as a drop in baseline values often occurs between ammonia and arginine.

STANDARDISATION OF COLUMNS AND CALCULATION OF RESULTS.

It was found that optimal resolution was only achieved after about 4 runs on a newly poured column. Flow rates may decrease slightly during this period but, with the columns used, adequate flow rates were obtained at pressures of between 20 - 30 cm. Hg. for the 150 cm. column and 15 - 20 cm. Hg. for the 15 cm.

column.

The columns were standardised by passing through a compound standard solution of amino acids prepared by dissolving the pure, desiccated amino acids in 0.8 N HCl. The composition of the standard is given in table XI.

The effluent was collected in fractions of 1.4 ml. and analysed by the ninhydrin procedure described before. The optical densities of the effluent fractions were plotted against the number of fractions, to facilitate assesment of the baseline and detect asymmetry in the amino acid peaks.

Calculation of Results:-

The ion exchange columns were standardised by determining the colour yield (C) in colorimeter units per 0.1 μ mole for each amino acid in the standard using the formula.

$$C = \frac{\sum_1^n (x) - n\bar{b}}{c} \quad \text{colorimeter units/0.1 } \mu \text{ mole.}$$

where n = number of fractions per peak.

x = colour value in colorimeter units per fraction

$\bar{b}$ = average blank value.

c = μ moles of amino acid applied to the column.

The amount (in μ moles) of the individual amino acids in

the/...

the unknown sample was calculated as follows:-

$$\frac{\Sigma_1^n(x) - n\bar{b}}{c} \mu \text{ moles.}$$

Reproducibility of chromatographic Procedure:

The method proved to be reproducible once operating conditions were standardised. The colour yields for individual amino acids expressed as colorimeter units per 0.1 μ mole of amino acids obtained at different times are given in table XII. The effluent fractions were 1.4 ml. and the final reaction mixture 7.4 ml.

It can be seen that reproducibility of results is excellent for most amino acids. The value for lysine obtained on 24.7.63. differed significantly from the values obtained earlier (table XII). This result was obtained with resin fraction B while in the earlier experiments the coarser resin fraction C was used. The isoleucine values could not be established as all the samples of this amino acid, which could be obtained, contained approximately 50% of the isomer allo-isoleucine. The leucine values were, therefore used to calculate concentrations for this amino acid.

Nor leucine/...

TABLE XI.

Composition of compound amino acid standard for standardisation of ion exchange columns.

Amino Acid.	Concentration μ moles/ml.
Aspartic acid.	0.539
Threonine.	0.499
Serine.	0.502
Glutamic acid.	0.754
Proline.	0.470
Glycine.	0.512
Alanine.	0.499
Cystine.	0.501
Valine.	0.518
Methionine.	0.516
Iso leucine.	0.499
Leucine.	0.504
Nor Leucine.	0.504
Tyrosine.	0.511
Phenyl alanine.	0.502
Lysine. HCl.	0.526
Histidine HCl.	0.522
Ammonia HCl.	0.515
Arginine HCl.	0.553

TABLE XII.

Colour yields for individual amino acids obtained at different times by chromatographic analysis of a compound standard.

Amino acid.	Colour Yield/0.1 μ mole amino acid.		
	Date of analysis.		
	26.6.62.	18.7.62.	24.7.63.
Aspartic acid	112.7	114.6	113.3
Threonine	111	111	113
Serine	118	117	116.5
Glutamic acid	118	121	120
Proline	42.7	40.9	41.4
Glycine	113	113.7	111
Alanine	115	117	115.8
Cystine	131	126.5	128.5
Valine	113	113.7	111.6
Methionine	120.9	120.5	119.2
Iso leucine	(120.6)	(118.2)	(118.5)
Leucine	120.6	118.2	118.5
Nor leucine	-	-	118.5
Tyrosine	115	115	115.3
Phenyl alanine	116.5	112	114.3
Lysine	128	129	135.6
Histidine	119.7	120	122.3
Ammonia			117.6
Arginine	116.7	117.6	118

TABLE XIII.

Colour Yields for different amino acids separated by chromatography of a compound standard, expressed on a molar basis relative to leucine.

Amino Acid.	Colour Yield.	
	By Chromatography.	Moore & Stein [190].
Aspartic acid	0.96	0.94
Threonine	0.95	0.94
Serine	0.98	0.95
Glutamic acid	1.01	0.99
Proline	0.35	0.225
Glycine	0.94	0.95
Alanine	0.98	0.97
Cystine	1.08	1.10
Valine	0.94	0.97
Methionine	1.01	1.02
Iso leucine	1.00	1.00
Leucine	1.00	1.00
Nor leucine	1.00	-
Tyrosine	0.97	1.00
Phenylalanine	0.96	1.00
Histidine	1.03	1.02
Lysine	1.14	1.10
Ammonia	0.99	0.97
Arginine	1.00	1.01

Nor leucine, a synthetic amino acid, was incorporated in subsequent standards as this amino acid serves as a useful standard to check on the hydrolysis procedure. A known quantity is added to each sample of protein before hydrolysis and chromatographic recoveries of this amino acid will indicate losses of material during the various stages of transfer.

That no selective losses of amino acids occur during chromatography is demonstrated by the close agreement between the values obtained on chromatography and the theoretical, expressed on a molar basis relative to leucine - Table XIII. It is again evident that the colour yield for proline is significantly higher than the value reported by Moore and Stein [190].

It is essential to re-standardise the columns each time a new syphon is used as differences in fraction volumes will affect the final reaction volume and therefore the colour value obtained per 0.1 μ mole of amino acid.

DETERMINATION OF NITROGEN BY A MICRO KJELDAHL PROCEDURE:

Nitrogen determinations were performed on each hydrolysed sample as the results of the amino acid analysis on the albumin samples were expressed as g./100 g. protein. To obtain the protein equivalent per unit volume of the hydrolysed sample the nitrogen value obtained was multiplied by a factor of 6.25 based on a nitrogen content in the albumin molecule of 16%.

According/...

According to Chibnall et al [203] the commonest sources of errors in the Kjeldahl procedure are incomplete digestion of protein and the use of socalled "anhydrous protein preparations" for determination of the weights of samples. Lysine and histidine are resistant to digestion and proteins rich in these amino acids require longer periods of digestion. Socalled "anhydrous protein preparations" are extremely hygroscopic and analysis based on weights so obtained may be completely erroneous. These workers advise the use of air dried preparations and the correct weight of the sample to be analysed should be obtained by determining the moisture and ash content on separate aliquots of the sample. Losses of nitrogen may occur during digestion as a result of high digestion temperatures which occur when the sample has a high salt content or is rich in carbohydrate and fat. The acid-protein ratio should be increased under these circumstances.[204]

METHODOLOGY.

The procedure adopted in the present study was based on the method of McKenzie and Wallace [204]. Analysis could not be performed on air dried samples as insufficient material was available for the separate determination of moisture and ash content. It was considered that the most reliable results would be obtained by measuring the nitrogen content in an aliquot of the hydrolysed sample, equal to that used for amino acid analysis.

Reagents/...

Reagents:

1. H_2SO_4, 36 N. (Merck's A.R.).

2. K_2SO_4. B.D.H. A.R.)

3. $HgSO_4$ Solution.

   Prepared by dissolving 10 g. red HgO in 18 ml. conc. H_2SO_4 and diluted to 100 ml. with dist. H_2O.

4. NaOH - $Na_2S_2O_3$ Solution.

   Prepared by dissolving 200 g. NaOH and 12.5 g. $Na_2S_2O_3 . 5 H_2O$ in distilled H_2O and diluted to 500 ml.

5. Indicator Solution:

   Solution A. 0.2% Methyl Red in 95% aqeous ethanol.

   Solution B. 0.2% Methylene Blue in 95% aqeous ethanol.

   This solution was prepared fresh once a month.

   The indicator solution was prepared by mixing, in a ratio of 2 vol. Sol. A to 1 vol. Sol. B.

6. Boric Acid Solution.

   The ammonia in the distillate was trapped in a 2% w/v solution of boric acid containing the mixed indicator. (10 g. H_3BO_3 (Merck's A.R.) + 5 ml. mixed indicator diluted to 500 ml. in ammonia-free distilled H_2O).

7. $KH(IO_3)_2$ Solution. (0.01N).

   This solution was standardised with NaOH (0.01N) before each set of determinations using phenolphthalein as an indicator.

Apparatus/...

Apparatus:

1. Pyrex micro Kjeldahl digestion flasks of 30 ml. capacity.

2. Microdistillation apparatus. (Markham type). Connections between distillation flasks and condensors were constructed of pyrex glass, in one piece with no intervening joints. The funnel of the distillation apparatus was shut off from the distillation chamber by means of a teflon stopcock.

Procedure:

The sample containing the equivalent of approximately 1 mg. protein (0.1 - 0.2 mg. of nitrogen) was transferred to a micro Kjeldahl flask followed by the addition of 1.5 ml. H_2SO_4 (36N); 1.5 g. K_2SO_4 and 0.5 ml. $HgSO_4$.

The excess H_2O was boiled off and digestion continued until the sample was clear (i.e. all charred matter removed). This usually occurred 5 min. after fuming commenced. Digestion is continued for a further 15 minutes. The digest was diluted with a few ml. of ammonia-free distilled H_2O and quantitatively transferred to the distillation flask with several washings of dist. H_2O taking care that the total volume did not exceed 25 ml. A light application of grease to the rim of the digestion flask will assist quantitative transfer. 10 ml. of NaOH - $Na_2S_2O_3$ solution was added to the distillation chamber and the teflon stopcock closed. Distillation was performed in the following manner.

The tip of the condensor was immersed in 5 ml. boric acid solution contained in a 50 ml. flask which had been graduated to indicate levels of 20 ml. and 25 ml. Distillation was continued until the 20 ml. mark had been reached when the flask was lowered so that the tip of the condensor was above the surface of the distillate. A further 5 ml. was distilled over and the tip of the condensor was then rinsed with dist. H_2O adding the washings to the distillate.

The distillate was titrated with 0.01N $KH(IO_3)$ till the lilac endpoint of the indicator was reached. Suitable blanks were carried through with each batch analysed.

In the calculation of results, 1 ml. $KH(IO_3)_2$ = 0.1401 mg. N.

RECOVERIES:

With each set of unknowns analysed, NH_4Cl and histidine standards were run in parallel to check on the distillation procedure and the completeness of digestion respectively. Duplicate recoveries of histidine and NH_4Cl obtained during 5 consecutive experiments are shown in table XIV.

