THE ROLE OF PEPTIDES AS INTERMEDIATES IN PROTEIN METABOLISM.

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RESUME.

This thesis is an account of investigations into some aspects of the role of peptides as intermediates and regulators of protein biosynthesis.

The underlying concept was fostered by my supervisor, Prof. J.E. Kench, who found a peptide subunit of serum albumin in the urine of persons suffering from Cadmium poisoning[1], and by the theoretical considerations which follow this observation.

There is much evidence in the recent literature that peptides may be intermediates in normal protein biosynthesis. This has also been inferred from certain disease states and other conditions under which protein biosynthesis is blocked at some point, e.g. cadmium, amino acid analogues or (in bacteria) antibiotics. The literature covering this concept will be presented.

The present studies have been carried out on children, who because they are suffering from chronic protein malnutrition, have very much lowered rates of protein synthesis and breakdown. In this unfortunate, but natural experiment, it was hoped that some factor or factors derived from protein synthesis might be found which influenced the synthetic mechanism as a whole.

Evidence from the literature has been summarised, which concludes that urine, apart from being convenient to collect, is the biological fluid most likely to contain high concentrates of peptides which are released during cellular metabolism.

To my wife,
Jacqueline Wendy.

This document sets out to describe the experimental work and new facts which have emerged from studies on urinary peptides in Kwashiorkor and their relevance to protein metabolism in the body as a whole.

The salient observations are ennumerated and summarised below:-

1. The partition of nitrogen in the urine of recovered and protein-depleted individuals was measured. Total nitrogen, urea nitrogen, ammonia nitrogen, free and bound α-amino nitrogen and creatinine were determined. It became clear that half of the bound amino nitrogen in the urine consists of nitrogen in the form of peptides.

2. Considerable quantitative and qualitative differences were found in the partition of urinary nitrogen of children suffering from acute Kwashiorkor when compared with the same individuals following recovery or with normal children. In particular, the bound α-amino nitrogen was much increased during protein repletion, especially at the outset.

3. The individual amino acids which contributed to the rise in bound α-amino nitrogen were identified and measured by an automatic column chromatographic procedure. A striking fact which was thus revealed, was the continued losses of dietary essential amino acids in bound form. Bound hydroxyproline was also maximally excreted in the protein-depleted state, and excretion of this amino acid was lessened by repletion.

4. The peptide nature of the bound forms of α-amino nitrogen

was studied using the chromatographic procedures for separation of the amino acids which were liberated by acid hydrolysis. The quantity of such acid labile compounds was raised during the acute phase of protein depletion.

5. A method was devised for the detection and measurement of neutral peptides present in urine. It then became possible to account for peaks due to previously unrecognised ninhydrin-reacting substances present in the elution diagram of free amino acids in the urine.

When these studies were first undertaken, no universally applicable method was available by which urinary peptides could be fully investigated and a number of procedures were tried out and found not to be satisfactory. The perfection of the assay procedure here described, made a great contribution towards the successful outcome of the study.

6. The overall picture of the disturbance in protein metabolism in the depleted child was that increased losses of protein fragments continued in spite of diminished dietary intake and depletion of protein in the body.

7. The biochemical mechanisms through which these peptides might emerge during the biosynthesis and breakdown of proteins in the body are discussed, and an attempt made to evaluate the importance of such fragments as an index of imbalance in the factors which control and influence the normal turnover of proteins in man.

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SOME ABBREVIATIONS USED IN THIS MANUSCRIPT.

AMINO ACIDS:

ala. - alanine

arg. - arginine

asp. - aspartic acid

cyss. - cystine

cys.SO_3H - cysteic acid

glu. - glutamic acid

gly. - glycine

his. - histidine

iso. - isoleucine

leu. - leucine

lys. - lysine

met. - methionine

phe. - phenylalanine

pro. - proline

OH pro. - hydroxyproline

ser. - serine

P-ser. - phosphoserine

tau. - taurine

tyr. - tyrosine

val. - valine

β-a.i.b.a. - β-amino isobutyric acid

ml.	-	millilitre
cm.	-	centimetre
mm.	-	millimetre
l.	-	litre
μa.	-	microampere
I.D.	-	internal diameter
O.D.	-	outside diameter, or optical density, depending upon context.
lbs. p.s.i.	-	pounds per square inch
E	-	optical extinction coefficient
T	-	transmission (of light)
mμ.	-	millimicron (wavelength)
μmole	-	micromole
(A.)	-	adenine
(C.)	-	cytosine
(G.)	-	guanine
(U.)	-	uracil
AMP	-	adenosine monophosphate
ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate

RNA	-	ribonucleic acid
RNP	-	ribonucleoprotein
DNA	-	desoxyribonucleic acid
NAD	-	nicotinamide adenine-dinucleotide
E.D.T.A.	-	ethylene-diamine tetra acetic acid
DYDA	-	diketohydrindylidene-diketohydrinamine
FDNB	-	1 - fluoro, 2 : 4 - dinitrobenzene
DNP	-	dinitrophenyl
DNP-OH	-	2 : 4 - dinitrophenol
DNP-NH_2	-	2 : 4 - dinitro-aniline
HAc	-	acetic acid
Bu	-	butanol
DEAE	-	di-ethyl-amino-ethyl

SECTION A.

HISTORICAL BACKGROUND

AND MOTIVATION.

CHAPTER I.

INTRODUCTION.

The past decade has illustrated more than ever before, how fundamental advances in the basic sciences have affected the more applied fields of biology, physiology and medicine. In particular, developments and applications of elegant physico-chemical techniques have opened up fields of investigation, and solved problems, which a few years ago seemed unsurmountable.

Protein chemistry is already a classical example of this dependence. Even in 1951 when Sanger, Tuppy and Thompson elucidated the complete structure of insulin;[2][3] workers in this field doubted if it would ever be possible to unravel the intricacies of the larger proteins. The primary structure of haemoglobin is now known[4] and the mechanism by which this large and complex molecule is synthesised is no longer a complete mystery. The biosynthetic pathways of macromolecules, including proteins, together with genetic codes and mode of transmission have received much attention recently, and a plausible hypothesis of synthesis has been postulated involving such concepts as genetic codes, templates, messenger RNA etc. Although we are now reasonably certain of the mechanisms of protein synthesis, little is known about their control. By analogy with other bio-syntheses, as of Cholestrol, it is reasonable to suppose that homeostasis is secured by negative feedback mechanisms from the end product or intermediates on an early synthetic reaction. Cholesterol inhibits a reaction, 17 enzymatic steps earlier; the first enzymatic step in its own synthesis[5].

Whilst investigating men exposed to Cadmium fumes for signs of poisoning, Kench[1][6] noted progressive proteinuria in those patients who eventually succumbed. Proteinuria is also characteristic of poisoning by other transition metals, such as lead, zinc and mercury. Cadmium is remarkable, however, in that even though the albumin present in the urine has the usual electrophoretic mobility, its molecular weight is about one-quarter that of normal serum albumin; probably about 17,000[6].

The production of low molecular weight protein by cadmium raised several important theoretical considerations:-

a) Why does cadmium differ from other heavy metals ?

b) Is the low molecular weight protein an intermediate in protein biosynthesis ?

c) What is the role of peptides in protein biosynthesis - intermediate or by-product ?

The present thesis describes a study of the urinary peptides chiefly of young children, either protein depleted or recovered, undertaken in an attempt to evaluate the importance of peptides in normal protein biosynthesis.

CHAPTER 2.

AN OUTLINE OF THE MECHANISM OF PROTEIN SYNTHESIS.[7][8]

This chapter will deal with those aspects of protein biosynthesis which are pertinent to the general hypothesis and later discussion regarding participation of peptides in protein biosynthesis.

Genetic Control of Protein Biosynthesis.

It has been established that the most important site of protein synthesis is an intracellular cytoplasmic particle, the ribosome. These are dense granules, 100 - 150 Ångström units in diameter, 80% by weight of which consists of ribonucleoprotein. The ribosomes are attached to the endoplasmic reticulum.

Ribonucleic acid (RNA) concentration in the cell correlates with the rate of protein synthesis and all evidence agrees that ribosomal ribonucleic acid is the template upon which amino acids are incorporated into the polypeptide chain.

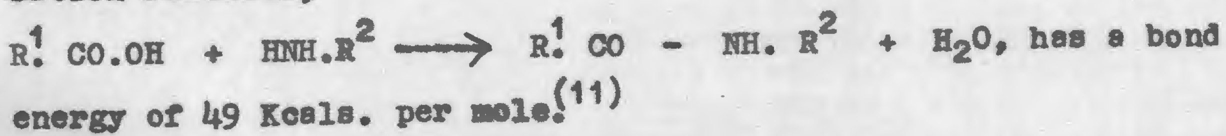
The code whereby the amino acid sequence in the protein is determined, is the sequence of bases in the nucleotides forming the ribonucleic acid. There is evidence that the code for an amino acid is a triplet of 3 adjacent, non-overlapping nucleotides. There are four nucleotides in RNA which differ only in their base; this being either adenine (A), cytosine (C), guanine (G) or uracil (U). There are thus n_3^4 or 64 possible triplets. Only 20 of these are necessary to "write" a complete protein, but an amino acid may be coded by more than one triplet.[9] Experiments with polyadenine RNA and poly-AC (RNA) indicate that two of the bases

in the triplet carry most of the information for amino acid specificity. This is called degeneracy of the code. Poly U incorporates phenylalanine, therefore one of the codes for phenylalanine must be U.U.U.[10]

Since the information for protein synthesis originally resided in the DNA of nuclear chromosomes, some mechanism must exist for the transfer of this information to the ribosome where protein synthesis takes place. It has been postulated that a hybrid (DNA/RNA) double helix is synthesised in the nucleus. The RNA portion of this splits off and migrates to the ribosome where it directs synthesis. This has been called Messenger RNA (Fig. 1 and Fig. 2.)

<u>Stages of Protein Synthesis</u>.

The peptide linkage -NH-CO- which is formed by the condensation reaction,



$R^1.CO.OH + HNH.R^2 \longrightarrow R^1.CO - NH.R^2 + H_2O$, has a bond energy of 49 Kcals. per mole.[11]

This reaction cannot therefore proceed either spontaneously or catalytically. The first stage in protein synthesis is one of activation of the amino acid. This reaction is between the amino acid and adenosine-tri-phosphate (ATP). Pyrophosphate is released.[12] The activated amino acid is the mixed anlydride between the $-PO_4$ of AMP and the carbonyl group.[13] (This is not an ester linkage). (Fig. 3).

The activation reaction is enzymically catalysed. The appropriate enzymes are to be found in a protein fraction which

FIG. I SYNTHESIS OF PROTEINS

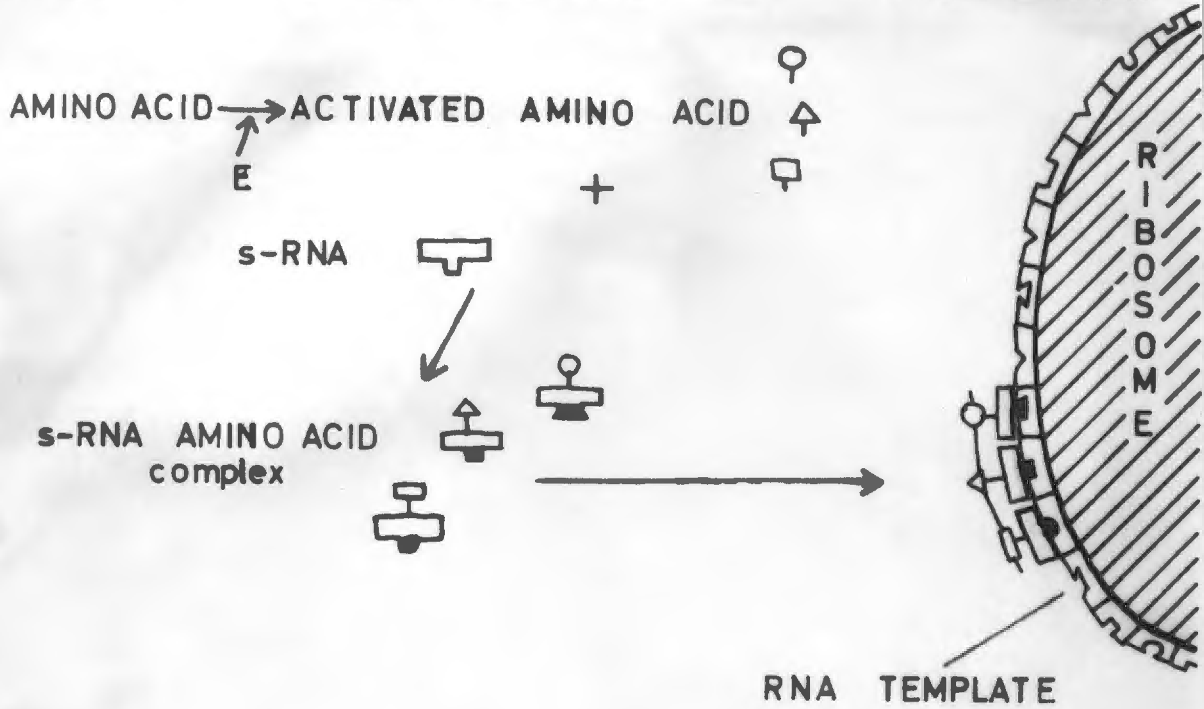
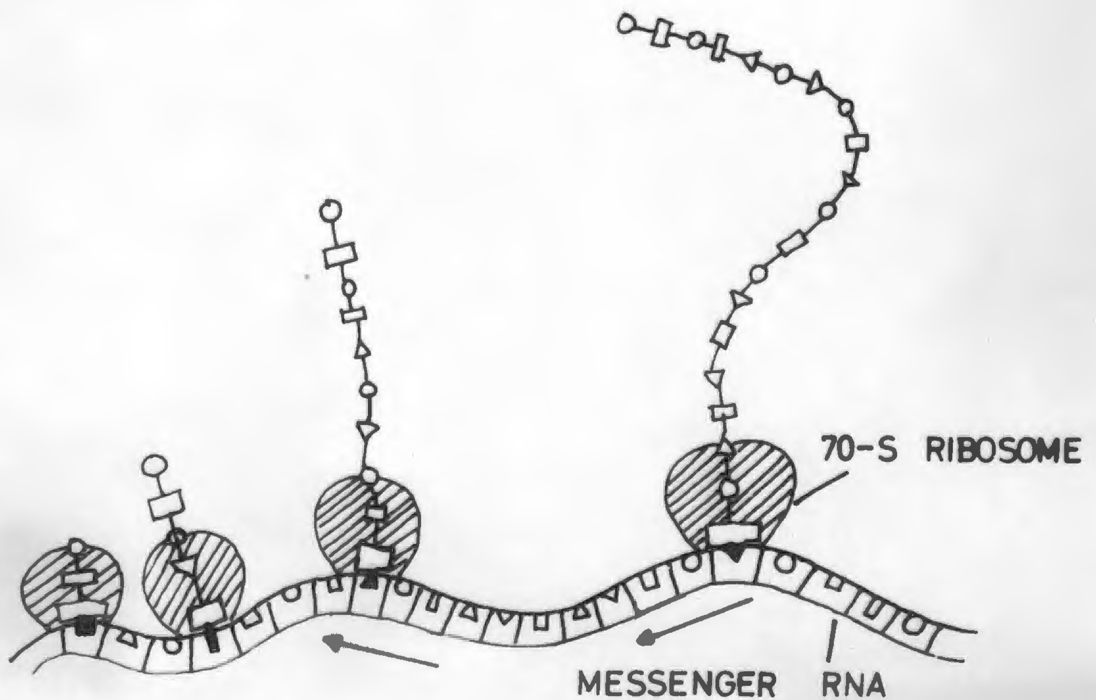