Low recoveries were almost invariably due to leakage from the distillation apparatus or too short a period of acid digestion, assuming that all reagents were properly standardised. The excellent recoveries obtained with histidine indicates that this digestion mixture is efficient, as histidine is a resistant amino acid for which 100% recoveries are difficult to obtain.

TABLE XIV.

Duplicate recoveries of NH_4Cl and Histidine standards during 5 consecutive micro-Kjeldahl analyses.

Experiment:		NH_4Cl. (% Recovery)	Histidine (% Recovery)
1.	a	99.9	100.8
	b	100.6	101.0
2.	a	101.7	100.8
	b	101.3	101.0
3.	a	99.8	100.7
	b	100.3	100.1
4.	a	97.8	99.7
	b	101.0	100.0
5.	a	100.5	99.6
	b	98.6	100.4

CHAPTER IX.

HYDROLYSIS OF PROTEINS AND

DETERMINATION OF AMIDE AMMONIA.

HYDROLYSIS OF PROTEIN SAMPLES.

In determining the amino acid composition of a particular protein, it is essential that the protein should be completely hydrolysed without destroying the liberated amino acids during the process. While the chromatographic procedure of Moore and Stein for amino acid analysis, possesses the sensitivity and accuracy required, the problems attending the hydrolysis of proteins have not been solved. In order to interpret the results obtained in a particular experiment, the method of hydrolysis should be carefully examined, as the best method for the hydrolysis of one protein may not be suitable for another. It is, therefore, necessary to consider some of the events occurring during the hydrolytic cleavage of proteins.

METHODS OF HYDROLYSIS:

1. Enzymic:

Although theoretically an attractive method, it suffers from the disadvantage that complete hydrolysis of a large protein molecule can not be achieved. The presence of enzyme protein in the hydrolysate further complicates subsequent analysis.

2. Alkaline Hydrolysis:

Proteins can be hydrolysed by alkalies but this procedure leads to extensive destruction of certain amino acids

with/...

with racemisation of others. Artifacts are produced in addition, as there is a tendency towards interconversion of amino acids. Serine may be converted to glycine and alanine and ∝ amino-butyric acid; cystine to alanine and arginine to citrulline and ornithine [205]. Alkaline hydrolysis is, nevertheless, still used in the determination of tryptophan as this amino acid is almost quantitatively lost during acid hydrolysis.

3. Acid Hydrolysis:

Hydrolysis in acid is the procedure most commonly employed in the determination of the amino acid composition of a protein. Many different acids or combinations of acids have been proposed but most workers prefer constant-boiling HCl (6N) [206]. Hydrolysis is performed either by boiling under reflux with 6N HCl for different periods of time [207, 208] or in sealed evacuated tubes at 105° - 110° [209, 210]. It is evident from the vast literature on this subject and the large number of different procedures used that uniformity of opinion has not been reached on the most suitable methods to be used. In order to appreciate these difficulties some of the factors involved in the hydrolytic process must be considered.

GENERAL COURSE OF EVENTS:

Lugg [211] studying the liberation of carboxyl- and amino groups, amide nitrogen and ammonia at different times of hydrolysis

with/...

with 6N HCl, made the following observations.

The ammonia liberated by deamidation of asparagine and glutamine reaches a peak within 10 hours. Thereafter, there is a slow increase in ammonia which is derived from deamination of threonine and serine. At the same time liberation of amino and carboxyl groups occurs rapidly reaching a peak between 20 and 30 hours. Thereafter the rate of liberation seems to parallel the rate of destruction - See fig. 49.

Determination of the optimum period of hydrolysis:

As pointed out before, hydrolysis should proceed until the maximum number of peptide bonds have been freed whilst destruction of amino acids is still minimal. This stage is usually defined as that point where the difference between amino-nitrogen and ammonia is maximal [211]. However, from the experience of past workers, it is evident that certain peptide linkages are resistant to hydrolysis while other amino acids are progressively destroyed on prolonged hydrolysis.

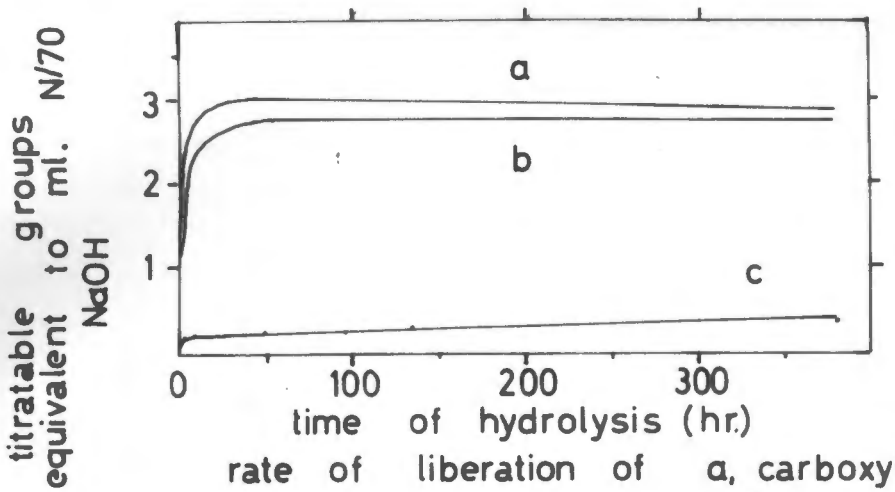
Destruction of amino acids during hydrolysis:

Recoveries of the hydroxy amino acids serine and threonine are known to decrease as the time of hydrolysis is increased. Rees [212] showed that a steady deamination, which was linear, occurred on hydrolysis of both threonine and serine

and/...

FIG. 49

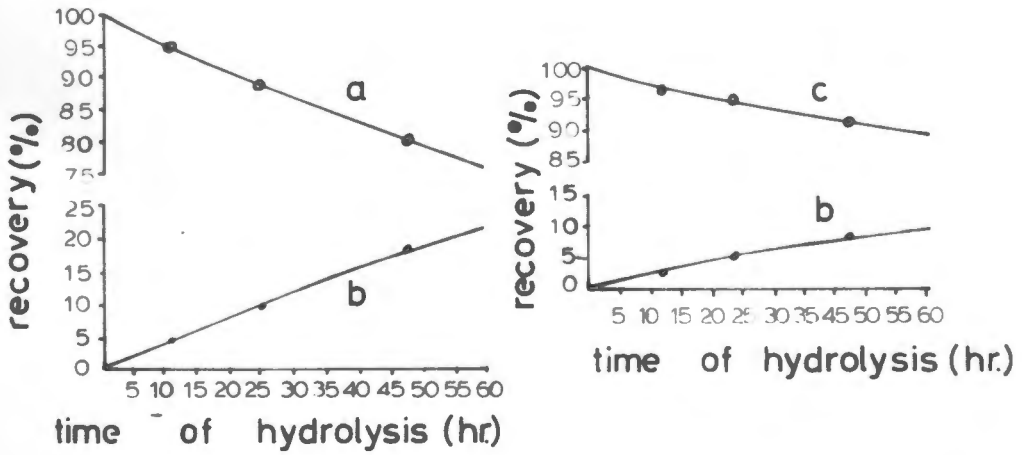
PROTEIN HYDROLYSIS IN MINERAL ACIDS.



rate of liberation of a, carboxyl groups., b, amino groups., c, volatile base.

FIG. 50

DESTRUCTION OF SERINE AND THREONINE DURING ACID HYDROLYSIS,



a. recoveries of serine at different times of hydrolysis.
c " " threonine " " " " " .
b ammonia produced during hydrolysis.

(data of Rees [212])

and gave rise to recoveries at 24 hours of 94.7% for threonine and 89.5% for serine. These losses could be quantitatively accounted for by the increase in ammonia - fig. 50.

Similar results were obtained by Smith et al on carboxypeptidase [209] and on crystalline papain [213] and by Hirs et al on ribonuclease [210]. However, Gordon [214], found no decrease in serine and threonine values even after 140 hours in the case of α lactalbumin - table XV. Gordon used hydrolysis under open reflux whereas the other workers hydrolysed their samples in evacuated sealed tubes.

Variable losses for tyrosine, cystine and methionine may also occur on prolonged hydrolysis especially in the presence of carbohydrate when humin formation is excessive.

Resistant peptide linkages:

The relative rates of hydrolysis of peptide bonds depend on the availability of the peptide bond to approaching hydrogen ions. This will be determined by the presence of charged groups in the neighbourhood of the bond and also by the size of the adjacent amino acid side chains. The bulky side chains of valine and also iso leucine probably account for the stability of the peptide bonds involving these amino acids [205, 214, 216, 217, 218].

Synge [215] demonstrated the differences in rates of hydrolysis of a number of dipeptides in strong acid - see table XVI.

TABLE XV.

Amino Acids Found in α-Lactalbumin Hydrolyzates.

(W.G. Gordon and J. Ziegler [214])

The amino acid values are given as grams amino acid yielded by 100 g. anhydrous ash-free protein. Hydrolyzate L-11 was prepared after oxidation with performic acid.

Amino acid	Time of hydrolysis.					
	20 hr.		70 hr.		140 hr.	
	Hydrolyzates L-5 and L-6.		Hydrolyzates L-7 and L-8		Hydrolyzates L-9 and L-11	
Aspartic acid	(17.69)[a]	18.45	18.89	18.80	18.45	
Threonine	5.43	5.39	5.54	5.63	5.51	
Serine	4.70	4.70	4.73	4.88	4.79	
Glutamic acid	12.61	12.73	13.00	13.01	12.91	
Proline	1.52	1.64	1.53	1.48	1.43	
Glycine	3.07	3.12	3.25	3.23	3.32	3.24
Alanine	2.01	2.13	2.13	2.10	2.34	2.15
Cystine	(4.37)	(5.60)	(3.58)	(4.33)	(3.05	(6.66)[b]
Valine	(3.20)	(3.25)	4.60	4.52	4.89	4.62
Methionine	0.91	0.92	0.90	0.95	0.95	
Isoleucine	(5.41)	(5.53)	6.78	6.53	7.06	6.82
Leucine	11.09	11.44	11.67	11.54	11.93	11.43
Tyrosine	5.20	5.30	5.02	4.83	4.47	
Phenylalanine	4.36	4.27	4.47	4.62	4.64	

a Omitted because of operational difficulties.

b Estimated as cysteic acid but calculated as cystine, assuming a 90% conversion of cystine to cysteic acid during performic acid oxidation.

TABLE XVI.

HYDROLYSIS OF PEPTIDES IN STRONG ACID.

Peptide.	Relative Velocity Rates.
glycyl - Glycyl	1.0
glycyl - Alanyl	0.62
Alanyl - Glycyl	0.62
Glycyl - Leucyl	0.40
Glycyl - Tryptophan	0.35
Glycyl - Valyl	0.31
Leucyl - Glycyl	0.23
Leucyl - Leucyl	0.048
Leucyl - Tryptophan	0.041
Valyl - Glycyl	0.015.

Taken from Synge [215].

The stabilising effect of the side chain seems to be less when it belongs to the residue which donates the amino-group to the bond.

The increasing yields of iso leucine and valine on prolonged hydrolysis have been confirmed by several workers:-

Gordon et al [214] (α Lactalbumin);

Smith et al [219] (rabbit antibodies);
Smith et al [213] (crystalline papain) and
Smith et al [209] (carboxypeptidase).

However in the experiments of Hirs et al [210] on ribonuclease A, an increase in isoleucine was demonstrated while valine concentrations remained the same between 22 and 70 hours - table XVII.

As a result of these observations, it has been recommended that hydrolysis of a particular protein should be carried out for different periods of time and the correct values obtained by extrapolation to zero time [210]. It is also evident that recoveries of individual amino acids vary with different times of hydrolysis for different proteins. It appears that the optimal conditions should be determined for each protein analysed, and the exact conditions should be specified by each worker.

METHODOLOGY.

The method adopted in the present study was based on the open reflux procedure using 6N HCl as recommended by Macpherson[207]. It is claimed by Dustin [220] that when a large excess of acid is used to hydrolyse a protein sample the maximum losses of any amino acid amounts to only 3%.

In principle, the method used was to hydrolyse the protein sample in 6N HCl under reflux. The hydrolysate was

TABLE XVII.

Amino Acid Composition of Hydrolysates of Ribonuclease A.

(Values of Hirs, Stein and Moore [210])

The values are in terms of the anhydrous ash-free protein containing 17.8 per cent nitrogen.

Amino Acid	Amino acid per 100 gm. protein					
	Time of hydrolysis					
	22 hrs	70 hrs	22 hrs	70 hrs	22 hrs	70 hrs
	gm.	gm.	gm.	gm.	gm.	gm.
Aspartic acid	(14.1)	(14.5)	14.5	13.5	14.3	12.8
Glutamic acid	12.0	11.5	12.3	11.8	12.1	11.7
Glycine	1.67	1.69	1.73	1.50	1.62	1.61
Alanine	7.80	7.63	7.83	7.39	7.71	7.68
Valine	7.55	7.65	7.66	7.15	7.46	7.46
Leucine	1.98	2.16	1.98	2.01	1.97	2.05
Isoleucine	(2.11)	2.84	(2.14)	2.59	(2.19)	2.57
Serine	9.57	7.52	9.96	6.71	9.19	5.66
Threonine	8.09	7.19	8.44	6.64	8.08	6.86
Cystine	(6.50)	(5.91)	(6.82)	(5.69)	(6.83)	(5.64)
Methionine	4.01	4.43	3.98	3.97	3.81	3.88
Proline	3.71	3.30	3.84	3.28	3.65	3.40
Phenylalanine	3.70	3.51	3.51	3.53	3.12	3.42
Tyrosine	7.31	6.55	7.26	6.20	6.90	6.12
Histidine	4.08	4.16	4.37	4.43	4.07	4.19
Lysine	10.6	10.9	10.6	10.2	10.4	(9.12)
Arginine	4.90	4.75	4.88	4.43	4.75	4.55
Ammonia	2.38	3.12	2.53	2.88	2.47	3.16

evaporated to dryness over KOH pellets and made up to volume in the appropriate buffer after oxidation of cysteine to cystine[210]. An aliquot of the hydrolysate was then taken for nitrogen estimation by a micro-Kjeldahl procedure and the remaining material used for amino acid analysis by the ion exchange procedure described.

APPARATUS AND REAGENTS.

Reagents:

1. HCl. 6N.

   This was prepared by diluting concentrated HCl (S.G. 1.118) to approximately 6N. with dist. H_2O followed by triple distillation of the constant boiling mixture in an all-glass assembly. The acid was stored in dark containers away from direct sunlight.

2. Phosphate Buffer (0.2 M : pH 6.5).

3. HCl. 1 N.

   Prepared immediately before use by dilution of the 6 N HCl with ammonia-free dist. H_2O.

4. Citrate Buffer (0.2M : pH 2.2)

   The composition of this buffer is as follows:-

Citric acid. H_2O	105 g.
NaOH	42 g.
HCl (conc.)	80 ml.
Final volume	5 L.

   Final pH adjustments are made with NaOH or HCl.

Apparatus/...

Apparatus:

Hydrolysis was performed in pyrex round-bottomed flasks of 50 ml. capacity fitted with B24 ground glass joints. The condensors used were of the ("cold finger type") fitted with side arms which were connected by means of polythene tubing to a reservoir containing HCl (± 1N) to trap athmospheric ammonia. Hydrolysis was performed on a sandbath heated by two 750 watt elements.

PROCEDURE.

The hydrolysis flasks containing pure silica (vitreosil) chips to prevent bumping of the boiling mixture during hydrolysis, were cleaned in between experiments by refluxing with 6N. HCl for 2 hours, followed by exhaustive rinsing with ammonia-free dist. H_2O.

An aliquot of the protein solution, containing approximately 5 mg. of protein, was transferred to the flask and taken to dryness in vacuo over KOH pellets. To the dried protein was added 5 ml. of 6N HCl. acid (equivalent to ± 200 times the weight of the protein). The hydrolysis flasks were connected to the condensors by means of the ground glass joints and assembled on the sandbath. No grease was used on any of the joints. Hydrolysis was continued for periods of 22 hours or longer as will be specified for each experiment. The hydrolysate was then transferred quantitatively to 50 ml. beakers using several washes with distilled H_2O. The contents of the beakers were evaporated to dryness under reduced pressure using pellets of KOH as the desiccant. The residue was taken up in 0.5 ml. of warm (± 50°) distilled water and the pH. adjusted to 6.5 by addition of 1.5 ml. phosphate buffer (0.2 M : pH 6.5). The sample was left exposed to atmospheric oxygen for 4 hours to allow oxidation of cysteine to cystine [210]. The pH of the hydrolysate was subsequently adjusted to 2.4 ± 0.1 by the addition of 0.1 ml. freshly prepared HCl (1N) and made up to 5 ml. in a volumetric flask with citrate buffer (pH 2.2 : 0.2M) containing 1% v/v thiodiglycol. Quantitative transfer of the contents of the beaker is affected by using repeated small washings of citrate buffer. The hydrolysates were stored frozen until examined.

EXPERIMENTAL NOTES ON THE METHOD OF HYDROLYSIS ADOPTED.

Recovery of a known mixture of amino acids after 22 hours hydrolysis.

To determine the losses of amino acids during the hydrolytic procedure adopted, a compound standard solution of amino acids was hydrolysed under the conditions described. As the standard solution contained no ammonia, any ammonia present in the hydrolysate must have been produced as a result of oxidative deamination during hydrolysis. The concentrations of the individual amino acids in the standard after hydrolysis was determined by the ion exchange procedure described. The results obtained are presented in table XVIII.

The results obtained during this experiment are somewhat anomalous. The ammonia recovered after hydrolysis exceeded the sum of the losses for the amino acids in the original sample. This finding suggested that during hydrolysis or prior to analysis the sample was contaminated with extraneous ammonia. The low recoveries of leucine and glutamic acid could not be explained, but may be due to technical errors as the sample was not analysed in duplicate. It could be suggested that some conversion of glutamic acid to proline may have occurred by cyclisation but the formation of diketopiperazines are not known to occur in mineral acids stronger than 1 molar.

TABLE/...

TABLE XVIII.

RECOVERY OF AMINO ACIDS FROM A STANDARD SOLUTION AFTER 22 HOURS HYDROLYSIS.

Amino Acid.	Composition of Standard μ moles/ml.	Recovery μ moles/ml.	Percentage Recovery.
Aspartic Acid	0.599	0.580	96.8
Threonine	0.586	0.566	96.6
Serine	0.584	0.546	93.5
Glutamic Acid	0.692	0.660	95.4
Proline	0.564	0.575	101.9
Glycine	0.574	0.572	99.7
Alanine	0.466	0.457	98.1
Cystine	0.662	0.663	100.1
Valine	0.544	0.554	101.8
Methionine	0.554	0.543	98.0
Isoleucine	* 0.574		
Leucine	0.609	0.571	93.8
Tyrosine	0.593	0.592	100.0
Phenylalanine	0.868	0.844	97.2
Lysine HCl.	0.591	0.580	98.1
Histidine HCl.	0.588	0.575	97.8
Arginine HCl.	0.602	0.574	95.3
Ammonia	0.0	0.429	
Total	9.676	9.922	

* Isoleucine recoveries could not be calculated due to the presence of approximately 50% of allo-isoleucine in the original sample.

Recovery/...

Recovery of an internal nor leucine standard during hydrolysis of the Protein Sample.

The synthetic amino acid nor leucine is a stable substance resisting boiling in 6N HCl for periods up to 140 hours[221]. It has the additional advantage of being well separated from the other leucines on chromatography, emerging between leucine and tyrosine in the eluent off the 150 cm. column. The addition of a known quantity of this amino-acid to the sample of protein before hydrolysis provides a useful reference standard for the whole procedure and will reveal losses during transfer and sampling in the chromatographic analysis.

During the latter part of the present study the practice of adding a known amount of nor leucine to the protein to be hydrolysed was adopted. The chromatographic recoveries expressed as a percentage of the original are given in table XIX and represent the results obtained in 6 consecutive experiments.

Amino acid composition of normal serum albumin hydrolysed for different periods of time.

In order to determine the best time for hydrolysis of serum albumin under the conditions adopted, a sample of albumin from a normal adult prepared by Method I, described in chapter VII, was hydrolysed for 22, 48, 72 hours. The amino-acid composition was determined by the Moore and Stein chromatographic procedure

and/...

and the results, expressed as grammes of amino acid per 100 grammes of protein, are given in table XX.

TABLE XIX.

Chromatographic recoveries of an internal nor leucine standard after hydrolysis for 22 hours.

Date of Experiment.	Percentage Nor Leucine Recovery.
27. 8.63.	97.7%
3. 9.63.	100.5%
10. 9.63.	97.7%
19. 9.63.	100.0%
25. 9.63.	102.1%
13.10.63.	97.3%

It can be seen that recoveries in these 6 experiments were almost quantitative 100 ± 2%. Poor recoveries were usually produced by sampling errors.

TABLE/...

TABLE XX.

Amino acid composition of normal serum albumin at different times of hydrolysis.