FIG. 2 GROWTH OF PEPTIDE CHAIN



precipitates from the cell sap at pH 5, and are therefore known as pH 5 enzymes*. Each amino acid requires a specific enzyme for its activation[14]. Tryptophan-activating enzyme has been prepared in a highly purified state from beef pancreas[15] and tyrosine-activating enzyme from hog pancreas.[16]

Amino acid activation occurs in the cytoplasm. During the second stage in protein synthesis, activated amino acid molecules are attached to low molecular weight (± 30,000) ribonucleic acid, also in cytoplasmic fluid, prior to transfer to the ribosomal template. It is probable that a specific RNA molecule is required to transfer each of the 20 different amino acids. AMP and activating enzyme are regenerated during the reaction (Fig 3).

The final stage of protein synthesis occurs on the ribosome. On the surface of this particle, nucleotide bases are arranged in a sequence which determines the eventual amino acid sequence in the protein to be synthesised. The evidence of Watson (1963)[17 suggests that the peptide chain elongates at the amino-terminal end and that the other (carboxyl) end is attached via the RNA amino acid complex to the ribosomal template.

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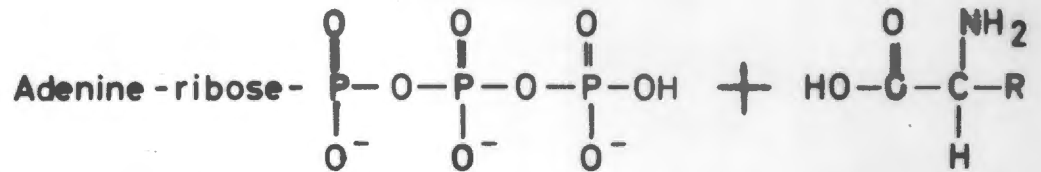
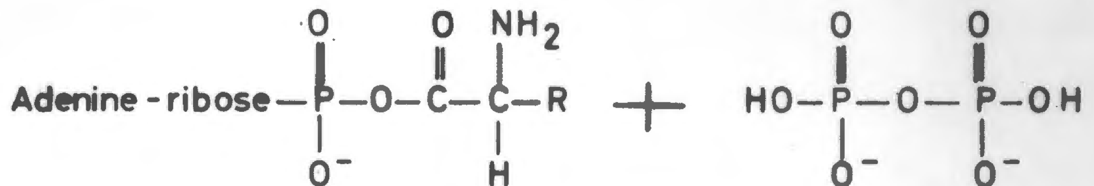
* This of course does not denote their optimum pH, as the nomenclature suggests.

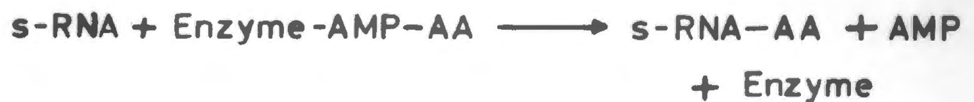
FIG. 3 AMINO ACID TRANSFER

1. Amino acid activation





2. Transfer to s-RNA



CHAPTER 3.

THE ROLE OF PEPTIDES IN PROTEIN BIOSYNTHESIS AND BREAKDOWN.

Once it had been established that the amino acids in a protein were arranged sequentially, being linked by peptide bonds, two main theories arose to explain how such a sequence might arise. Even before the structure and function of DNA and RNA had been proposed, a mechanism had to be postulated to guide the numerous amino acids into their correct positions in the peptide chain.

The first theory proposed that all the amino acids of a particular molecule were linked together at once. The other considered it more likely that peptide intermediates were first formed, which then linked together to form the final protein structure (fig. 4). Although the peptide theory was favoured, the evidence on which it was based was mainly indirect.

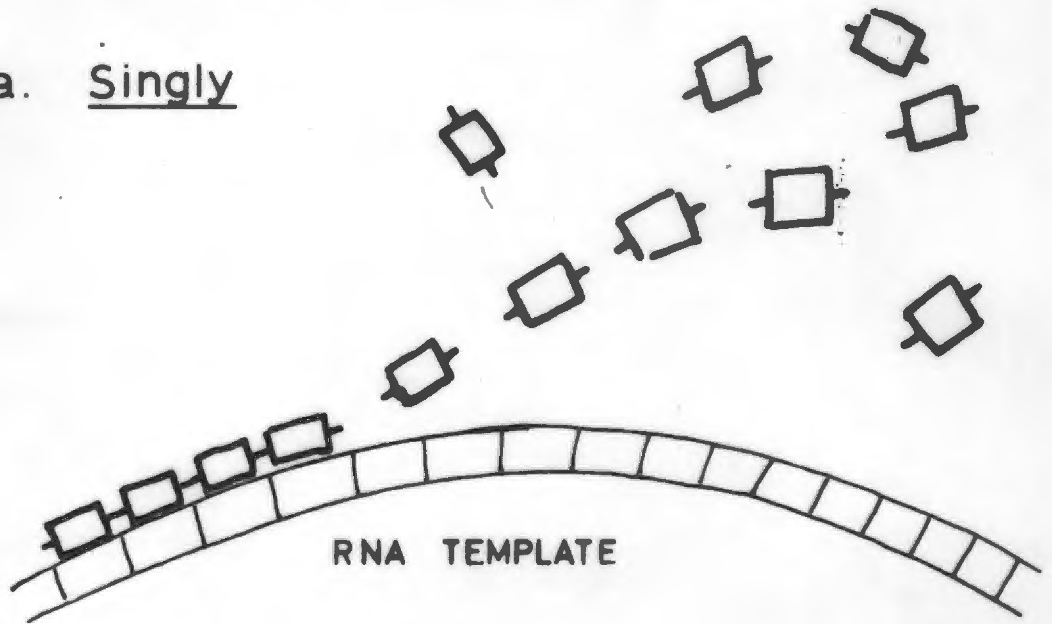
There are many reports that amino acids present in peptides are more readily incorporated into protein than are free amino acids. This is certainly true for plants[18] and bacteria[19]. The growth of Lactobacillus casei in synthetic amino acid mixtures is greatly enhanced by small amounts of partial casein hydrolysates. The growth promoting factor is peptide in nature and similar results for peptide utilisation with peptides from globin has been noted in duck erythrocytes[20].

Leach and Snell have studied the uptake of peptides by L. casei and confirmed these findings.[19][21][22]

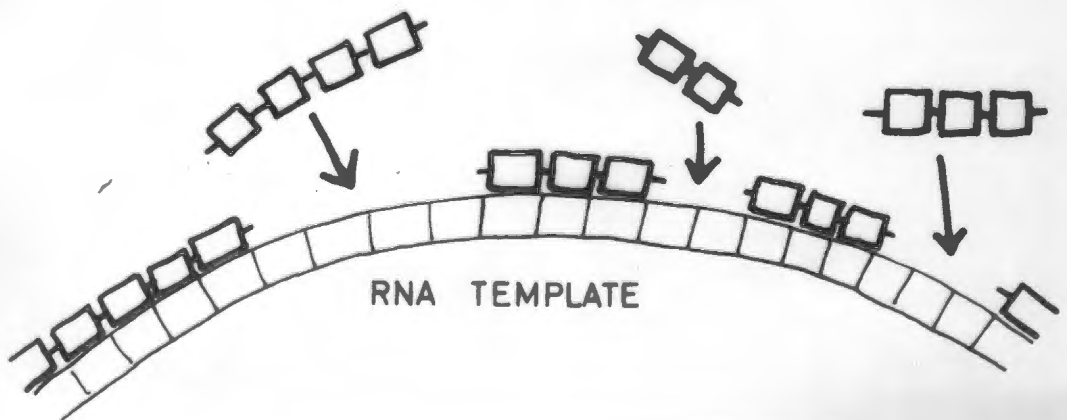
The uptake of label from L-alanyl-C^{14} glycine was ten times that of the free amino acid. There is evidence that

FIG. 4 THEORIES OF AMINO ACID INCORPORATION

a. Singly



b. As Preformed Peptides



different mechanisms exist for the uptake of free amino acids and for peptide. D-alanine, which inhibits the retention of L-alanine in Vitamin B_6 deficient rats*, did not interfere with the retention of this amino acid when it was supplied in peptides[23]. Both mechanisms were inhibited by 2,4,-dinitrophenol, which uncouples oxidative phosphorylation[21].

There are other possible mechanisms, besides selective uptake of peptides, which appear to favour the incorporation into proteins of peptides or of larger molecules. The free amino acid may be utilised in non-protein synthetic mechanisms e.g. decarboxylation of tyrosine to tyramine. Assimilation of the free amino acid, but not of its peptide is inhibited by analogues e.g. D series of amino acids or p-fluo-phenylalanine[24].

The use of whole proteins as substitutes for resynthesis has also been investigated. Frances and Winnick[25] measured the uptake of proteins of embryo extract into embryonic heart cultures and found that they were utilised in preference to free amino acids. The analogue p-fluo-phenylalamine, which inhibits phenylalamine uptake and blocks protein synthesis, did not interfere with utilisation of whole protein.

Although peptides are more readily assimilated by bacteria and cells, there is little evidence that they are incorporated as such into proteins, in fact, that available is that they are first degraded to free amino acids within the cell.

---

* In the presence of the cofactor Vitamin B_6, alanine can be synthesised by transamination of pyruvic and glutamic acids.

In the experiments of Leach and Snell[19], C^{14} glycine taken up by the cell as the free amino acid, or from a glycine-containing peptide, could be exchanged with free glycine or free peptide; but faster with the latter. Furthermore after rupture of the cells all the exchanged C^{14} activity was present in the supernatant as free glycine. This suggests that all the peptide glycine is hydrolysed within the cell prior to incorporation into protein. In the capsules of certain bacteria, amino acids bound as peptides, act as storage form of nitrogen. Prior to utilisation, these are split into free amino acids[21]. The in vivo studies on human subjects by Christensen et al[26] suggest that the above conclusions apply equally well to the intact animal. These authors found that a partial acid hydrolysate of casein, fortified with tryptophan, in which two-thirds of the amino acids were in bound form, was a better source of nitrogen than an equal amount of completely digested protein when administered intravenously.

Opposition to the peptide theory has in the past stemmed from failure to isolate peptide fragments in cells. With modern techniques, peptides have now been identified in plants, bacteria and mammalian cells.

In the experiments of Steinberg et al[27], incorporation of C^{14} amino acids into liver proteins, resulted in non-uniform labelling which indicated the participation of amino acid conjugates as intermediates in protein synthesis but their experiments provided no information regarding the chemical nature or molecular size of these intermediates.

The nature of these cellular peptides has been investigated in a wide variety of systems including yeast,[28] Chlorella,[29] Staph. aureus,[30] liver and muscle[31] and ascites tumour cells[32].

The peptides are bound to a low molecular weight nucleic acid fraction; from which they can be split and isolated as the hydroxamate. Free carboxyl activated adenylates couple readily with hydroxylamine. The coupling reaction is associated with an increase in periodate reacting material.

$$R.CO.Nucleotide + NH_2OH \longrightarrow$$

$$R.CO.NH.OH + AMP$$

Unmasking ov vicinal -OH groups led Van der Grinten to propose the following structure for carboxyl-activated peptides[33].

```
                  O
                /   \
Adenine — CH         CH
            \       /
             CH — CH
             |     |
             O     O
              \___/
                |
   Peptide — CO    H
```

It is not certain whether the ester bond of the carboxyl group of the peptide is to the 2' or 3' OH group of the AMP-ribose residue. Since 1957, when nucleotide-bound carboxyl activated peptides were first described in yeast by Koningsberger et al[34], similar compounds have been described in other micro-organisms e.g. streptococcus faecalis[35]. The acidic peptide

conjugates from mammalian liver have been shown to contain AMP, ADP, ATP, adenosine, guanosine, uridine and cytidine.[36]

Activated peptides, whose hydroxamates behave similarly on chromatography and with similar amino acid composition, have been found associated with particulate fractions of ruptured cells and also in the 100,000G supernatant.[37] The exact site or mode of production is not known.

Raake in his studies on pea seedlings[38] found that with ripening, the amount of peptide material increased, whilst free amino acid concentration fell. At the end of the ripening period, a sudden drop in peptide concentration was accompanied by an increase in the protein fraction.

Davies and Harris[39] have investigated the dynamic aspects of the peptidyl-nucleotide pool of brewers yeast during various phases of its growth cycle. The pool was maximum at the onset of the logarithmic growth phase and declined to a low value during this phase. As the rate of growth decreased, tne amount of peptidyl-nucleotide again increased. They suggested that this material participated in the synthesis of nucleoprotein and was organised early for rapid turnover during the logarithmic phase. When these intermediates were no longer integrated, they accumulated. This very reasonable hypothesis explains the following:-

a) the purpose and reason for the lag phase i.e. accumulation of peptide intermediates for use during the phase of rapid growth.

b) a pyrimidine derivative stimulates protein synthesis in particulate fractions of pea seedlings.[40]

c) induction of β-galactosidase activity in a mutant of E. coli requires pyrimidine.[41]

d) unbalanced growth of L. acidophilus results in accumulation of a pool of uracil ribonucleotide derivatives.[42]

Pulse labelling experiments on yeasts[43] showed that up to 3 minutes after the introduction of the label, activity was greater in the peptide fraction, but later protein activity increased rapidly. In a similar system using L-leucine C^{14}, McManus[44] isolated an acidic leucine-containing peptide, whose leucine had the same activity as that of free leucine, whilst the protein fraction had relatively little activity. These kinetic experiments are strong evidence that peptides are intermediates in protein formation.

The amino acids of activated peptides include all those present in proteins, but glutamic and aspartic acids are especially prominent[45] (table 1). This contrasts with the relatively few amino acids found in mucopeptide in cell walls, for which precursor activity in protein synthesis is unlikely.[46]

It was hoped that the study of glutathione synthesis might be useful since this is a well-defined intracellular peptide which is synthesised in the cell by the formation of a true peptide bond between γ-glutamyl cysteine and glycine. This synthesis differs in many respects from those mechanisms involved in protein synthesis. Although the activating enzyme requires ATP, Mg^{++} and Mn^{++}, the split occurs at the terminal phosphate bond, liberating ADP and $PO_4^{\equiv}$ in contrast to liberation of AMP and pyrophosphate, and unlike protein synthesis, glutathione

Table 1.

Amino Acid Composition of some Peptide Hydroxamic Acids.

(Van der Grinten, 1959).[45]

Rf of the peptide Hydroxamate	0.26	0.47		0.75	0.96
Source	D	D	M	D	M
Cys. SH.	-?	-?	-?	-?	-?
Lys.	+?	++	++	++	++
His.	-	-	-	-	-
Asp.	+	++	++	++	++
Arg.	-	-	-	-	-
Ser.	+?	+?	+?	+?	+?
Gly.	-	-?	-?	-?	-?
Glu.	+	+	+	+?	++
Thr.	-	-	-	-	+
Ala.	+?	++	++	++	++
Pro.	++	++	++	++	++
Tyr.	+	-	+?	-	-
Val.	+	++	++	+	++
Met.	-?	-?	-?	-?	-?
Try.	-	-	-	-	-
Iso.	+	++	++	-	+?
Phe.	+	++	++	-	-
Leu.	+	++	++	-	+?

D = Dialysate.
M = Microsomes.

++: present in rather high concentrations
+: present.
+?: probably present.
-?: probably absent.
-: absent.

can be formed in a particle free solution.[47]

Intracellular breakdown of proteins.

In an intact animal there is no doubt that protein turnover occurs. There is, however, no clear indication as to the site of protein breakdown. The enzymic action of the cathepsins liberated by rupture or death of the cell was one of the first to be studied. These enzymes are proteases and peptidases, but there is little evidence that they act intracellularly since they occur in pro-enzyme form and have a pH optimum far removed from the physiological value. Based on this, and the fact that he could detect no turnover of protein in growing E. coli, Monod[48] concluded that normal protein breakdown occurred extra-cellularly, but others have found intracellular turnover as well in resting bacteria[49] and liver[50]. There is no demonstrable link between turnover and cathepsin activity, especially since Simpson[51] has shown that intracellular release of labelled amino acids from protein is inhibited by those agents which interfere with energy-controlled reactions. Intracellular protein breakdown can also be inhibited by inhibitors of protein synthesis, e.g. amino acid analogues.[52]

The above observations indicate that protein breakdown in cells is not by simple proteolysis, and implicate possible mechanisms whereby protein turnover may be regulated.

Mechanisms controlling protein turnover.

During studies on induction of β-glucosidase activity in yeast protoplasts by maltose, de Kloet et al[53] found two compounds in yeast dialysates, one of which could stimulate

synthesis of the enzyme and the other caused inhibition. Both compounds were peptides, and that which stimulated synthesis was adsorbed onto activated charcoal, which removed all nucleotide material.

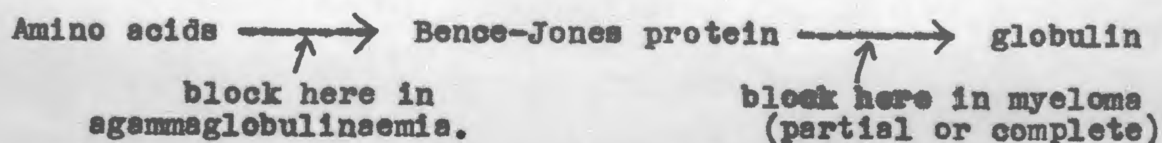
Protein homologues.

Nature provides several examples of proteins which have similar properties and functions, and whose size relationships suggest that the larger member of the pair may be a polymer of the smaller.

Haemoglobin (M.Wt. 68,000) is twice the size of Myoglobin (35,000), which has similar chemical properties enabling these haemoproteins to function as oxygen transporters. Haemoglobin molecules of vertebrates are composed of four polypeptide chains linked by bonds which are weaker than true peptide bonds. Each chain consists of approximately 150 amino acid residues and a haem group containing an attached iron atom which is capable of reversibly binding oxygen. Although their amino acid sequences differ markedly, haemoglobin and myoglobin both show similar and highly specific secondary structure (manner in which the peptide chain is folded in space)[54]. Although myoglobin peptide chains do not usually associate as do those of haemoglobin, to produce a tetramer, Manwell has found evidence of dimers of invertebrate myoglobin in aqueous solution.[55] Human and bovine lactalbumin (17,000) bears the same simple size ratio to mammalian serum albumin (68,000). These albumins have similar low iso-electric points (pH 4), electrophoretic properties and amino acid composition. It is tempting to suggest that the smaller molecule

was the phylogenetic precursor of its polymer analogue, and that this sequence of events persists in the mechanisms of synthesis of the larger molecule. None of the peptides so far isolated and implicated as precursors or intermediates of normal protein synthesis, have been of a size approaching that of lactoglobin or myoglobin. In two disease processes, the existence of such high molecular weight precursors is, however, likely.

Multiple myelomatosis is a neoplastic disease characterised by uncontrolled activity of a clone of plasma cells of the reticulo-endothelial system which normally produces γ-globulins. In well-developed cases, the serum contains an excess of a particular γ-globulin. The urine of some (± 25%) of these patients contains a low molecular weight protein (Bence-Jones)[56] of molecular weight 35,000, which has similar electrophoretic mobility, antigenic properties and amino acid composition to that of the abnormal excess γ-globulin. It is generally inferred from these findings that the urinary protein is an abortive fragment arising out of, and during uncontrolled synthesis of the serum globulin, which has been partially blocked by factors as yet unknown. It is significant from this point of view that some cases of myelomatosis have been described in which there is Bence-Jones proteinuria with diminished serum globulins. This represents a block in the assembly of 'peptide' (Bence-Jones protein) into γ-globulin.



Bence-Jones originally detected the urinary protein because of its remarkable reversible precipitation through the temperature range 56° - 90°C. This method is still used to detect this protein. It is conceivable that other intermediates of protein synthesis, having less bizarre physical properties, might also be present in the urine of these patients. This possibility will be investigated at a later date.

The urine of men and animals poisoned by Cadmium, contains an albumin, which has a molecular weight of 17,000. In other respects it resembles serum albumin electrophoretically and antigenically. It has been postulated that this is an abortive fragment and intermediate of normal albumin synthesis.[1][6] Vallee[57][58][59] has some evidence that Cadmium ions inhibit carboxypeptidase. Zinc ions, bound to the apoenzyme, probably as the mercaptide, are necessary for peptidase activity. When these are displaced by mercury, cadmium or lead, peptidase activity is lost but surprisingly these metal-apoenzyme complexes have marked esterase activity. It is thus possible that the low molecular weight albumin, released by cadmium ions, may be on the degradative pathway, in spite of the labelling experiments of Kench et al.

<u>Release of protein and peptides from ribosomes</u>.

The mechanisms whereby peptide chains are released or stripped off the ribosomal RNA template have been the subject of many recent investigations.

Obviously, under normal in vivo conditions, complete protein must be liberated from the ribosome, but in the early in vitro

studies, although amino acids were incorporated into ribosomal protein, these were not released into the supernatant as soluble protein. This is certainly true for most tissues studied, including liver and tumour cell ribosomes[60] but ribosomes from E. coli and reticulocytes do release some protein[61], but amounts of protein recovered are affected by the precipitating agent. Use of TCA/tungstate mixture results in an apparent four-fold increase in protein release, probably because basic peptides are released by the ribosome[62].

Stripping off of protein appears to be an active process. Labile protein can be rapidly released from pea seedlings[63] and haemoglobin from reticulocytes[64] by incubation with ATP and Mg^{++}. The latter also requires a soluble enzyme fraction.[65] NAD^- (DPN) also causes release of labelled protein from liver microsomes.[66] These findings suggest that energy is required to release protein from ribosomes of the intact cell.

The integrity of the ribosome is also in some manner maintained by magnesium ions, since when those are removed by chelating agents such as pyrophosphate, citrate and EDTA, protein enzymes and much of the ribosomal RNA is released.[67] When Mg^{++} is replaced by a basic polyamine such as spermine, protein but not RNA is released.[68] This is an intriguing possible role for these amines.

Normally in vivo, only complete proteins are released from their sites of synthesis, although there is ample evidence that partially formed polypeptide chains are always present.[69][70] Puromycin is structurally very similar to activated phenyl-alanine

and blocks protein synthesis presumably by displacement of phenyl-alanine by its p-methoxy analogue on the C-terminal end of the growing polypeptide chain. When puromycin is incubated with reticulocyte ribosomes, polypeptides chains are released, having the same N-terminal amino acid (valine) but they are incomplete and neither ATP nor supernatant enzymes are required for their release.[71]

The above brief summary illustrates several possible mechanisms whereby peptide intermediates of protein biosynthesis may be released from cells. This is pertinent to the present investigation since it will be postulated that if synthesis were blocked due to lack of amino acids, mechanisms are available for the release of these incomplete or abortive fragments of protein synthesis.

Since it is now generally accepted that proteins are synthesised sequentially, at any one time, there must be unfinished peptide chains. The crucial problem is whether these chains are present in amounts in excess of what would be expected from interruption of sequential addition of amino acids, and whether these can be stored and utilised later.

CHAPTER 4.

DISTURBANCES OF NITROGEN METABOLISM IN KWASHIORKOR - A PROTEIN DEPLETED STATE IN MAN.

Kwashiorkor (lit. - a deprived child)[72] is the name applied to a clinical syndrome which occurs in children who have been subjected to long periods of dietary protein restriction. The typical patient is undersized and apathetic - features which are expected in any chronic illness. The characteristic signs, however, are depigmentation of the skin and hair, oedema of the extremeties and ascites.

There can be no doubt that the features of kwashiorkor are the direct result of chronic malnutrition of protein during the stages of rapid growth, since the condition is completely reversible by feeding protein alone.[73] Marasmus is the name applied to a similar syndrome in children, also due to protein malnutrition, but it differs from kwashiorkor in that oedema is absent. There is no satisfactory explanation for this, but the important feature is that children with kwashiorkor have a higher intake of calories (from carbohydrate). These differences are not pertinent to the present discussion.

PROTEIN CONTENT OF THE DIET OF CHILDREN SUFFERING FROM KWASHIORKOR.