Amino Acid	Composition expressed in g./100 g Protein.		
	22 hours	48 hours	72 hours
Aspartic Acid	9.90	10.1	10.0
Threonine	5.10	4.41	4.05
Serine	3.50	3.05	2.67
Glutamic acid	17.32	17.06	17.03
Proline	4.24	4.60	4.26
Glycine	1.59	1.63	1.53
Alanine	7.94	8.3	8.09
Cystine	5.82	5.73	2.84
Valine	6.8	6.98	6.99
Methionine	1.25	1.23	1.18
Iso leucine	1.55	1.72	1.65
Leucine	10.88	10.6	11.2
Tyrosine	4.86	4.75	4.2
Phenylalanine	7.17	6.85	7.26
Lysine	11.90	12.01	12.62
Histidine	3.44	3.47	3.64
Ammonia	1.26	1.42	1.74
Arginine	6.13	5.95	5.82

It/...

It is evident that significant losses of threonine and serine occurred on prolonged hydrolysis. This is accompanied by a progressive increase in ammonia values from oxidative deamination. Cystine and tyrosine also show losses at 72 hours. Increases in valine do not appear significant on prolonged periods of hydrolysis. The increase in isoleucine is difficult to evaluate as the accuracy of the amino acid determinations are less for those present in very low concentrations.

Reproducibility of results obtained after hydrolysis of albumin for a specific length of time.

To test the reproducibility of the results obtained after a certain time of hydrolysis, a sample of albumin prepared from a patient with kwashiorkor, by the trichloracetic acid procedure described in chapter VII, was hydrolysed and analysed, with a period of 14 days in between the experiments. This was done to determine whether experimental conditions could be adequately controlled over a period of time. The results obtained are given in table XXI.

It is clear from this experiment that reproducible results could be obtained by this procedure of hydrolysis and amino acid analysis.

TABLE/...

TABLE XXI.

Reproducibility of results obtained on the serum albumin of a patient with kwashiorkor analysed at different times.

Amino Acid.	Amino Acid composition (g./100 g. protein) Date of analysis.	
	10-12/9/63.	25-27/9/63.
Aspartic Acid	10.1	9.97
Threonine	4.8	4.9
Serine	3.46	3.5
Glutamic acid	17.6	17.45
Proline	4.46	4.66
Glycine	1.55	1.7
Alanine	8.03	7.86
Cystine	5.52	5.79
Valine	6.8	6.96
Methionine	1.25	1.23
Isoleucine	1.57	1.61
Leucine	11.19	11.28
Tyrosine	4.58	4.3
Phenylalanine	7.05	6.9
Lysine	12.32	12.3
Histidine	3.5	3.5
Ammonia	1.17	1.2
Arginine	6.19	6.0

COMMENTS/...

COMMENTS:

The present experimental observations suggest that certain amino acids are progressively lost during prolonged hydrolysis whereas isoleucine and valine values may increase. However, the values obtained after 22 hours hydrolysis were not corrected by extrapolation to zero time as the validity of this procedure remains doubtful. It is known that amino acids are less stable when linked in peptide form [222]. It can not, therefore, be assumed that the destruction of amino acids occurs as a first order reaction. In view of the reproducibility of the results obtained when a protein sample was hydrolysed in duplicate for a specific period of time, significant differences between normals and abnormals should be detectable by this procedure. In addition, the analysis by the manual Moore and Stein technique of a large number of samples hydrolysed for three different periods of time would have been a formidable task.

DETERMINATION OF AMIDE AMMONIA IN ALBUMIN.

The ammonia measured in a protein hydrolysate is derived mostly from the amides, glutamine and asparagine, and, to a lesser extent, from threonine and serine as a result of oxidative deamination. In the present study, the ammonia values determined chromatographically showed rather wide variations from case to case.

It was consequently essential to determine true amide ammonia values by a different procedure.

The side chain amide groups in a protein are attached to the terminal carboxyl groups of aspartic acid and glutamic acid. Under mild conditions of hydrolysis the amide ammonia can be liberated before any ammonia is produced by deamination of other amino acids. Acid hydrolysis is preferable to alkaline hydrolysis as the latter produces rapid deamination of, especially, threonine and serine, yielding unreliable results.

HCl is the acid most commonly used under different experimental conditions, ranging from 12N acid at 37° C for 10 days to shorter times at 100° C using more dilute acid [223]. Moore and Stein [210] demonstrated that the amide ammonia in ribonuclease A could be completely liberated by normal H_2SO_4 in 4 hours at 100° C. These workers measured the liberated ammonia using Conway units. This method was modified in the present study where the ammonia was trapped in boric acid after distillation in a micro Kjeldahl apparatus. The ammonia was subsequently measured by the ninhydrin procedure described.

METHODOLOGY.

Reagents:

1. H_2SO_4 1N. (ammonia free).
2. H_3BO_3 2% w/v in ammonia-free distilled H_2O.

3./...

3. Saturated K_2CO_3 (112 g. dissolved in 100 ml. ammonia-free dist. H_2O).

4. Stock NH_4Cl standard solution (0.2675 g./litre dist. H_2O).

   A working NH_4Cl standard containing 0.2 u moles/ml. was prepared by adding 1 ml. of stock standard to 5 ml. of Boric acid solution and diluted to 25 ml. with deionised H_2O.

   This standard was used to determine the colour yields for ammonia in boric acid by the ninhydrin technique and recoveries were based on the values so obtained.

5. Asparagine Standard 5.0 μ moles/ml.

   Prepared by dissolving 0.37535 g. asparagine in deionised H_2O to a final volume of 500 ml.

Method:

With each batch of amide ammonia determinations, asparagine and ammonium chloride standards were examined to check the hydrolysis and distillation steps respectively.

GENERAL PROCEDURE.

To the sample to be analysed was added the appropriate quantity of H_2SO_4 (1N) in a 50 ml. glass-stoppered centrifuge tube and heated for 4 hours in a boiling water bath. After 4 hours the contents were quantitatively transferred to the distillation chamber of a micro Kjeldahl flask. The free ammonia in the hydrolysate was steam distilled into 5 ml. of boric acid (2% w/v) after the addition of 5 ml. saturated K_2CO_3 to the distillation chamber. The distillation was continued until a volume of 20 ml. had been reached.

The distillate was then transferred quantitatively to a 25 ml. volumetric flask and made to volume with dist. H_2O. The ammonia content of the distillate was determined in duplicate on 1 ml. aliquots by the ninhydrin procedure. Appropriate blanks consisting of deionised water instead of the standard or unknown were analysed by the same procedure.

Determination/..

Determination of amide ammonia in albumin samples:

A sample of albumin containing approximately 12.5 mg. of protein was hydrolysed with 9 ml. of H_2SO_4 (1N) as outlined above. 5 ml. aliquots of the hydrolysate were then taken for amide nitrogen and Kjeldhal nitrogen estimation respectively. The Kjeldahl nitrogen value so obtained was used in the calculation of the amide ammonia content in g./100g. protein.

Asparagine Standard Recovery:

1 ml. asparagine standard containing 5 μ moles/ml. was hydrolysed as before and the ammonia content measured in 1 ml. aliquots of the distillate.

NH_4Cl standard recoveries:

1 ml. undiluted NH_4Cl standard (5 μ moles/ml) was steam-distilled, following the addition of 5 ml. K_2CO_3 and the distillate examined by the ninhydrin procedure as outlined before.

RESULTS:

The results of duplicate recovery experiments are presented in table XXII.

TABLE XXII.

Duplicate recoveries of NH_4Cl and Asparagine in the procedure for amide ammonia determination.

Standards.	μ moles/ 1 ml distillate.		% Recovery.
	Expected.	Recovered.	
NH_4Cl.	0.2	0.201	100.6
Asparagine.	0.2	0.196	98.5

The figures present the average values obtained during an experiment performed in duplicate.

CHAPTER X.

CASE MATERIAL

AND

GENERAL OUTLINE OF

EXPERIMENTAL PROCEDURE.

CHAPTER X.

CASE MATERIAL:

The criteria adopted for the diagnosis of kwashiorkor were the same as those described in part A of this thesis. In the majority of cases, blood samples were collected at the time when the patients first attended the Outpatient Department. From two patients admitted to the hospital, blood was also collected one week after initiation of treatment. This was done in order to determine whether alterations in amino acid composition can occur during this very active period of protein synthesis. The normal adult controls were both laboratory workers, while the normal children represented cases admitted for elective surgery and judged to be otherwise healthy. Three of the children were convalescing after attacks of acute poliomyelitis, the samples being collected after all signs of activity of the disease had subsided. The relevant data applying to the case material are presented in table XXIII.

Collection and subsequent processing of the blood samples.

Blood was collected by venepuncture and the serum separated by centrifugation. Total protein and albumin-globulin ratios were determined by the biuret procedure (123) and by the Na_2SO_4 fractionation method of Howe [160] respectively. The serum

samples/...

samples were then dialysed against distilled water for 4-6 hours and lyophylised. The dried protein samples were stored at 4° C in sealed ampoules until further processing. Prior to the preparation of albumin by one of the methods described in chapter VII, the dried protein was dissolved in phosphate buffer (0.06M : pH 7.0).

The albumin preparations were dialysed exhaustively against distilled water and stored in the frozen state until hydrolysis. It was considered essential to dialyse the preparations before hydrolysis in order to remove any remaining traces of free amino acids or ammonia which could have been present in the albumin samples.

An aliquot of the purified albumin containing approximately 5 mg. of protein, to which was added the internal nor leucine standard (3 μ moles), was then hydrolysed under the conditions already described. The hydrolysed sample was evaporated to dryness over NaOH pellets in a vacuum desiccator to remove the acid and made up to 5 ml. in a volumetric flask. The hydrolysate was stored frozen until the amino acid analysis could be performed. Each 1 ml. aliquot of the hydrolysate, therefore, contained the equivalent of approximately 1 mg. protein and 0.6 μ moles of nor leucine.

Amino acid analyses were performed on 1 ml. samples and the nitrogen content was determined on an equal volume of the hydrolysate by the micro-Kjeldahl procedure. The corrected

TABLE XXIII.

CASE MATERIAL.

SUMMARY OF RELEVANT DATE.

Source	Sample	Age years	Sex	Race	Serum Proteins (g./100 ml)		
					Total Protein	Albumin	Globulin
Normal Adults:	Pim.	27	M	E	7.4	4.70	2.70
	Car.	35	M	C	6.9	4.00	2.90
Normal Children:	Kis.	$1\frac{9}{12}$	M	N	6.5	3.85	2.65
	Park.	$4\frac{6}{12}$	M	C	7.73	4.47	3.26
	Shew.	$1\frac{5}{12}$	F	N	6.74	4.74	2.00
	Ab.	$1\frac{4}{12}$	F	C	6.20	4.30	1.90
	Fled.	$1\frac{6}{12}$	M	C	6.16	4.16	2.00
	Jenk.	2	M	E	5.62	3.90	1.72
Kwashiorkor (before treatment)	For.	1	F	C	4.0	1.8	2.2
	Toy.	$1\frac{6}{12}$	M	C	4.1	2.2	1.9
	Han.	$1\frac{3}{12}$	M	C	2.7	1.6	1.1
	Jon.	$2\frac{1}{12}$	F	C	3.0	1.6	1.4
	Gam.	3	F	N	3.7	2.3	1.4
	Ziz.	$1\frac{6}{12}$	F	C	4.52	2.84	1.68
	Ock.	$1\frac{9}{12}$	M	C	4.16	2.71	1.45
	Con.	$2\frac{4}{12}$	M	C	4.5	2.9	1.6
	Is.	$\frac{9}{12}$	M	C	3.7	2.1	1.6
Kwashiorkor (after one week on treatment)	Con.	$2\frac{4}{12}$	M	C	5.0	3.3	1.7
	Is.	$\frac{9}{12}$	M	C	5.4	3.3	2.1

N = Native (African) M = Male. F = Female.
C = Cape Coloured. E = European.

nitrogen value was obtained by subtracting the nor leucine nitrogen, determined by chromatographic analysis, from the total nitrogen figure obtained by the Kjeldahl estimation.

When unsatisfactory results were obtained as a result of technical problems which arose during the chromatographic procedure, the analysis was repeated on a further aliquot of the same hydrolysate or, if insufficient material was available, a sample of the original albumin was again hydrolysed and examined.

CHAPTER XI.

THE AMINO ACID COMPOSITION OF SERUM ALBUMIN FROM NORMAL INDIVIDUALS AND PATIENTS WITH KWASHIORKOR.

RESULTS.

CHAPTER XI.

CALCULATION AND EXPRESSION OF RESULTS.

In order to render the results obtained comparable to those reported by other workers, the amino acid composition of albumin was expressed in grammes of amino acid per 100 grammes of protein. The nitrogen values, obtained by Kjeldahl estimation and by summation of the amino acids recovered during chromatographic analysis of the hydrolysate, were converted to its protein equivalent by using a factor of 6.25, based on the reported nitrogen content of human albumin which is given as 16% w/w. In the calculation, the actual nitrogen recovered during the chromatographic procedure was used and not the theoretical value computed from Kjeldahl analysis. This is essential as, although the ratios of the amino acids to one another remain the same, results expressed in g./100 g. protein will vary according to the actual recoveries of nitrogen. For instance, when the nitrogen recoveries during two experiments are 93% and 101% respectively, the results of the former will be 8% lower than those of the latter when based on the theoretical 100% and expressed as g./100 g. of protein.

No attempts were made during the present investigation to determine the tryptophan content of the albumin samples as usually insufficient material was available. Cystine was expressed as half cystine and probably includes cysteine since the latter

was/...

was oxidised to cystine after hydrolysis.

The results obtained during the analysis of albumin samples from normals and patients with kwashiorkor are presented in table XXIV. In the abbreviations, the first three letters of the amino acid are used as the symbol for each in the following tables.

Asp = Aspartic acid.	Ala = Alanine	Tyr = Tyrosine
Thr.= Threonine	Cys = Cystine	Phe = Phenylalanine
Ser = Serine	Val = Valine	Lys = Lysine
Glu = Glutamic	Met = Methionine	His = Histidine
Pro = Proline	Ileu = Isoleucine	NH_3 = Total Ammonia
Gly = Glycine	Leu = Leucine	Arg = Arginine

The three methods used for the preparation of albumin are described in chapter VII. Method I (C.M.C.) refers to chromatography on C.M.C. without preliminary purification. Method II (P.e.g. C.M.C.),preliminary purification with polyethylene glycol followed by chromatography on carboxy methyl cellulose. In this method the albumin was separated into two components of different electrophoretic mobility where fraction I refers to the faster, and fraction II to the slower moving component.

From inspection of the results presented in table XXIV it is evident that there is some scatter of the results for each amino acid in both the normal and kwashiorkor groups as well as

the/...

TABLE XXIV.

Amino-acid composition of serum albumin from normals and patients with kwashiorkor expressed in g./100 g. protein.

Source.	Preparation.	Asp.	Thr.	Ser.	Glu.	Pro.	Gly.	Ala.	Cys.	Val.	Met.	Ileu.	Leu.	Tyr.	Phe.	Lys.	His.	NH_3	Arg.	% Recovery. Nor Leu.	% Recovery. Nitrogen.
NORMAL.	Method I CMC																				
Kis. Child	" "	10.01	4.93	3.40	17.40	4.47	1.66	7.55	5.70	6.86	1.102	1.66	10.55	4.88	6.86	11.81	3.40	1.34	6.43	100.7	95.6
Park. "	" "	9.74	4.87	3.58	17.29	4.31	1.69	8.02	5.56	6.51	1.31	1.70	10.80	4.65	7.36	12.32	3.76	1.23	5.54	100.7	99.9
Pim. Adult	" "	9.90	5.05	3.5	17.32	4.24	1.59	7.94	5.82	6.80	1.25	1.55	10.88	4.86	7.17	11.90	3.44	1.26	6.13		93.0
KWASHIORKOR.																					
Jon.(untreated)	" "	9.94	5.03	3.75	16.88	4.34	1.94	7.37	5.54	6.73	1.28	2.18	10.57	4.61	7.00	12.03	3.78	1.30	5.96		97.0
Ziz. "	" "	9.73	4.75	3.64	16.90	4.43	1.52	7.98	5.70	6.45	1.14	1.53	10.52	4.36	7.12	11.86	3.8	1.43	6.19		100.1
Ock. "	" "	10.00	4.77	3.42	17.78	4.46	1.51	8.60	5.42	7.40	1.28	1.62	11.20	4.59	7.17	11.60	3.52	1.24	5.89		94.2
Gam. "	" "	9.92	4.84	3.84	16.50	4.12	1.98	7.75	4.75	7.40	1.34	1.77	10.78	4.74	7.17	11.63	3.36	1.69	5.64		100.1
NORMAL.	Method II Pap. CMC																				
Jenk. Child	Fraction I	10.21	4.64	3.47	17.3	4.35	1.58	7.93	5.91	6.60	1.25	1.62	11.03	4.42	7.30	12.46	3.8	1.21	6.20	97.7	92.4
" "	" II	10.18	4.85	3.59	17.46	4.40	1.51	8.01	6.14	6.67	1.29	1.58	11.00	4.55	7.38	12.20	3.7	1.12	5.97	100.5	95.4
Fled. "	" I	10.03	4.84	3.60	17.00	4.61	1.58	8.00	5.64	6.65	1.27	1.50	10.70	4.52	7.30	12.20	3.70	1.17	6.40	104.1	97.5
" "	" II*																				
J.C. Adult	" I	10.02	5.03	3.64	17.21	4.38	1.66	7.75	5.67	6.68	1.26	1.78	10.60	4.60	7.00	12.31	3.62	1.46	5.90	103.0	96.1
" "	" II	9.99	4.82	3.71	16.83	4.45	1.78	7.74	5.68	6.49	1.23	1.72	10.72	4.47	7.30	12.39	3.73	1.32	5.82	97.7	99.1
KWASHIORKOR.																					
Han.(untreated)	" I	10.1	4.78	3.59	17.3	4.62	1.59	7.92	5.56	6.85	1.28	1.64	11.14	4.47	7.20	12.22	3.63	1.13	6.18	98.7	102.5
" "	" II	10.0	4.90	3.50	17.23	4.50	1.53	7.94	5.46	6.86	1.28	1.71	11.20	4.58	7.03	12.20	3.69	1.18	6.14	102.5	104.0
Con. "	" I	9.80	4.73	3.50	17.20	4.60	1.61	7.83	5.68	6.62	1.25	1.61	10.60	4.30	7.00	12.27	3.63	1.38	5.96	98.3	95.4
" "	" II	10.10	4.75	3.53	17.24	4.63	1.60	7.86	5.70	7.10	1.26	1.52	11.00	4.56	7.12	12.27	3.45	1.19	6.17	97.7	94.0
" (1 week after starting treatment)	" I	9.70	4.75	3.70	17.60	4.36	1.61	8.00	5.68	7.10	1.27	1.68	10.90	4.55	7.10	11.70	3.69	1.30	6.02	98.5	101.6
	" II	9.95	5.05	4.00	17.00	4.60	1.91	7.50	5.37	6.90	1.29	1.80	10.80	4.34	7.09	11.30	3.40	1.50	5.96	99.7	102.2
NORMAL.	Method III TCA																				
Shew.	"	10.0	4.80	3.65	17.55	4.36	1.57	8.00	5.55	7.01	1.28	1.57	11.01	4.64	7.42	12.18	3.52	1.25	5.80	98.0	98.2
Ab.	"	10.2	4.90	3.54	17.50	4.20	1.61	8.20	5.65	6.70	1.27	1.50	11.00	4.55	7.30	12.23	3.31	1.20	6.19	101.2	97.7
KWASHIORKOR.																					
For.(untreated)	"	9.77	4.55	3.56	17.13	4.53	1.59	7.83	5.78	6.71	1.23	1.46	10.68	4.52	7.15	11.99	3.64	1.37	6.20	99.7	94.9
Toy. "	"	9.84	4.67	3.57	17.30	4.49	1.62	7.96	5.63	6.53	1.26	1.59	10.91	4.50	7.15	11.96	3.32	1.43	6.18	98.3	96.7
Is. "	"	9.99	4.85	3.50	17.52	4.56	1.62	7.95	5.65	6.88	1.24	1.59	11.23	4.44	6.97	12.31	3.50	1.19	6.09	99.8	98.4
Is.(1 week after starting treatment)	"	10.00	4.75	3.51	17.70	4.56	1.68	8.24	5.61	6.70	1.22	1.61	11.50	4.74	6.97	12.00	3.30	1.05	6.50	100.0	93.0

* Sample spoilt.

between the different methods of albumin preparation. As no obvious differences in the amino acid composition between albumin from normal individuals and patients with kwashiorkor or between the different preparations could be detected the results were subjected to a test for homogeneity using the X^2 method of analysis. The data from the following groups were compared.

1. Amino acid composition of albumin from normal individuals with that from patients with kwashiorkor for each method of albumin preparation.
2. Amino acid composition of normal albumin prepared by three different methods.
3. Amino acid composition of albumin from children with kwashiorkor prepared by three different methods.
4. Amino acid composition of the two albumin fractions obtained by Method II (P.e.g. C.M.C.) from both the normal groups and from patients with kwashiorkor.
5. The amino acid composition of albumin from untreated patients with their albumin one week after initiation of treatment.