The diet of children in populations in which marasmus and kwashiorkor are endemic have been extensively studied. In typical African diets, all the essential amino acids are present in quantities which exceed basic requirements for adequate growth,

but the margin is least for methionine and tryptophan.[74] Collis & Olamiyan[75] studied the diets of children who developed kwashiorkor and found that these were similar to non-kwashiorkor diets qualitatively, but that the total intake of nitrogen was low.

The minimum protein requirement of growing infants is much greater than the sum of the requirements of individual essential amino acids. The growth of children on marginal diets can be increased by a non-essential amino acid such as glycine, and even by urea.[76]

Harper and Kumpta[77] have also presented facts which suggest that protein malnutrition may result from amino acid imbalance. Addition of a small amount of the limiting amino acid increased the nitrogen balance and requirement. Dietary amino acid imbalance is more likely to occur in adults.[78]

In certain geographical areas, amino acid imbalance, rather than total nitrogen deficiency, appears to be the predominant factor responsible for kwashiorkor. In Central America lysine is the limiting dietary factor and addition of this amino acid to the diet completely restores its nutritional value.[79]

Since amino acids may now be synthesised on a commercial scale, it should be possible to correct imbalanced diets economically. The amino acid lacking in soya is methionine, as is a diet derived predominantly from peanuts.[80] Zein, from maize is deficient in lysine and tryptophan.[81]

CHANGES IN PROTEIN METABOLISM DURING PROTEIN MALNUTRITION.

Compared with normal children, the most obvious change in

protein-depleted infants is lowered total body protein, which is expressed clinically as retardation or abscence of growth. Since there is failure of protein secretion, this should be regarded as a protein deficit rather than depletion: protein has not been lost unduly - it has never been laid down.

Protein deficiency does not affect all body tissues and organs to a similar extent. Addis et al[82] measured the percentage of original protein lost from different organs during a seven day fast, and found the following:-

Organ.	% protein lost on (a) 2-day	(b) 7-day fast.
Liver.	20	40
Kidney	4	20
Heart	4	18
Other organs (average)	4	10

These results can be expected on the basis of the known rates of turnover of the principal proteins in these organs. It has been useful to consider body proteins arbitrarily as either mobile or fixed. The mobile proteins are generally intra-cellular (parenchymatous cells) and have rapid turnover rates. The extra-cellular supporting and connective tissue proteins are 'fixed' and have relatively slow turnover. Brain proteins are anomalous in that they are intra-cellular, but fixed. The two protein types are relatively equal in amount; fixed +/- 46% and mobile +/- 54%, both in children and adults.[83] As expected, mobile proteins are reduced more than fixed proteins in protein depletion.

A. Protein content of blood.

The most striking consequence of dietary protein deficiency is a reduction in the serum albumin concentration to 40% or less of the concentration which is attained after recovery. Both extra- and intra-vascular albumin pools are equally affected when measured by the I^{131}-albumin dilution technique.[84] Globulins are diminished to a lesser extent, whilst the γ-fraction is normal or even slightly increased, since in many cases of kwashiorkor there is superimposed infection.

It has been suggested that the anaemia of kwashiorkor is due to protein deficiency which interferes with haemoglobin synthesis at the stage of globin formation.[85] This has not been proven and is unlikely, since protein repletion alone does not correct the anaemia.[86] Macrocytic anaemia is sometimes found and both vitamin B_{12} and folic acid deficiencies have been implicated.[87]

B. Protein in the liver.

The liver in marasmus is small and shrunken, whilst in kwashiorkor it is enlarged. This distinguishing feature is due to loading with excess neutral fat. Microscopically, it is obvious that the liver cells in kwashiorkor are also atrophic.[88]

Composition of the liver in marasmus and kwashiorkor.
% of normal weight (Waterlow, 1956)[88]

	Kwashiorkor	Marasmus	Control.
Total protein	67	51.5	80
Total fat	145	38.0	53

C. Muscle Protein.

Muscle wasting is obvious clinically, but absolute measure-

ments of muscle protein are difficult to make. However, Mendes and Waterlow [89] found that the ratio of nitrogen to DNA was lower than normal in protein deficiency states. Montgomery[90] who studied transverse sections of sartorius muscle in Jamaican infants who had died from protein malnutrition found that the muscle fibre was reduced to a size which was comparable with that of the foetus. There was also evidence of loss of cells and nuclei which implied that there had been loss of muscle nitrogen.

It is unfortunate that more accurate methods of measuring muscle protein are not available, since products of muscle catabolism form a large proportion of the amino nitrogen which can be incorporated into the proteins synthesised by other organs. This concept was stressed in the now classic paper of Shemin and Rittenberg[91] who found a 'reservoir' of protein in muscle which acts for protein metabolism just as adipose tissues do for fat. Muscle protein is a highly labile pool of amino nitrogen and acts as a buffer to tide the organism over short periods of acute protein inadequacy.

Children, suffering from kwashiorkor, who have depleted muscle mass and hence depleted muscle protein, are ill equiped to withstand the stress of short periods of protein starvation, whereas in the normal child this situation would be of no consequence. It is therefore understandable that the full blown picture of kwashiorkor usually is precipitated by infection, vomiting and diarrhoea.

PROTEIN TURNOVER IN KWASHIORKOR.

Virtually all the studies on protein turnover in kwashiorkor have been made on serum albumin. Increase in its catabolic rate has been excluded as a cause of the low levels of this protein found in states of malnutrition. (Table 2.)

Table 2.

Rate of Albumin turnover in Protein depletion.

Author.	Subjects.	Diagnosis.	Half-life (days)	Range.
Gitlin et al (1958)(84)	8	Malnourished	9.7	6.6 - 14.5
	8	Treated	9.8	7.0 - 11.3
		Normal	±12	9 - 17
Garrow & Waterlow (1952)(92)	2	Malnourished	9.7	10.1 & 9.3
	2	Recovered	11.0	12.4 & 9.7
Purves & Hansen (1962)(93)	4	Malnourished	12.3	8.7 - 16.0
	4	Recovered	6.3	5.5 - 7.5

Several difficulties in interpretation probably account for the discrepancies between the result of different workers. No correction can be applied to account for recycling of break-down products, which will increase the apparent half-life of the

protein. Purves and Hansen[93] using I^{131} labelled albumin for their turnover studies, showed doubling of the half-life of serum albumin in kwashiorkor. This method has the advantage that recycling of label is eliminated, which is a drawback of C^{14} and N^{15} labelled markers. These authors also corrected for gastro-intestinal loss of labelled albumin which was greater than normal. The absolute catabolic rate of labelled albumin was definitely reduced and both synthesis and catabolism of the protein balanced at low serum levels.

NITROGEN METABOLISM.

(a) Nitrogen absorption from the gastro-intestinal tract.

The term 'absorption' is defined as the difference between total nitrogen intake and faecal loss, and its value is affected by those processes which are collectively termed 'digestion'.

Gastro-intestinal absorption is surprisingly efficient in malnourished infants, and is equal to, or only slightly less than normal. Ninety to 95% of dietary nitrogen is absorbed. Mitchell and Bert[94], Gomez et al[95] found that blood amino acid concentration rose normally after test feeds of protein. Diarrhoea does not lower the efficiency of absorption significantly.[96] These findings are at variance with those of Hansen et al[97] who noted malabsorption of nitrogen on a milk diet.

The exocrine pancreas is atrophic in advanced kwashiorkor, and the duodenal juice contains less trypsin, amylase and lipase than normal[98]. It is probable that the lowered amounts of enzymes are adequate to deal with the small load of substrate

contained in the average kwashiorkor diet. No figures are available concerning concentrations of digestive enzymes during treatment (protein repletion) nor of their rate of return to normal, neither is it known whether digestion is complete at this phase. In fact, oub knowledge of the metabolic turnover of these and other enzymes participating in human metabolism is virtually negligible. It is not unlikely, therefore, that in acute kwashiorkor, especially during the early stages of treatment when protein repletion is in progress, that significant quantities of incompletely digested protein fragments (peptides) or other forms of bound amino acids may be absorbed into the portal blood.

(b) Nitrogen retention.

Nitrogen retention (N intake - urinary N - faecal N) and positive balance imply either growth or repletion of nitrogen stores.

Retention is naturally influenced by the balance of amino acids in the diet. Nitrogen of cows milk is 79% retained, whilst a comparable figure for a vegetable diet of corn and beans is only 29%. The latter figure could be increased considerably by adding lysine - the limiting amino acid (Cravioto)[99].

The average retention of nitrogen, in those children who recover, as determined by several independant observations, is 58%.[99][100][101][102] This figure is higher than that obtained for normal children on the same diet (Stearns et al)[103], but as the nutritional state improves, retention approaches normal.

Allison[104] recorded similar findings in dogs.

Synthesis of protein is the only mechanism whereby nitrogen may be retained, therefore the above studies on nitrogen retention indicate that in reversible malnutrition there is no impairment of the mechanisms of protein synthesis.

FREE AMINO ACIDS IN PLASMA.

Free amino acids in plasma have been studied by numerous workers, with varied results. In Nigerians, Edozien[105] found that the total free α-amino nitrogen of plasma filtrates was diminished to 50% of normal, with relatively greater fall in essential amino acids, with the single exception of lysine. Sarnoy[106] working in the Congo, found normal or high values of plasma amino acids and commented on the high phenylalanine to tyrosine ratio.

Artificial diets, which are deficient in an essential amino acid cause the plasma level of that amino acid to drop sharply (Snyderman et al).[107] Holt and his co-workers[108] have studied the free amino acid pattern of plasma of kwashiorkor patients from nine different countries and have found a uniform pattern regardless of diet. The essential amino acids were not preferentially affected, but both essential and non-essential acids were reduced in concentration. This evidence implies that the first limiting factor in kwashiorkor is deficient dietary nitrogen, rather than any one or a group of essential amino acids.

Partition of nitrogen in Urine.

Studies of the partition of nitrogen in urine amongst excretion forms of this element should yield information con-

cerning protein metabolism in the depleted state. Several such studies have been performed with this object in view.

In the acute phase of the protein depletion, before commencement of therapy, total nitrogen excretion is low. Values are from 500 to 1,000 mgm. per day[109]. Understandably this figure will vary since it is impossible to determine the exact diet at the time of admission to hospital. Dean[110] maintains that total nitrogen excretion is related to the severity of the illness, but workers in India could not confirm this and there seems to be difficulty in assessing grades of illness[111]. In spite of these and other discrepancies, there is general agreement that most, if not all of the nitrogen excreted during the acute phase is of endogenous origin. Fifty per cent of acute phase nitrogen is excreted as urea. This is significantly lower than during recovery and in normal children (± 85%). The contribution of creatine, creatinine and uric acid did not change appreciably during treatment.

Ammonia, amino and undetermined nitrogen were relatively increased, owing to low urea concentration[112]. The high ammonia excretion continued and became absolutely greater than normal if children were treated on a biologically inferior diet of peanut protein only[110].

Several independent studies have noted increased excretion of free amino acids in the urine of malnourished children, before ahd whilst receiving therapeutic diets. (Senecal et al [113] Sarnoy, 1957[106], Schendel and his co-workers[114] were able to study amino acid excretion, whilst there cases were on milk and

on synthetic diets of pure amino acids. They found increased excretion of five amino acids on both regimes. The amino aciduria diminished during treatment. They suggested that the mechanism was a temporary renal defect in reabsorption from the tubule. Edozien[105] also noted generalised amino aciduria and in addition detected increased amounts of β-aminoisobutyric acid and ethanolamine in the urine of acute cases. Although these two substances are not known to take part in protein synthesis, their excretion returned to normal with protein therapy. In later observations, Edozien and Phillips[112] found increased excretion of bound as well as of free forms of amino acids, and in most cases, excretion of bound forms exceeded those of the free. These authors also suggested (based on the biuret reaction) that much of the increase in bound forms was due to peptides. Whitehead and Matthew[115] working with East African children also found increased excretion of bound amino acids, although they do not state by what method their values were derived. In three of their cases, bound α-amino nitrogen was 4, 3 and 1½ times that of the free form. In normal children, approximately equal amounts of the two forms are excreted.

The East African workers were particularly impressed by high values of undetermined nitrogen in the urine of their acutely ill patients, and this fraction was greater in severe cases (26%) than in milder ones (10%). Absolute amounts of undetermined nitrogen decreased more than 4-fold with treatment. Preliminary investigations suggested that at least

part of the undetermined nitrogen fraction was a nucleic acid derivative, but another investigation failed to show increased excretion of purine derivatives.[116]

CHAPTER 5.

PEPTIDES IN URINE.

Urine is perhaps the most complex of all biological fluids, since it reflects and is influenced by every metabolic process.

Urea has been known to be the main form of nitrogen excretion since ±1800 , but it was soon realised that other nitrogen containing compounds were present in not inconsiderable amounts. Fractionation, identification and measurement of these substances has closely paralled development of methods, and in many instances new techniques have been developed to solve this problem, and these have since found application in other fields. It is perhaps true to say that chemical analysis of urine has always been a problem for the last 150 years, and every advance has followed application of newer and more elegant techniques. As will be shown, there does not exist at present a general procedure for the identification of all peptides present in urine.