In each case a value for $P > 0.99$ was obtained. Although by treating the results in this way differences of one or two residues per molecule of protein may not be detected, the X^2 test suggests that the albumins from the normal controls and patients with kwashiorkor form a homogeneous group with no detectable

differences/...

differences in amino acid composition.

Amide Ammonia values of albumin:

The total ammonia concentrations presented in table XXIV are the values obtained by chromatographic analyses of the albumin samples hydrolysed for 22 hours. It can be seen that wide variations occur in all the groups. The ammonia measured in this way represents true amide ammonia in addition to ammonia presumably derived by oxidative deamination of mainly serine and threonine. The results of amide ammonia determinations on albumin from four patients with kwashiorkor and four normal individuals are presented in table XXV and compared with the total ammonia values found on chromatographic analysis of the same preparations.

The number of samples analysed for true amide ammonia concentrations are too small to draw valid conclusions, in view of the wide scatter of the results obtained. Certain observations are perhaps justified. In all cases, the amide ammonia values were lower than the total ammonia values but this difference was by no means constant. It has to be concluded, therefore, that significant losses of amino acids occur even after 22 hours of hydrolysis. However, the source of this secondary ammonia is not so obvious.

It will be noticed that the biggest differences between true amide ammonia and total ammonia concentrations were found in

patient/...

patient Gam, although the values for threonine and serine were, if anything, higher than the mean normal for these amino acids. In this particular case there appears to be a net gain of ammonia. The possibility of contamination with atmospheric ammonia during preparation of the sample must be considered but this does not appear to be the sole cause. This possibility was investigated by exposing a sample of the phosphate buffer (0.2 M : pH 6.5) to the atmosphere for four hours under identical conditions with those present during the oxidation of cysteine to cystine. There was no significant difference in the ninhydrin colour value before and after the four hour period. It is interesting to note that Leach and Parkhill [222] made similar observations and could also not demonstrate any correlation between this secondary liberation of ammonia and threonine and serine concentrations. In addition, this factor varies from protein to protein. These findings further strengthen our contention that values for threonine and serine obtained by extrapolation to zero time are not always valid. Although this problem of hydrolysis of proteins has exercised the minds of many workers it is by no means settled. Recently Hill and Schmidt [224] described a method for the complete enzymic hydrolysis of proteins employing only proteolytic enzymes. It may be necessary to re-examine our concepts by applying a more physiological method such as the latter to the elucidation of this problem.

TABLE/...

TABLE XXV.

Amide ammonia values and total ammonia values obtained on human serum albumin prepared by different methods.

Source of Albumin	Preparation	a Amide Ammonia g./100 g. protein	B Total Ammonia g./100 g. protein	b-a g./100 g. protein
NORMALS:				
Shew.)	Method III (TCA)	0.993	1.25	0.257
Ab.)Children	Method III (TCA)	1.08	1.20	0.120
Jen.)	Method II (Peg.CMC.)	1.003	1.12	0.117
Pim. Adult	Method I (CMC)	1.04	1.26	0.220
Average.		1.02		
KWASHIORKOR:				
Toy.	Method III (TCA)	1.194	1.43	0.236
Han.	Method II (Peg. CMC)	1.026	1.18	0.154
Gam.	Method I (CMC)	1.200	1.69	0.490
Jon.	Method I (CMC)	1.130	1.30	0.200
Average		1.14		

NITROGEN RECOVERIES.

It can be seen from table XXIV that recoveries of nitrogen

varied/...

varied between 93% and 104%. Low recoveries can not be explained by losses during transfer of samples only, as the nor leucine recoveries should then also be less. It is more likely that in some cases the low recoveries were due to the presence of unhydrolysed peptides which would not be detected during the chromatographic analysis as the colour yields given by peptides are usually much less than those of the free amino acids. Again there is no apparent correlation between recoveries of nitrogen and the isoleucine and valine values found. These observations again illustrate the importance of using the quantity of nitrogen recovered and not the theoretical value when calculating results expressed in g./100 g. of protein.

Amino acid composition of albumin from kwashiorkor and normals compared with values reported in the literature.

In view of the fact that no statistically significant differences could be demonstrated between the albumins from normal persons and patients with kwashiorkor, or between albumins prepared by different methods, the average values in each group (normal and kwashiorkor) are presented in table XXVI.

The values of Brand [225], although obtained by a variety of microbiological and chemical procedures are still regarded as the best available to date. In the present investigation, the molecular weight of albumin was assumed to be 67,000 whereas

TABLE XXVI.

Amino acid composition (average value) of serum albumin from patients with kwashiorkor and normals, compared with reported values.

Amino acid	g./100 g. protein.			Residues/mole of protein.		
	Normals	Kwashiorkor	Brand	Normals	Kwashiorkor	Brand
Asp.	10.00	9.92	10.4	50	50	55
Thr.	4.88	4.80	5.0	27	27	29
Ser.	3.57	3.61	3.7	23	23	25
Glut.	17.29	17.24	17.4			
Prol.	4.38	4.49	5.1	26	26	31
Glyc.	1.62	1.67	1.6	15	15	15
Ala.	7.91	7.91	-	59	59	-
½Cys.	5.73	5.54	5.58	31	31	32
Val.	6.69	6.87	7.7	38	39	46
Meth.	1.25	1.26	1.28	6	6	6
Ileu.	1.62	1.66	1.7	8	8	9
Leu.	10.83	10.92	11.9	55	56	64
Tyr.	4.61	4.52	4.66	17	17	18
Phe.	7.24	7.09	7.8	29	29	33
Lys.	12.20	11.95	12.3	56	55	59
His.	3.60	3.55	3.5	15	15	16
Total NH_3	1.26	1.31	-			
Amide NH_3	1.02	1.14	1.07			
Arg.	6.04	6.08	6.15	23	23	25
Free Glut.				39	34	39
Glut-NH_2				40	45	44
Cysteine			0.70			4
Tryptophan			0.19			1
Total	**110.72**	**110.4**	**107.7**	**557**	**558**	**551**

Assumed Molecular weight of Albumin:-

Present Author = 67,000.

Brand [225] = 70,000.

Residues per mole corrected to the nearest integer.

Brand [225] used a figure of 70,000. All the amide ammonia was assigned to glutamine, and the free glutamic acid value represents the difference between total glutamic acid obtained by chromatographic analysis and amide ammonia (glutamine) determined independently. The amide ammonia values represent the average of the four normals and four patients with kwashiorkor given in table XXV.

It is evident that the results found in the present study agree remarkably well with those of Brand [225] with the exception of valine, proline and leucine. The difference in number of residues between our results and those of Brand can largely be accounted for by the different molecular weights used. To our knowledge, no other results on the composition of human serum albumin, as determined by modern chromatographic procedures, have been published. However, the values found by the isotope dilution method of Shemin [226] who determined the concentrations of glutamic acid, aspartic acid, tyrosine and glycine in human serum albumin, are in good agreement with the present results (table XXVII).

It can be seen from table XXVI that the number of residues/mole of albumin in normals and kwashiorkor is identical for all amino acids except valine, leucine and lysine where there is a difference of one residue. It is felt that the detection of one residue difference in a molecule of 67,000 (M.W.) is probably

beyond/...,,

TABLE XXVII.

Concentration of four amino acids in normal human serum albumin determined by ion exchange chromatography (present author) and the isotope dilution method (Shamin [226]).

Amino Acid.	Method of Analysis.	
	Chromatographic.	Isotope Dilution.
Glutamic Acid	17.29	17.03
Aspartic Acid	10.00	9.77
Tyrosine	4.61	4.73
Glycine	1.67	1.60

beyond the accuracy of the method in our hands and these differences are not regarded as significant.

The significance of the difference in the number of glutamine and glutamic acid residues between the albumin of normals and patients with kwashiorkor can not be dismissed so readily.

Here the differences appear more clearcut but, evenso, one of the results in each group falls well within the range of the other. A small number only of samples have so far been examined and it is essential that more determinations should be performed before this question can be resolved. It is also well known that the amide content of a protein may be affected by isolation and

purification procedures [151]. Although the samples examined are representative of different methods of isolation it can not be stated with confidence that conditions were absolutely identical during each experiment. Further speculations are, therefore, unjustified on such a small number of samples. It must be concluded that, with the methods employed in the present investigation, no differences in the amino acid composition of albumin from normal persons and patients with kwashiorkor could be demonstrated, with the possible exception of the amide ammonia concentration.

CHAPTER XII.

DISCUSSION.

CHAPTER XII.

DISCUSSION.

No differences in the amino acid composition of serum albumin from normal children and patients suffering from the protein depleted state, kwashiorkor, could be detected by the methods used in the present investigation. The validity of this conclusion depends on certain assumptions and additional factors which have to be considered.

1. That the albumin samples are representative of the body pool of albumin synthesised at a particular time.

It has to be assumed that the albumin isolated from serum is representative of the albumin synthesised by the cells at the time of collection of the specimen, and that the composition of intracellular and extracellular albumin is the same. It is known that chemically altered protein molecules are degraded by an animal into which they are injected, at a much higher rate than untreated molecules [227]. Similarly, foreign proteins in their native state are not recognised by the recipient but are destroyed through the antibody mechanism. Theoretically, therefore, protein molecules significantly different from those produced normally may be broken down rapidly intracellularly without ever reaching the extracellular environment. This mechanism, however, would defeat the object of adaptive alterations in protein synthesis for

survival/...

survival of the animal under conditions of stress, and has no other evidence to support its existence.

In the condition of multiple myelomatosis we have an example in which the circulating serum proteins are not representative of the molecular species synthesised. This disease which is regarded as a neoplasm of the plasma cells which synthesize globulin, is characterised by the production of the so-called myeloma protein which can be demonstrated in the serum and by the excretion of Bence-Jones protein in the urine. The latter which is a low molecular weight protein (37,000-45,000), is regarded as a precursor of the myeloma protein found in the serum but is excreted in the urine at such a rate that its presence in the serum can not usually be detected [228]. Furthermore, the amino acid composition of a number of well characterised proteins, analysed by the Moore and Stein technique, varied significantly, although the general pattern resembled human γ globulin, being notably rich in threonine and serine. The content of a given amino acid often varied within a twofold range for the fourteen Bence-Jones proteins studied [229]. The amino acid composition of the myeloma protein isolated from the serum may, therefore, not be representative of the pool synthesised intracellularly, and no valid conclusions can be drawn with regard to alterations in protein synthesising mechanisms from data so obtained.

There is no evidence to suggest that a similar state of

affairs/...

affairs exists in kwashiorkor. It is well known that total nitrogen excretion in the urine is low and proteinuria is not a feature unless complications are present. The studies of Berman and Kench [230] revealed an increased excretion of bound amino acids in the untreated state, but these peptides may be derived from tissue protein catabolism and need not necessarily represent intermediates of protein synthesis.

2. Selectivity of preparative methods.

It has already been pointed out that the method of isolation and purification of a particular protein from a complex mixture should yield a product which is representative of the entire molecular species. If only a fraction of the original protein is recovered in the final preparation, conclusions based on the amino acid composition of the final product cannot be valid. For this reason, it was considered essential to use different methods for the preparation of albumin in the present investigation. At least one of these methods gave yields approaching the theoretical. However, it still can not be argued that no differences in the amino acid composition of albumin were present, even if the material analysed represented the bulk of the original protein. If there is a true microheterogeneity of the primary structure of albumin, the protein deficiency state may produce an alteration in the relative amounts of different molecules

synthesised/...

synthesised. Unless the amino acid composition of these groups of molecules is significantly different, analysis of the mixture will reveal no discrepancies.

Theoretically, therefore, the isolated protein should be separated into the largest number of fractions possible with the available physico-chemical procedures, and the amino acid composition of each fraction determined individually. The two albumin fractions of different electrophoretic mobility examined in the present study showed no difference in amino acid composition. Rejnek et al [231] subjected preparations of human serum albumin to chromatographic analysis on modified cellulose and studied the fractions obtained by immunoelectrophoresis. Certain differences in the antigenic properties were demonstrated, which were ascribed to true microheterogeneity of the original sample. Again these observations cannot be interpreted as indicating variations in primary structure. These workers did not examine the different fractions of albumin for the presence of isomers by ultracentrifugation, neither was the amino acid composition of the individual fractions determined. The antigenic differences could possibly be due to masking of antigenic groups on the surface of some molecules as a result of dimer or polymer formation. If this is so the technique is exposing differences, not in the primary amino acid composition but in the secondary and tertiary structure of the molecules. Further evidence that antigenic differences do not

necessarily/...

necessarily reflect differences in amino acid composition was provided by Smith et al [219]. These workers examined the amino acid composition of rabbit antipneumococcal antibodies to types I, VII, VIII and XIV but could demonstrate no differences between these. Variations in physico-chemical or even biological properties, therefore, do not necessarily reflect differences in amino acid composition. Alternatively, similarity of physico-chemical properties cannot be accepted as evidence of similarity in primary structure of proteins. The physico-chemical parameters, sedimentation constant, diffusion constant, intrinsic viscosity, molecular weight and shape of prothrombin and albumin molecules are remarkably similar but the amino-acid composition of the two proteins is entirely different [232].

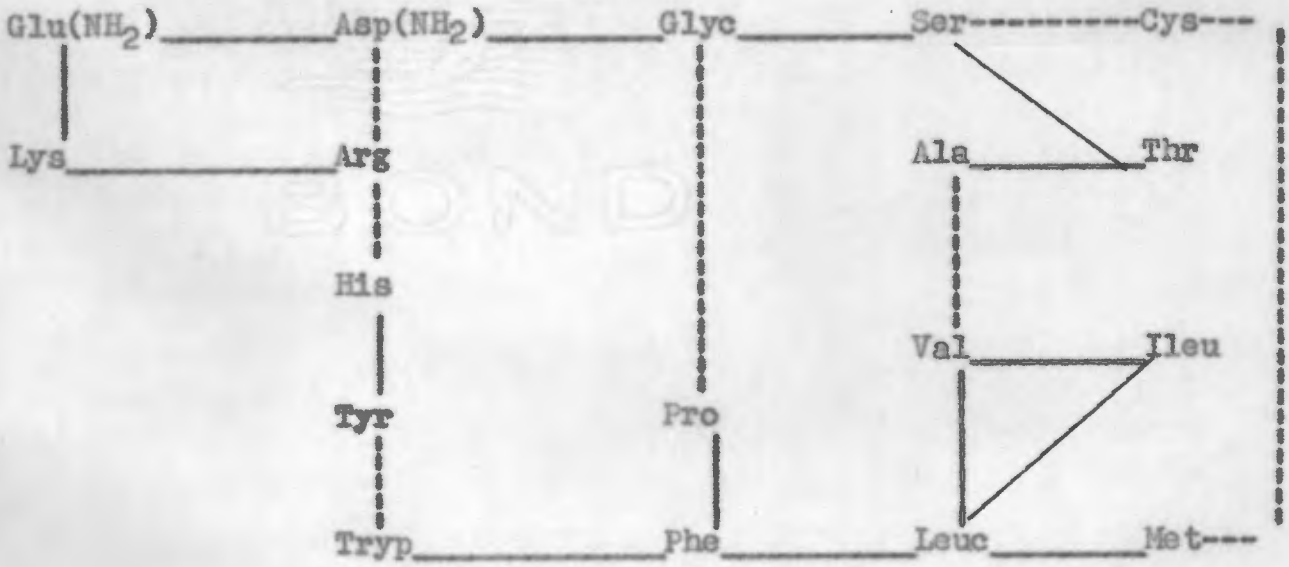
It must therefore be concluded from the available evidence on the amino acid composition of albumin fractions under normal conditions and from our observations in the protein-depleted state that no detectable differences exist. The validity of the observations on the amide nitrogen content of albumin have already been discussed but no further conclusions can be drawn until more samples have been examined.

It appears, therefore, that protein molecules within a given species are reproduced with almost unfailing accuracy. However, certain interchanges of amino acids have been demonstrated in homologous proteins from different species. This subject was

recently reviewed in great detail by Sorm and Keil [233]. Although these considerations cannot be fully dealt with in the present discussion, some mechanisms whereby one amino acid may be replaced by another within a given protein molecule will be outlined.

From a comparison of the amino acid sequences in biologically related proteins from different animals it became evident that certain amino acids are quite regularly replaced by other amino acids during phylogeny. These interchanges as envisaged today are given in fig. 51 taken from the review of Sorm and Keil [233].

Fig. 51.



Amino acid Interchanges.

Full lines directly connecting the symbols of two amino

acids indicate standard amino acid interchanges, i.e. interchanges which have been observed quite consistently; dotted lines represent interchanges which are probably also standard but have not been reliably established.

As outlined already the specific sequential incorporation of amino acids into a protein molecule depends on the sequence of code words, on the messenger R.N.A. tem plate. As yet no overlapping of the code words for different amino acids has been demonstrated. On the assumption that each code word consists of three bases, one of which appears invariably to be uracil, Smith [234], [235] postulated that only one of the two remaining bases need be replaced to interchange the amino acids incorporated at a particular site in the peptide chain.

To facilitate discussion it is necessary to present the stages of protein biosynthesis in a simplified form and consider the possible mechanisms whereby the primary structure of a protein may be altered at these different stages. Such a simplified scheme is presented in fig. 52. The different stages are indicated by capital letters A-D.

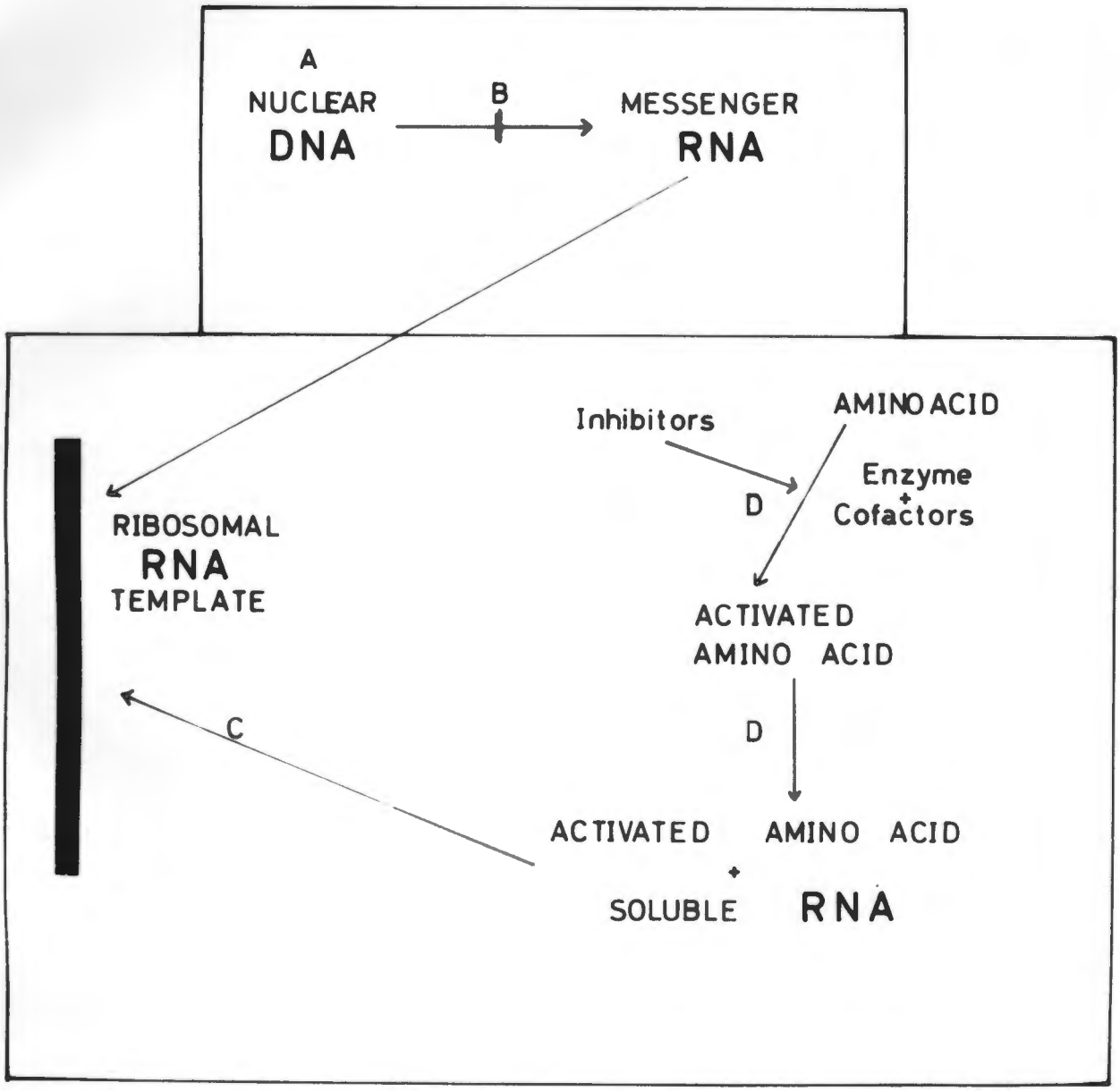
Stage A:

Changes in the base sequence or genetic information of nuclear desoxyribonucleic acid (DNA).