It has long been known that free amino acids can be found in normal urine. In 1810, the Reverend Wollaston[(117)] described the presence of cystine and later Frerichs & Staedele (1854)[(118)] identified leucine and tyrosine. At about the same time, glycine, bound to benzoic acid as hippuric acid was found. On the other hand, even in 1911, Anderson[(119)] concluded that up to that time only glycine had been conclusively identified in normal urine. The chief deterrent was the lack of efficient methods both for the detection of amino acids and in obtaining their quantitative values. Formol titration of amino acids,

when applied to urine showed that this fraction constituted about 1% of total nitrogen.[120][121] Although the method is questionable on grounds of specificity, α-amino nitrogen when so measured showed a marked increase after acid hydrolysis. Bound forms were also demonstrated later by the gasometric method of Van Slyke.[122] The so-called 'CO_2' method developed by the same worker, has proved to be the most specific for α-amino nitrogen.[123] Microbiological methods[124] agree with the others that the average α-amino nitrogen excretion in the adult lies between 100 and 180 mgm. per day. Chromatographic procedures using paper as a support and later ion exchange resins have replaced others.

Recent measurements of amino acids in urine have been the subject of several reviews, emphasising rapid advances in this field.[125][126][127] All those amino acids present in proteins have been found in urine. Aspartic and glutamic acids, proline value and cystine are present in minute quantities but are released after acid hydrolysis. A normal adult excretes about 1 gram of free amino acids whilst another two grams are released after hydrolysis.[128] A considerable part of the bound amino acid fraction is composed of low molecular weight substances. Hippuric acid accounts for 70% of bound glycine[129] and half of the bound glutamic acid comes from phenylacetylglutamine.[130] There remains a considerable fraction of bound forms which consist of slowly dialysable polypeptides.

'Peptones' and 'albuminoses' have been described in the older literature in the urine of subjects suffering from fevers

and wasting diseases. As early as 1897, Bondzynski precipitated a so-called "oxyproteic" acid fraction from urine with barium salts.[131] These were shown to be polypeptides and have more recently been characterised. A substance, isolated from urine was found to be a polypeptide by Hári[132] which showed some resemblance to the oxyproteic acids.

Methodology.

Up to the present time, there is no general method available either for measurement of the total amount of peptide material, nor for the identification of individual peptides present in urine. (The number of procedures used almost equals the numbers of investigators.)

Determination of Total Mass of Peptide Material in Urine.

The 'bound amino acid' fraction is defined as the difference between total α-amino nitrogen measured before and after hydrolysis.[121][133] It is not possible to equate this fraction with peptides, since more than half may be accounted for by non-peptide conjugates such as hippuric acid and phenylacetylglutamine.

A peptide fraction may be prepared by precipitation with phosphotungstate after proteins have been removed with trichloracetic acid. The precipitate is dissolved and measured by Folins phenol reagent.[134] Uric acid contaminates the precipitate and gives a colour with this reagent. The biuret reaction has been applied directly to

urine.[135][136] The disadvantage of the latter two methods is that since the composition of the colour-yielding peptides is not known, no suitable reference substance or mixture can be used. Results are therefore of comparative value only.

Amino acids liberated after hydrolysis of urine have been individually measured by microbiological means[137][138][139] paper chromatography[133] and column chromatographic methods.[128] The validity of microbiological methods for this purpose is certainly suspect since it has been shown that amino acids bound in peptides may be preferentially utilised by the test organisms.[140]

It was hoped that determination of the amino acid content of some nitrogenous fractions prepared from whole urine would give a more accurate estimation of peptide content. The peptide nature of the 'oxyproteic acids' prepared by barium precipitation was established[141][142][143] but other theories of their nature were held.[144][145][146] At about the same time a nondialysable fraction prepared from urine was shown to be a polypeptide.[147] Recently modern and more selective methods have been applied. Boulanger[148] subjected deproteinised urine to dialysis and selective precipitation with cadmium hydroxide, yielded three polypeptide fractions: undialysable, dialysable and cadmium precipitate.

Several ion exchange methods have been used to produce materials for further study. Ling[149] desalted urine and freed it from amino acids on a cation exchange column of

1R 112 (H^+). Peptides which were not absorbed appeared in the effluent which was fractionated in IRA anion exchange resin by successive elution with 0.16M acetic acid, 0.08 N formic acid, 0.25 N formic acid, 0.08 N hydrochloric acid, and finally 0.16 N formic acid, all containing 10% acetone. Each fraction was hydrolysed with hydrochrotic acid and liberated amino acids were identified by paper chromatography. Low molecular weight peptides have been noted during qualitative studies on urine for free amino acids. Dent(150)(151) noted several acid labile spots with two dimensional paper chromatography. This method although sensitive has low capacity. Moore and Stein's system of column chromatography applied to urine(152)(153) allowed the use of a bigger load of material and many other ampholytes, including acid labile oligopeptides, were detected in urine. Carsten(154) desalted urine on a cation exchanger (1 R - 100). Adsorbed material was eluted with 2M aqueous ammonia and then applied to 1R - 400 The ampholytes which were retained could be eluted with 1M HCl and then fractionated on Dowex 50 with 2M and 4M HCl. Two dimensional paper chromatography showed free amino acids and di- and tetra-peptides both in normal and in pathological urine. Westall(155)(156)(157) using similar ion exchange methods, but on a much larger scale, fractionated 83 litres of urine and detected numerous peptides of varying complexity.

The preceeding methods all suffer from a common disadvantage in that they do not separate amino acids from peptides Hanson and Fittkau(158)(159), by a combination of charcoal

absorption and countercurrent distribution were able to prepare a fraction from 100 litres of urine, 80% of the total nitrogen of which was peptide nitrogen. This fraction was further resolved by high voltage electrophoresis and paper chromatography into ten peptides.

Using a procedure based on a modification of Bondzynski's original precipitate of oxyproteic acid, Saruecka-Keller[160] separated an acid, a neutral and two alkaline peptide fractions by high voltage electrophoresis. These were subjected to single dimension paper partition chromatography in a butanol/acetic acid/water mixture and individual peptides were characterised by amino acid composition, amino- and carboxylic-end terminal residues.

Results of Studies on Urinary Peptides.

Owing to the wide diversity and inadequacy of methods which have hitherto been employed to assay urinary peptides, estimates of the total amount of peptide present in urine varies according to the procedure used. For similar reasons differences in peptide pattern obtained by various authors are certainly due to their method of extraction and isolation.

Many measurements have been made of bound forms of amino acids in urine i.e. amino acids liberated after acid hydrolysis. The results with methods etc., are given in table 3.

Generally, no corrections for losses during hydrolysis can be made. Those methods which are applied to whole urine lack specificity and give high results. For the same reason no suitable standard is available. Formol titration

TABLE 3.

BOUND AMINO ACIDS IN URINE

Author(s)	Method	Result α NH_2 N mg/24 hrs.	Reference
Moore and Stein	Column Chromatographic	356 and 455	(161)
Muting	Column Chromatographic	356 367	(133)
Eckhardt and Davidson	Ninhydrin $-CO_2$	575	(138)
Rubinstein and Pryce	Colorimetric Ninhydrin	289 ± 69	(198)

gives high results[121] as do colorimetric ninhydrin procedures due to the presence of other ninhydrin-positive constituents of urine e.g. urea, taurine and creatinine. Van Slykes ninhydrin CO_2 method[123] is perhaps the most specific available for α-amino carboxylic acids. The reaction occurs at high temperature and high acid concentration which favours hydrolysis, thus giving low figures for the bound amino acid fraction. This method gives reliable results when applied to deproteinised plasma, since this fluid only contains 1 - 2% of bound forms.

The most acceptable results for total bound amino acids are those derived from estimation of individual amino acids before, and after, hydrolysis. Stein[161] using column chromatography found a range 1761 to 2459 mgm. of amino acid excreted in the bound form per 24 hrs. in normal subjects.

Muting[133] confirmed these figures and found averages 2221 and 2288 mgm. per 24 hrs. in 20 males and 20 females respectively.

NATURE OF BOUND AMINO ACID FRACTION IN URINE.

Since amino acids are present in compounds, otherwise than in peptide linkage, from which compounds the amino acid may be liberated by inorganic acids, the bound amino acids fraction bannot be equated with the peptide fraction. Stein[162] found that 580 mgm. per 24 hrs. of bound glycine existed as hippuric acid, whilst 550 mgm. of bound glutamic

acid was derived from phenlyacetylglutamine. Eades et al[163] isolated a complex of glysine with an unidentified steroid and demonstrated the presence of aspartic and glutamic glucoronides.[164] No quantitation was attempted but the substances were barely detectable in 100 ml. of urine and therefore do not contribute appreciably to total bound amino acids. Dunn et al have confirmed these findings.[165] No other significant quantities of bound amino acids could be accounted for. It can be deduced therefore that approximately 1 gram of amino acids exist as peptides in the urine. The amino acid composition of the bound forms is shown in Table 4.

A number of amino acid conjugates have been found in tissues which have not, up to now, been detected in urine but which may contribute to the bound fraction to a greater or lesser extent. Heyns and Paulsen[166] isolated a number of fructosyl and glucosyl amino acid derivatives from aqueous extracts of mammalian liver which possessed the general structures N - (D - fructosyl - 1) amino acid and N - (D -gly-cosyl - 2) amino acid respectively.

On a quantitative basis, glycine, glutamic acid, aspartic acid, histidine and proline are the most important bound amino acids. Serine, cystine, threonine, alamine, valine, phenylala-nine and leucine occur less abundantly. Only traces of bound arginine, isoleucine and methyl histidine were detected. These findings were confirmed by later workers but there were discre-pancies. Whilst Stein[161] found very little bound methionine, Muting[133] and Albanese et al[167] found large increases in

Table 4.

Free and Bound Amino Acids in Normal Urine. (Mg/24 hrs).[161]

Specimen.	Free.			Bound.		
	A.	D.	G2.	A.	D.	G2.
Tau.	134	294	180	13	8	0
Asp.	0	0	0	192	194	251
Thr.	24	27	53	34	37	82
Glut.	0	8	0	470	475	640
Pro.	0	0	0	67	89	94
Gly.	153	116	184	750	680	940
Ala.	41	38	71	28	24	45
Cys.	0	11	0	52	50	42
Val.	0	0	0	22	28	40
Iso.	21	14	28	5	8	-3
Leu.	14	18	24	5	14	7
Tyr.	24	48	49	24	20	62
Ph. al.	14	26	31	10	32	23
His.	-	236	320	-	33	130
Met. His.	130	104	47	10	3	-1
Lys.	11	18	17	37	39	60
Arg.	20	20	20	-	-	-

this amino acid after hydrolysis. Wellreff et al[168] did not find more than 0.12 mgm. of bound methionine per 24 hrs.

There is some evidence that the dicarboxylic amino acids exist as glucuronides.[165][164] Muting[133] found 11-86 mgm. of bound tryptophan after alkaline hydrolysis.

Ion exchange chromatography has discredited many of the earlier investigations performed on systems having inferior resolving power. An instance was the report by Thomson and Kirby[169] of relatively large amounts of bound arginine, which was measured by microbiological assay.

AMINO ACID COMPOSITION OF PEPTIDE FRACTIONS ISOLATED FROM URINE.

In an attempt to determine the nature of the bound forms of amino acids in urine, various 'peptide' fractions have been isolated and their amino acid composition determined.

Alderhalden and Pregl[170] determined the composition of the non-dialysable fraction, and detected leucine, alanine, glycine, glutamic and aspartic acids in the hydrolysate. Boulangers'[148] non-diffusable fraction contained these amino acids, and in addition, serine, threonine and the basic amino acids were present. This fraction could be separated on 'decaldite' resin into an acidic and an alkaline component. The former was composed of glutamic and aspartic acids, leucine and cystine, and the latter, glycine, alanine and basic acids. Albanese[167] determined the following amino acids quantitatively in the non-diffusable fraction from one litre of urine: Tryptophan 32.8 mg., cystine 15.2 mg., arginine 13.1 mg.,

histidine 6.7 mg. and tyrosine 3.9 mg.

Cadmium precipitation of urine produced an alkaline (glycine, alanine and basic amino acids) and an acidic fraction (chiefly glutamic acid and glycine). The diffusable fraction yielded, on hydrolysis, amino acids which had not previously been found in the free form viz. proline, valine, leucine and phenylalanine.[148]

Ling's procedure[149] yielded five peptide fractions by elution from Amberlie 1RA 400 with acids. These had the following amine acid composition:-

Fraction I (0.16N acetic acid)

Alanine, β-alanine, γ-amino butyric acid, aspartic and glutamic acids, glycine, proline, sarcosine, serine and valine.

Fraction II (0.08N Formic acid)

Alanine, aspartic acid, glycine, serine, threonine, sarcosine, proline, valine, leucine and isoleucine.

Fraction III & IV (0.16N & 0.25N Formic acid) - glycine and glutamic acid.

Fraction V (0.08N Hydrochloric acid)

β-alanine, aspartic acid, glutamic acid, valine, tyrosine and leucine.

Westalls technique[157] separated at least ten peptides composed of glutamic and aspartic acid, glycine, alanine, valine, leucine, serine, threonine, tyrosine, phenylanine, proline and β-alanine.

Neither Ling nor Westall found any sulphur-containing amino acids, arginine, histidine or lysine in hydrolysates of

their peptide fractions. Westall found a peptide containing β-amino-isobutyric acid, a fact which was not confirmed by Ling.

COMPOSITION AND HOMOGENEITY OF GROUPS OF PEPTIDES ISOLATED FROM URINE.

All the methods yet devised to detect individual peptides in urine involve initial preparation of a peptide fraction or a group of peptides. Although each procedure results in fairly uniform groups, the groups themselves, from various methods differ and results are not comparable. Another disadvantage is that, taken all together, these peptide groups do not include all the peptides present in urine.