Knowledge concerning the synthesis of D.N.A. is too incomplete to warrant speculation but certain factors are worthy of discussion.

FIG. 52.

SCHEMATIC PRESENTATION OF PROTEIN BIOSYNTHESIS.



Mutations:

Genetic changes are known to occur frequently in phylogeny and produce the driving force for the evolution of new functions in proteins by the addition or alteration of reactive groups. These will modify or change the specifity and reactivity of enzymes and other proteins or peptides at their active sites. For instance, the substitution of phenylalanine for isoleucine in the primitive vasotocin of the frog produces the much more active vasopressin of higher animals. This again involves only a single change in the code words U_2A (isoleucine) to U_3 (phenylalanine) [234]. Some mutations may of course be deleterious involving the loss of an enzyme.

It becomes necessary then to consider some factors which will increase the rate of mutations in a particular organism. The treatment of tobacco mosaic virus with nitrous acid results in the deamination of nucleotides [236] and the formation of mutant strains of the virus [237]. Cytidine is converted to uridine, adenosine to hypoxanthine and guanosine to xanthosine. The protein isolated from such mutant strains is, in many cases, different from the protein produced by the original or wild type of virus [238].

The classical example of the production of a protein with specific properties resulting from a specific stimulus or change in environment is the formation of antibodies. It is wellknown that the antibodies of the newborn, at least in man, are derived from

the/...

the mother by placental transfer. The production of new antibodies are stimulated by specific antigens. It is postulated that as a result of the primary stimulus, antigen enters the nucleus of potential antibody forming cells and produces a modification of desoxyribonucleic acid (DNA). This leads to the reproduction of templates (possibly ribonucleoprotein) which are specifically related to the antigen. However, it is necessary again to stress that differences in amino acid composition of antibodies have not been demonstrated and Paulings [239] suggestion that the specifity of antibody is a function only of folding of the peptide chain, is still not disproven.

Very little is known as to how alterations in the composition and concentrations of the substrate pool will effect the rate of mutations. It is interesting in this respect to compare the amino acid composition of albumins from different mammalian species - Table XXVIII. It can be seen that certain definite differences are present. Whether these represent point mutations induced by the different diets consumed by these species must remain only a speculation.

B. ERRORS IN TRANSMITTING THE NUCLEAR D.N.A. CODE TO MESSENGER R.N.A.

Although this hypothesis has not been tested experimentally, to our knowledge, it is theoretically possible that non-

genetically determined changes in amino acid composition may be produced by incorrect transcribing of nuclear code to the ribosomal R.N.A. template. This is unlikely to be a common error and may be undetectable.

TABLE XXVIII.

Amino acid composition of serum albumin from different mammalian species in g./100 g. protein.

Amino Acid	Species.			
	a Human	b Bovine	c Dog	d Rat
Aspartic Acid	10.00	10.91	8.95	10.07
Threonine	4.88	5.83	3.55	5.72
Serine	3.57	4.23	3.36	3.77
Glutamic Acid	17.29	16.5	16.18	17.46
Proline	4.38	4.75	4.51	5.14
Glycine	1.62	1.82	1.99	1.96
Alanine	7.91	6.25	6.55	7.89
Cystine/2	5.73	-	5.12	5.75
Valine	6.69	5.92	6.11	5.85
Methionine	1.25	0.81	0.78	1.32
Isoleucine	1.62	2.61	0.99	2.04
Leucine	10.83	12.27	10.99	10.71
Tyrosine	4.61	5.06	5.26	6.00
Phenylalanine	7.24	6.59	6.66	5.81
Lysine	12.20	12.83	11.30	11.14
Histidine	3.60	4.0	2.50	3.25
Amide Ammonia	1.02	0.95	0.87	0.99
Arginine	6.04	5.90	5.35	6.05
Cystine	-	5.91	-	-

a = Present author.

b = Stein and Moore [246].

c = Allerton et al [162].

d = Peters [247].

NON-GENETICALLY DETERMINED FACTORS WHICH MAY OPERATE AT STAGES C AND D.

In genetically determined substitution of one amino acid for another all, or a large fraction, of the protein molecules contain the specific error. On the other hand, there could be errors in reading the genetic code, in attaching amino acids to appropriate soluble R.N.A. molecules and in assembling amino acids. These non-genetically determined errors have been referred to as a kind of "noise" in transmitting genetic information and arise from the finite ability of chemical surfaces to distinguish among closely related chemical substances [240]. Valine, for example, might substitute for isoleucine 5% of the time on theoretical grounds.

This hypothesis was tested by Loftfield [240] who incubated oviduct tissue with different labelled amino acids. The radioactivity in the isolated ovalbumin peptides indicated that valine was substituted for isoleucine not more than once in a thousand times. This again demonstrates the remarkable precision of the biosynthetic mechanism and it is obvious the laws governing conventional chemical reactions cannot directly be applied to biological systems.

Incorporation of amino acid analogues into proteins.

The biological significance of amino acid analogues with

special reference to protein synthesis was discussed by Vaughan and Steinberg in a recent review [149].

The term amino acid analogue is used loosely to refer to a group of compounds which are closely related to, but not identical with, naturally occuring amino acids. Examples are tryptophan and 5-methyl tryptophan, methionine and ethionine.

Among the amino acids found in naturally occurring proteins, those most similar in structure are distinguished from one another by the presence or absence of

1. a methylene group (valine-isoleucine, glycine-alanine, serine-threonine, aspartic acid-glutamic acid, asparagine-glutamine);
2. a hydroxyl group (alanine-serine, phenylalanine-tyrosine, proline-hydroxyproline, lysine-hydroxylysine); or
3. an amide group (aspartic acid-asparagine, glutamic acid-glutamine).

The remaining amino acids differ more obviously from one another, apart from leucine and isoleucine, where only the configuration of the carbon chain is different. These amino acids can therefore be distinguished by the synthetic mechanisms, but it appears as if differences between, for example, a hydrogen atom and a fluorine atom can not be recognised. It has now been convincingly demonstrated that ethionine can replace methionine in α

amylase of Bacillus subtilis without effecting the enzyme activity[241]. Similarly Black and Kleiber [242] showed that nor leucine can be incorporated into the casein of milk from cows.

Nevertheless, these observations can not be used in support of the argument that such errors in interpreting the code should occur with naturally occurring amino acids. In kwashiorkor we are dealing only with a reduced pool of normal amino acids or probably an altered composition of the pool.

Inhibition or absence of amino acid activating enzymes.

Before amino acids can be transferred to soluble R.N.A., they are activated by specific enzymes. It is theoretically possible that these enzymes may be deficient in a protein-depleted state. However, to produce alterations in the composition of the protein to be synthesised one must postulate that some enzymes are relatively more affected than others or are even absent. Absence of an activating enzyme for one amino acid will then result in the production of an incomplete protein. This hypothesis is unlikely to be correct and has no evidence to support it.

A much more attractive theory is that certain enzymes, required for the activation of amino acids or subsequent steps in protein synthesis, may be inhibited by a lack of essential cofactors or the presence of enzyme inhibitors.

The observations of Kench et al [243] on the biochemical

effects produced by cadmium poisoning are particularly relevant. It is well known that many heavy metals inhibit certain enzymes by combining with the sulphydryl groups in the active centres of these enzymes. The isolation of a low molecular weight protein resembling albumin in amino acid composition, from the urine of men and rabbits poisoned with cadmium suggests that such a mechanism might indeed operate in this case.

The inhibition of one or more enzymes required in the biosynthetic process may lead to the production of an incomplete protein molecule which is eliminated rapidly in a manner analogous to Bence-Jones protein in multiple myelomatosis.

Absence of Code Words for certain amino acids.

Although the code words for a large number of the different amino acids are probably correctly known, the exact code words for others are still in dispute. There is also evidence that some amino acids are not coded for. For instance, proline is only hydroxylated after incorporation into the polypeptide chain to form hydroxyproline and cystines are probably formed by the interaction of two peptide-linked cysteines [244].

Ascorbic acid deficiency inhibits the conversion of proline to hydroxyproline resulting in the formation of collagen with altered physical properties.

The experiments of Zubay [245] who studied the incorporation

of uniformly labelled C^{14} glutamic acid and C^{14} glutamine into soluble R.N.A. showed that glutamine was incorporated at a much faster rate than glutamic acid. When soluble R.N.A., incubated with C^{14} glutamic acid, was isolated and the amino acid separated from the R.N.A., it could be shown that all radioactivity which was incorporated could be accounted for as glutamine. These results strongly suggest that glutamic acid is first converted to glutamine before incorporation and a code word is only provided for glutamine and not for glutamic acid. Similar observations have been made on the aspartic acid - asparagine system. These results further suggest that the aspartic and glutamic acid residues in protein molecules are formed by deamidation of the corresponding amides after incorporation into the protein molecule. If these observations are substantiated it becomes necessary to regard the mechanisms determining the hydrolysis of the amides as additional factors whereby the primary structure of a protein molecule may be altered.

It is tempting to speculate that these controlling mechanisms may be interfered with in kwashiorkor but more data must be collected before the validity of this concept can be discussed.

It must be concluded from our studies, and from the theoretical considerations which have been discussed, that a change in the composition, or a reduction in the available substrate pool

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such as occurs in kwashiorkor, does not lead to alterations in the amino acid composition of serum albumin. The substitution in a protein molecule of non-essential amino acids for essential amino acids appears therefore to be an unlikely mechanism for the conservation of nitrogen in protein depleted states.

SUMMARY/...

SUMMARY.

The amino acid composition of serum albumin from normal children and adults, and from children suffering from kwashiorkor, prepared by three different methods, was determined by the Moore and Stein chromatographic technique. No differences could be demonstrated between normals and abnormals for most of the amino acids. Amide ammonia determinations were made on albumin from four normal individuals and four patients with kwashiorkor. The mean amide ammonia concentration in kwashiorkor was higher than in corresponding normals but, in view of the small number of samples analysed, the significance of these results is doubtful.

The specificity of protein biosynthesis is discussed and certain mechanisms by which alterations in the primary structure of a protein molecule could occur, are considered.

It is concluded that the replacement of essential amino acids by non essential amino acids in the protein molecule does not operate as a mechanism for conservation of essential amino acids in a state of protein depletion.

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