Hansen and Fittkau[158] isolated 17 different peptides from normal urine. Glutamic and aspartic acids, glycine and alanine were common to most of these peptides. Twelve peptides contained lysine, and eight valine. Threonine, tyrosine, leucine, phenylalanine, proline and hydroxyproline were less infrequently encountered. One peptide, containing γ-NH_2 N-butyric acid was identified.

Studies on normal adults (three male and one female) by Ansorge et at[159] yielded 20 peptides, 17 of which were common to all four subjects.

Sarneca-Keller[160] prepared a well-defined peptide fraction from urine, which constituted 0.68% to 1.27% of total nitrogen and 16.1 to 20.9% of all biuret-positive substances. High voltage electrophoresis and paper chromatography resolved 22 ninhydrin positive components, 14 of which were peptides. The compositions and N-terminal amino acids of these were deter-

mined, and were different to those of the peptides isolated by Hansen and Fittkau. Each peptide contained at least 5 different amino acids and their composition was similar to bound urinary amino acids i.e. large amounts of glycine, histidine, glutamic and aspartic acids.

The peptides found by Sarnecka-Keller differed from those of Hansen and Fittkau. The preparations of the latter workers contained no histidine, whereas 10 peptides of Sarnecka-Keller did. In fact two peptides, isolated by the latter, were composed almost entirely of histidine and another of lysine. Glycine and alanine were the main N-terminal amino acids, but the polybasic peptides had no free terminal $-NH_2$ group.

Urinary pigment, urochrome, appears to be another distinct polypeptide fraction which is characterised by attached indoxyl groups.[171] The fraction is not homogeneous and consists of various peptides combined with a dipryrrole.

INDIVIDUAL PEPTIDES ENCOUNTERED IN URINE.

Apart from studies on specially prepared peptide fractions, several distinctive peptides have been identified in urine.

Whilst studying urine from children suffering from amino-aciduria of the Fanconi type, Dent[150] found [illegible] serylglycylglycine. Carsten[154] found several peptides in normal urine, each of which contained at least one dicarboxylic acid residue. Two tetra-peptides had the following molar ratios:

1. aspartic acid; glycine (1 : 1).
2. glycine, alanine, glutamic (2 : 1 : 1).

Westall[157] found five peptides:-

1. arginine; lysine; histidine ; glycine (1:1:1:1)
2. arginine; lysine; glycine; glutamic acid.
3. glutamic acid; glycine; lysine; X (1:1:1:1)
4. glutamic acid; aspartic acid; lysine.
5. glutamic and aspartic acids,

and one peptide containing only proline and hydroxyproline.

Seven non-diffusable peptides were separated by Boulanger.[148] Their composition revealed a more varied amino acid pattern than those of Carsten and Westall reflecting the greater number of residues per molecule.

SOME CHANGES IN THE TOTAL QUANTITY AND PATTERN OF PEPTIDES EXCRETED IN PATHOLOGICAL STATES.

Many apparently unrelated disease states have been found to be associated with increased excretion of peptides, or peptide-like material in the urine.

Increased quantities of peptide-like substances have been demonstrated in the urine of recently burned patients[172], cirrhosis of the liver and in leukaemia.[173] Hyperparathyroidism[174] and Marfan's syndrome,[175] a congenital connective tissue defect, are also characterised by pathological peptiduria.

Stein[176] found increased quantities of bound leucine, isoleucine and valine in the urine of patients suffering from Wilson's disease (Hepatolenticular degeneration). Uzman and Hood[177] have found that peptides excreted in the urine of these patients are rich in the di-carboxylic acids and have postulated that the increased quantity of copper that is present in the tissue, especially in the liver and basal ganglia of the

brain, interferes with metabolism of peptides by forming chelates.

Alteration in peptide excretion have been noted in some pathological conditions which are known to have increased excretion of free amino acids (aminoaciduria) as in diffuse hepatic necrosis[178] and cirrhosis of the liver.[173]

Therapeutic doses of x-rays to malignant tissue produced two urinary peptides, which have not seen found in normal urine. One consisted of asp., glut., ser., gly., and traces of thr., ala., and cysteic acid and the other of val., leu., ala., gly., thr., asp., glut., and lys.[179]

Dent's nephrosis peptide[150] was found in the deproteinised urine of two patients who had nephrosis. The amino acid composition of this peptide is similar to that of normal serum proteins. The presence of the peptide has since been confirmed in the blood and urine of nephrotic patients by Ellis et al,[180] who also demonstrated that it exhibited pressor activity similar to that of vasopressin.

It is only during the last decade that systematic studies on peptides have been made in urine. As will be gathered from the previous brief resume, there is much confusion, owing to the diversity of the procedures which have been used, and their lack of specificity and reproduceability.

Certain general facts, however, have emerged from these investigations. Peptiduria is a physiological phenomenon, and peptides account for 1 to 2% of total urinary nitrogen. Approximately the same quantity of nitrogen is present as non-peptide

conjugated forms of amino acids. In spite of the difficulties of quantitation, there is a wide normal range of peptide nitrogen, and marked qualitative and quantitative changes occur in disease states.

The precise metabolic origin or origins of the urinary peptides are as yet unknown. The peptide hormones, vasopressin, oxytocin, glucagon, adrenocorticotrophin and melanocyte stimulating hormone are known to occur in urine, and have been detected by biological methods, but these peptides occur in amounts which are not expected to be detected chemically with the methods under discussion. Other peptides, not derived from protein metabolism have been demonstrated in urine by their pharmacological activity, e.g. bradykinin, hypertensin and the group of so-called 'inflammatory' peptides of Menkin.[181] Since peptiduria increases as the result of tissue injury (malignancy, radiation damage) it is reasonable to suppose that many of the peptide fragments found in normal urine come from cathepsin-like activity or turnover of tissues and cells.

The presence of hydroxyproline peptides in urine is, however, incontraverible evidence of collagen breakdown. This amino acid is not assimilated into procollagen, but hydroxylation of proline, which has already been incorporated in the procollagen peptide chain, occurs by transfer of -OH groups from ascorbic acid.[182]

The amino acid composition of the collagen of skin, connective tissue and bone is similar; glycine accounts for 35% and alanine, proline and hydroxyproline, 10% by weight.[183]

The three sequences gly., pro., gly.,/gly., pro., ala., and gly., pro., hydroxypro., account for 94% of the proline and 74% of glycine.[184] Collagen is the most chemically inert protein, as has been shown by the turnover studies of Neuberger, Perrone and Slack[185] but collagen of bone turns over quicker than collagen from any other site. This would explain why bone disease is associated with increased excretion of bound hydroxyproline, e.g. Paget's disease[186], hyperparathyroidism[174] and an unusual bone disorder, described by Searkins[187]. Meilman et al have partially characterised 7 or 8 peptides which occur in 5 peaks on ion exchange chromatography of normal urine and have found that the pattern of excretion of these is altered characteristically in different bone diseases[188]; generally increased amounts are excreted in disease states.

CHAPTER 6.

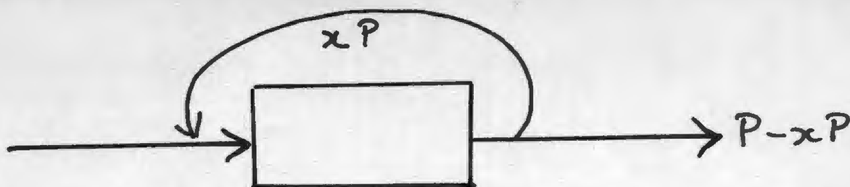
WORKING HYPOTHESIS.

Perhaps the most exciting concepts which are emerging from current day biological research, are those which explain perpetuation of information (genetic) responsible for preservation of the species and mechanisms whereby such information is transformed into macromolecules. These concepts raise other issues of equal importance. One of these is the mechanism or mechanisms whereby the amount of material synthesised is controlled. This thesis is concerned with such mechanisms.

Except for adaptive changes and growth, the amount of protein in an organism is held constant within narrow limits. This is well exemplified in the constancy of serum albumin concentration in the plasma of mammals. Since half of the total circulating albumin is renewed every 8 to 10 days, constancy of concentration implies equal rates of synthesis and breakdown. It is almost categorical that such accurate homeostasis must be mediated by a negative feedback mechanism.

In dogs, in whom almost all the intra- and extravascular protein has been removed by plasmaphoresis, serum albumin concentration returns to normal if the animal survives.[189] There is much indirect evidence that both synthesis and catabolism may be modified as the need arises.

Negative feedback implies that the output level (protein concentration) P, limits input (synthesis) by a feedback signal, whose magnitude is proportional to output i.e. xP where x is the feedback constant.



In terms of protein synthesis, this would mean that a small percentage of the total protein output of the biosynthetic mechanism would be diverted to damp down or deaccelerate the rate of synthesis. The basis of our working hypothesis has been that the feedback pathway, controlling synthesis rate can be equated with a fragment of the protein in question.

Various techniques are available whereby protein turnover may be modified. The method of plasmapheresis has already been mentioned. The reticulo-endothelial system may also be stimulated to produce globulins by suitable antigens e.g. those derived from capsular material of certain bacteria. In vitro systems, which synthesise proteins may be blocked by amino acid analogues and puromycin.

The hypothesis is as follows:-

1. Peptides are intermediates in protein synthesis and catabolism. Once formed, these peptide fragments are linked together to form the protein molecule. If any stage at which the fragments are joined together, is blocked, it should be possible to detect these intermediates of protein synthesis.
2. Protein synthesis is controlled by a peptide intermediate. The rate of synthesis is inversely proportional to the concentration of the peptide or peptides which control it.

We have available in the condition of kwashiorkor, a unique system in which protein synthesis may be limited by

deficiency of a relatively few essential amino acids, the concentrations of which are rate deciding. This experimental subject has the advantage of being physiological and reversible. Evidence has been presented (Chapter 4) that all mechanisms of protein synthesis are intact. Study of the disease and the effect of treatment allows observations to be made on protein synthesis in three phases of activity:

(a) in the acute phase of protein depletion, synthesis rates are approximately one half of that of normal.

(b) repletion stimulates synthesis and rates of synthesis are much accelerated.

(c) study of the phase of recovery allows comparison to be made with the preceeding two states.

The urine of kwashiorkor patients has been selected as the fluid most likely to contain the peptides fragments in question. (peptides are concentrated in urine, since they cannot be absorbed by the renal tubules).

Several practical considerations have also dictated the choice of this experimental model. Kwashiorkor is a world wide social and economical problem, which is in itself an adequate stimulation for these investigations. In addition, the disease is particularly prevalent in South Africa, and local clinical experience has helped in planning the investigation. The excellent nursing facilities of the Red Cross Children's Memorial Hospital, in which the children were treated, greatly facilitated collection of specimens. The

diets of these children, could for similar reasons be more easily controlled. The proposal was, therefore, to examine the urine of children suffering from kwashiorkor, before, during and after protein repletion for peptide fragments derived from modified protein synthesis.

One of two sets of circumstances may be operative:

1. If the quantity of dietary hitrogen is adequate but there are insufficient essential amino acids, one expects either:

a) a rate pf protein synthesis limited by the available essential amino acid moleculES (and apprently it is not in any case limited at the enzyme or even co-enzyme level). In this case we might get slow turnover and very small pools of peptide intermediates, but incomplete (abortive) peptides might arise in which the continued growth of the peptide chain was prevented because the messenger RNA had no more peptide bricks available. These peptides would be stripped off and excreted and would be lacking in the essential amino acid which had been exhausted; alternatively

b) perhaps non-essential amino acids would substitute for so-called essential ones on the RNA template or perhaps to some extent these so-called essential amino acids might be synthesised in vivo.

or c) sparing of essential amino acids in substrates, just as creatine and creatinine will be automaticaally spared because the muscle protein mass is, in any case, much less than normal.

or d) the catabolic enzymes may be rich in essential amino acids or use a cofactor which is rich in essential amino acids

and first reserve of action might be slowing (to 50%) of turnover due to falling catabolic rate. Recycling also might be expected to be more efficient and less catabolic peptides might therefore accumulate with less inhibition of synthesis by feedback mechanisms. The upshot of this situation is that relatively few essential amino acid-containing peptides should be available in the catabolic pathway for negative feedback control; all peptides liverated would be in the anabolic pathway which should be of that composition discussed overleaf.

If this latter occurs, they should disappear (providing anabolic enzymes are more than adequate) when the proportion of essential amino acids to the total is brought to normal on repletion; then we might expect that with overmuch amino acids available, a catabolic peptide controlling mechanism could be brought into operation.

In this situation one would expect mainly peptides containing non-essential amino acids since gluconeogenesis is limited by the high carbohydrate diet.

2. If the turnover is limited by total nitrogen, one might expect to find peptides in the urine containing essential amino acids throughout and theoretical lack of non-essential amino acids terminally, as the restrictive factor.

It was hoped, that whatever the outcome of the investigation, the findings might throw some light on the disease process of kwashiorkor, and on the mechanisms of control of protein synthesis in general.

SECTION B.

EXPERIMENTAL AND RESULTS.

CHAPTER 7.

CASE MATERIAL.

A. Selection of Cases.

The children, whose urine specimens were studied, were first seen as out-patients at a large urban Paediatric Hospital and were admitted for investigation and treatment.

The diagnosis of kwashiorkor was made on clinical grounds and was based on the presence of characteristic skin lesions and generalised oedema. The severity of the illness was assessed by the presence of systemic disturbances, such as inability to sit or walk, loss of muscle tone or cerebral dysfunction. Only those cases who were regarded as moderately or severely ill were studied, since only these were admitted to hospital for treatment.

The group of cases consists entirely of male subjects, because collection of urine from female children was a difficult nursing problem.

Extremely ill patients were also excluded, since collection of urine from these imposes a burden which cannot be justified. However, in one case, urine collection had to be abandoned due to clinical relapse after one specimen had been collected. This patient died one week later. All other cases showed clinical improvement and were discharged after 3 - 5 weeks' stay in hospital.

The group of cases studied, therefore, consisted of 6 male children, who suffered from moderate to severe grades of kwashiorkor, and in whom protein depletion was reversible.

Table 5. Summary of clinical and laboratory findings in the cases studied.

No.	Age months	Severity	Wt. (lbs.) Adm.	Wt. (lbs.) Disch.	Hb G.%	Serum Proteins g./100 ml. Alb.	Serum Proteins g./100 ml. Glob.	Other.
1. (H.L.)	15	Severe	$16\frac{15}{16}$	$17\frac{14}{16}$	14.0	2.1	2.7	
2. (A.O.)	21	Moderate	$14\frac{8}{16}$	$15\frac{10}{16}$	12.0	2.7	1.5	
3. (J.C.)	28	Moderate	$20\frac{5}{16}$	23	13.5	2.9	1.6	
4. (F.B.)	48	Severe	$23\frac{14}{16}$	$25\frac{13}{16}$	10.0	1.8	1.9	
5. (T.I.)	21	Moderate	20	$20\frac{11}{16}$	9.5	1.4	2.6	
6. (S.G.)	17	Mild	-	-	9.5	3.2	3.1	
7. (P.J.)	12	Severe	$13\frac{8}{16}$	-	7.0	-	-	Serum:- Na^{+}: 165m.eq./l. Cl^{-} : 146m.eq./l. Urea 49 mg./100ml. Ca^{++}: 6.6mg./100ml.

Some data were also obtained from the case who succumbed from irreversible protein depletion.

A summary of the clinical and routine biochemical findings appears in Table 5.

B. Treatment.

In all cases, milk feeding was the only treatment required to induce clinical improvement of the features of kwashiorkor. The regime of feeding was as follows:-

Day of admission : clear fluids (Darrows and glucose solution).

Day after admission = Day 1 : clear fluids.

Days 2 and 3 : skim milk.

Days 4 and 5 : half-cream milk.

Day 6 and later ; full-cream milk.

The amounts given were calculated as 2½ fl. ozs. per lb. body weight.

The only exception to the above, was in case 6 (S.G.) who had clear feeds on the day of admission, and the two following days (2 and 3).

All cases routinely received antibiotics. These included Penicillin, Streptomycin and the tetracycline derivatives.

C. Collection of urine specimens.

Throughout the period of collection (24 hrs.), the patient was on a metabolic frame. A glass receiver was taped over the penis, and from this a rubber tube led to a collection bottle on the floor. A few ml. of toluene acted as a preservative. As coon as collection was complete, the urine was stored at $-10^{\circ}C$ until required for analysis.

CHAPTER 8.

THE PARTITION OF URINARY NITROGEN IN KWASHIORKOR.

Partition of nitrogen amongst the major nitrogen fractions, normally occurring in urine, was studied in order to assess quantitatively the changes which might occur during protein depletion, repletion and recovery.

The following nitrogen fractions have been measured:-

1. Total nitrogen.
2. Urea nitrogen.
3. Ammonia nitrogen.
4. Creatinine.
5. Free α-NH_2 nitrogen.
6. Bound α-NH_2 nitrogen.

A suitable aliquot of the pooled 24-hour specimen was analysed for the compound in question. Any sediment (urates, phosphates) was stirred and well mixed before sampling in order to include any adsorbed or precipitated material.

METHODOLOGY:

1. Total Nitrogen.

The total nitrogen content of the urine sample was determined by the micro-Khjeldahl procedure[190]. An aliquot, containing approximately 0.2 mg. of nitrogen (0.2 - 0.5 ml.) was digested.

Recovery: A histidine standard containing 0.2 mg. of nitrogen was assayed 34 times. Average nitrogen recovery was 100.2%, with a range of 98.8 to 101.7%.

2. Urea Nitrogen.

Urinary urea was measured by the colorimetric diacetyl-monoxime reaction, and the complex with ferric ions was measured on the Technicon Auto-Analyser at 470 mu[191]. Urine samples were prediluted (1 in 10) and the results read from a standard curve.

Recovery of added urea (Urea N):

Urine	0.588 G/100 ml.
Urine + 0.234 G.%.	0.830 G/100 ml.
Recovered	0.242 G/100 ml., i.e. a recovery of 104%.

3. Ammonia Nitrogen.

A modification based on the micro-Khjeldahl procedure was employed.

Reagents: Tri-n-butyl phosphate.

Saturated K_2CO_3 solution.

Indicator: a mixture of 2 vol. of methyl red and 1 vol. of methylene blue. Both indicators were kept as 0.2% (w/v) solutions in 95% (w/v) ethyl alcohol.

Boric acid solution: 10 gm. boric acid + 5 ml. of mixed indicator solution were made up to 500 ml.

0.01 n. $KH(IO_3)_2$ solution.

Method: One ml. of urine and 2 - 3 drops of tri-n-butyl phosphate (to prevent frothing) was placed in the Khjeldahl distillation apparatus. Saturated K_2CO_3 solution (4 ml.) was added and steam distilled into 5 ml. of boric acid solution until 20 ml. of distillate had been collected. NH_4^+ was titrated

with 0.01 N $KH(IO_3)_2$ to a lilac end point. A blank on 1 ml. of water was performed.

Calculation: 1 ml. of 0.01 N $KH(IO_3)_2 \equiv$ 0.1401 mg. N.

Recovery of 0.2 mg. N in an ammonium chloride standard was 99.9% (average of 17 recoveries) with a range of 98.0 to 101.7%

Contribution of Amide Nitrogen to result: one ml. of solution containing 2.347 mg. of Asparagine (0.249 mg. amide N) was assayed for free ammonia by the above method. None could be detected, i.e. less than 0.5% amide N could be recovered as ammonia. The results obtained on urine were therefore not affected by decomposition of amides.

4. Urinary creatinine.

The Jaffe alkaline picrate method of Bosnes and Taussky[192] was employed.

Method: 3 ml. of a 1 in 100 dilution of urine was mixed with 1 ml. each of 0.04M picric acid and 0.75N NaOH, allowed to stand for 15 minutes and read against a blank at 520 mu. A standard solution containing 0.01 mg. creatinine per ml. was assayed concommitantly.

THE NINHYDRIN REACTION.

Many of the methods presently being used to assay or detect amino acids, utilise their reaction with ninhydrin. This reaction will be discussed, with special reference to its specificity since this bears particular importance in the problem of measuring amino acids in complex mixtures such as biological fluids.

Triketohydrindene hydrate (ninhydrin) forms a blue complex with α-amino carboxylic acid. The reaction is fairly specific and has therefore replaced Sorenson's formol titration[120] and the nitrous acid reaction of van Slyke.[122]

The exact mechanism of the ninhydrin reaction is uncertain but that shown in fig. 5 is accepted as a working hypothesis.[193]

The typical violet colour of the products of the ninhydrin reaction which has maximum absorption at 570 mu is due to the anion of diketohydrindylidene - diketohydrinamine (DYDA). Ammonia (NH_3) will only complex with ninhydrin if a reducing agent is present, but even under these conditions, the reaction (with NH_3) is slower and less complete than with amino acids, suggesting an intermediate complex prior to de-amination.

The gasometric method of van Slyke[123] is based upon the release of CO_2 and is highly specific for α-NH_2 Nitrogen. This is also a convenient method for removing the carboxyl carbon atom.

Initially the photometric method was unreliable owing to atmospheric and self oxidation of intermediate hydrindantin. Moore and Stein[194] introduced stannous chloride into the reaction and described a rapid and convenient method for estimating amino acids. Colour development can be accelerated and increased by organic solvents, especially phenol and pyridine.[195] The presence of phosphates in biological fluids precludes the use of stannous chloride as a reducing agent, but this may be replaced by exogenous hydrindantin*[196] or cyanide (CN)[197]. The latter method of Yemm and Cocking is particularly useful.

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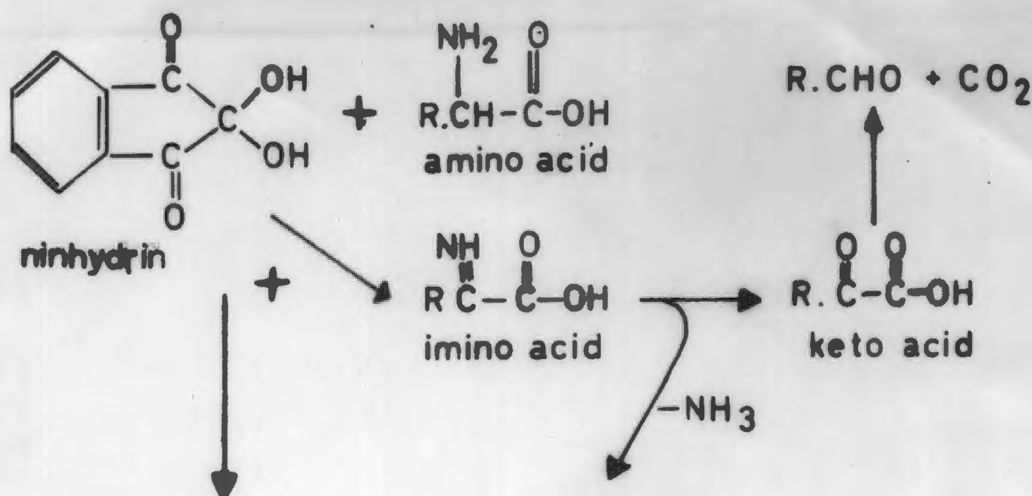
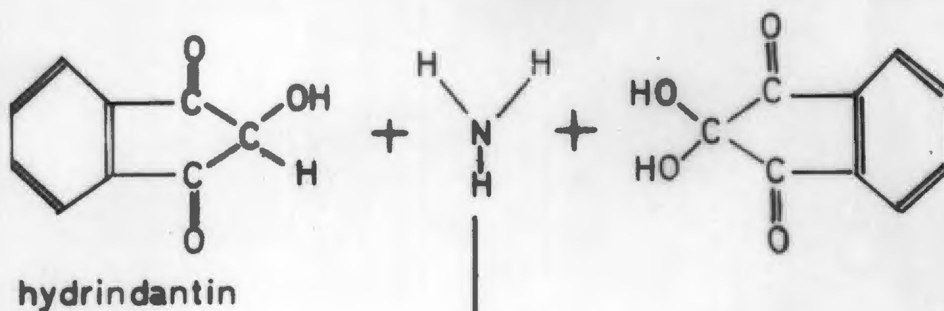
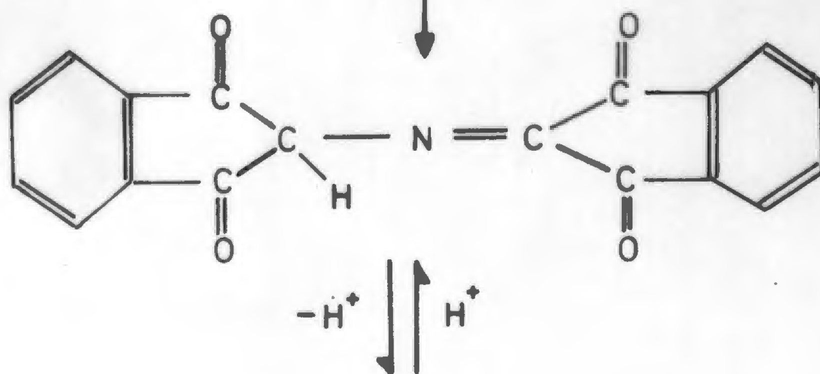
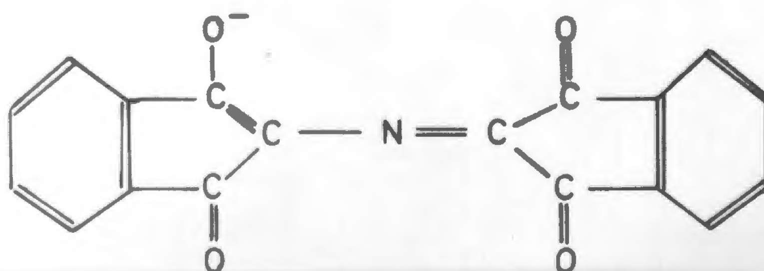
* prepared by reducing ninhydrin with ascorbic acid.

FIG. 5 THE NINHYDRIN REACTION









anion of diketohydrindylidene-diketohydrinamine (DYDA)

Rubinstein and Pryce[198] replaced pyridine with E.D.T.A., which removed trace metals and improved reproduceability. This method has been used to determine free and bound α-NH_2 nitrogen in urine, cerebro-spinal fluid and plasma.

The imino acids react with ninhydrin rapidly at room temperature and pH 7, but reducing agents have no effect on rate or amount of coloured complexes formed. Carbon dioxide is evolved but there is no oxidative de-amination and the imino residue condenses directly with ninhydrin yielding a yellow compound, which has a broad absorption maximum at 440 mu. Red coloured intermediates of this reaction have been isolated by benzene extraction and were identified as di-(diketohydrindylidene) - pyrroles. When these are placed in a system containing ninhydrin in aqueous medium, typical yellow compounds form rapidly.

The colour yields per mole, of most amino acids in these methods, are between 97 and 102% of the E value of DYDA, but tryptophan (70%) and lysine (110%) are exceptions. The increased colour yeeld of the latter amino acid is probably related to the ε-amino group. The colour yield of peptides decreases with increasing chain length; 95% for di- and 85% for tetra-peptides.

Other non α-NH_2 acids and miscellaneous compounds having amino or imino groups, yield coloured complexes with ninhydrin which absorb maximally at 550 to 570 mu, although the molar colour yields are considerably less than those of the free α-amino acids. Other ninhydrin-positive compounds include

the following, which are of considerable biological importance:-

β-amino acids e.g. β-alanine $NH_2.CH_2.CH_2.COOH$,

and β-amino isobutyric acid $NH_2.CH_2.\underset{CH_3}{CH}\,COOH$

γ-amino acids e.g. γ-amino butyric acid $NH_2.CH_2.CH_2.CH_2.COOH$

ethanolamine $NH_2.CH_2.CH_2.OH$

urea $NH_2.CO.NH_2$

creatinine

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NH = C - NH
      \    \
CH3 - N      CO
       \CH2
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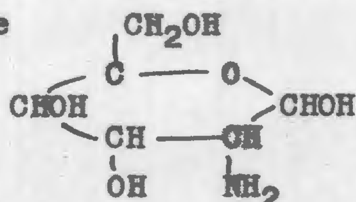
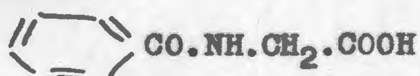
hexosamines e.g. glucosamine



Taurine, $NH_2.CH_2.CH_2.SO_3H$, which is not a true amino acid has a high colour yield, and in addition, occurs in urine in relatively high concentration. The exact colour yields of these amino compounds will be reported in the experimental section.

The common factor in this wide range of ninhydrin-positive compounds is an $-NH_2$ or an $-NH-$ group. If, however, one or both hydrogen atoms are substituted, no reaction is possible. The $-NH-$ group forming a peptide bond does not contribute to the ninhydrin colour produced by peptides or proteins. The acetylated amino acids $CH_3.CO.NH.\underset{R}{CH}.COOH$ are ninhydrin negative, as is hippuric acid.



Steric hindrance by adjacent groups has been suggested as an explanation for these discrepancies.[199]

5. Free and Bound alpha amino nitrogen.

The colorimetric ninhydrin procedure, especially adapted for biological fluids by Rubinstein and Pryse[198] has been used. In this method free ammonia is removed by alkaline distillation in vacuo, and interference by non-alpha amino acids circumvented by using the cyanide reducing reagent of Yemm and Cocking[197].

REAGENTS.

1. Phenol reagent: 400 g. of freshly distilled phenol was melted and dissolved in 100 ml. of absolute ethanol. After cooling, 5 g. of Zeokarb 225, in the H^+ phase was added and the reagent stirred for 10 minutes. The solution could be kept in a dark bottle, without removing the resin, for several months, or until blank values rose above 0.150 O.D. units.

2. Ninhydrin reagent: Six g. of ninhydrin (BDH)* were dissolved in 100 ml. of absolute ethanol. Two g. of Zeokarb 225 (H^+ cycle) were added and the suspension was stirred. The reagent was filtered into and stored in a dark bottle. Colour yields with a standard amino acid solution were constant for up to three months with this solution.

3. Buffer: 300 ml. of 2M sodium acetate (164 g. of the anhydrous salt per litre) were added to 700 ml. of 2M acetic acid (120 ml. of glacial acid per litre). Five g. of EDTA sodium and 240 mg. of KCN were then added. A few drops of chloroform acted as preservative. The pH of the buffer was 5.10.

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* British Drug Houses.

4. <u>0.05M Na_2CO_3</u> and <u>0.2M K_2CO_3</u>.

5. <u>Leucine standard solution:</u> which contained 70.0 µg. of α-amino nitrogen per 1.00 ml. was made up in 10% (v/v) acqueous isopropanol.

<u>PROCEDURE</u>.

<u>Free α-amino nitrogen.</u>

0.2 ml. of urine was pipetted into a 15 ml. beaker which contained 2.0 ml. of 0.05M Na_2CO_3 solution. A standard and blank were prepared with leucine standard and water respectively. These solutions were evaporated to dryness over conc. sulphuric acid in a vacuum desiccator at room temperature.

Ten ml. of water was added to each beaker and mixed with a glass rod. 0.5 ml. aliquots of these test, standard and blank solutions were added to:-

0.50 ml. of pH 5.1 buffer,

5.00 ml. of phenol reagent

and 0.50 ml. of ninhydrin reagent and well mixed by shaking in a boiling tube (6" x 1"). The solutions were then heated in a vigorously boiling water bath for exactly 5 minutes and cooled rapidly in water. When cold, the tubes were shaken in a mechanical shaker for 2 minutes to oxidise any hydrindantin which remained. After standing in the dark for 30 minutes, the optical densities of the solutions were read in a Beckman DU spectrophotometer at 570 mu. The slit width varied between 0.02 and 0.03 mm. The blank reading ideally had an optical density of less than 0.100 units. A reading of more than 0.125 indicated contamination of reagents usually by absorbed ammonia.

Calculation: α-NH_2 nitrogen = $\frac{\text{Test - blank}}{\text{Std. - blank}} \times \frac{3.5}{1} \times 10$ mg/100 ml. urine.

Total -NH_2 Nitrogen.

Proteins were removed by tungstic acid precipitation. Total α-NH_2 nitrogen was determined after hydrolysis in 6 N HCl by the colorimetric ninhydrin reaction as for the free form.

To 10.0 ml. urine was added 1.0 ml. of 0.67 N H_2SO_4 and 1.0 ml. of 10% (w/v) sodium tungstate solution. Any precipitate was removed by centrifugation. Two ml. of protein-free filtrate and 2 ml. of conc. HCl were sealed into a 4" x $\frac{5}{8}$" glass ampoule and hydrolysed for 18 hours at 105°C in a thermostatted oven. The hydrolysate was filtered through a plug of glass wool and 0.1 ml. of the filtrate was pipetted into 2 ml. of 0.4 M K_2CO_3 in a 15 ml. beaker over conc. H_2SO_4 in a vacuum desiccator. The residue was then treated exactly the same as for free α-NH_2 nitrogen.

Calculation:-

Total α-NH_2 nitrogen (mg.)

$$= \frac{\text{Opt. density of test - O.D. of blank}}{\text{Opt. density of std. - O.D. of blank}} \times \frac{3.5}{1} \times \frac{40}{1} \times \frac{12}{10} \text{ mg./100 ml. urine}$$

The blank was higher than for free α-NH_2 nitrogen. (Usually 0.130 to 0.150)

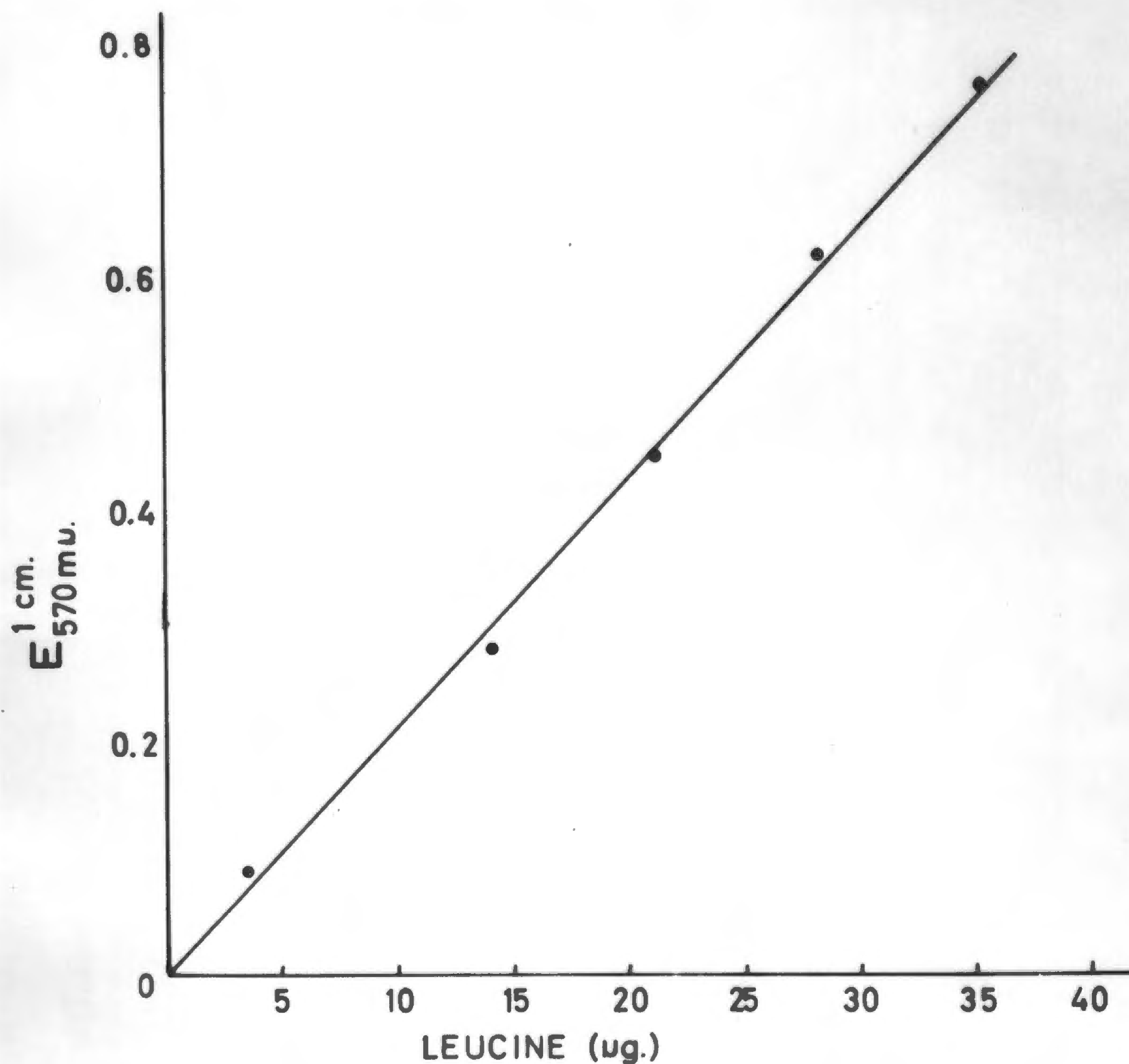
A calibration curve was constructed for the range of α-amino nitrogen encountered in urine. Beers' law was obeyed up to a concentration of 3.5 µg in the total reaction mixture (Fig. 6).

Recovery of standard amount of free amino acids:-

Std. leucine recovery. (3.5 µg. α-NH_2 N)

FIG. 6

CALIBRATION CURVE α-NH_2 NITROGEN



O.D. Std. 0.764. (Av. of two readings).

O.D. of recovered Std. = 0.759 (Av. of two readings).

Recovery = 99.3%.

Recovery of a simple peptide.

O.D. Values.		
	Std. 0.353 μg. leucine	0.866.
	Urine alone (0.2 ml./20.)	0.310.
	Bacitracin (5.40 μmoles/20.)	0.294.
	Urine + Bacitracin	0.628.

Recovery of Bacitracin : $\frac{0.628 - 0.310}{0.294} \times 100 = 108.1\%$

The bound α-NH_2 nitrogen fraction is the difference between total and free α-NH_2 nitrogen. The ratio of bound to free forms was also calculated.

RESULTS.

The results of the quantitative tests of partition of nitrogen in the urine are shown in the following tables. The first specimen for each case was taken before protein feeds were administered. The next one or two specimens were taken immediately after, and up to 6 days after protein repletion commenced. In most cases three specimens were collected during a 5 day period immediately prior to discharge from hospital. The initial 2 or 3 specimens were labelled acute phase, and the last three, recovery phase.

Tables 6 - 11 include results on nitrogen partition in 6 cases. In one of these the acute phase only has been studied. In the 7th case (P.J.) the patient became too ill to allow

TABLE 6.

Case 1. (H.L.) Partition of urinary nitrogen (mg./24 hrs.).

PHASE	DAY	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hr.	α-NH_2 N. Bound	α-NH_2 N. Free	Ratio Bound / Free
ACUTE	1	23.5	241	57	96	- *	7.4	5.9	1.2
	3	239	1,410	336	378	82	27.0	24.1	1.1
	5	887	4,200	1,812	434	128	152	113	1.3
RECOVERY	15	557	3,403	2,060	986	96	42.3	55.1	0.8
	17	370	2,580	1,280	1095	65	23.3	24.0	1.0
	19	438	3,066	628	2203	89	44.5	23.0	1.9

* Insufficient.

TABLE 7.

Case 2. (A.O.) Partition of urinary nitrogen (mg./24hrs.)

Phase	Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hr.	α-NH_2 N. Bound	Free	Ratio. Bound/Free
ACUTE	2	190	551	222	181	56	29.3	14.4	2.0
	4	846	1,286	614	255	64	94.0	64.0	1.5
RECOVERY	14	460	1,359	496	160	21	25.9	13.6	1.9
	15	281	1,206	894	123	23	16.5	29.0	0.6
	16	236	2,556	1,412	520	36	11.5	10.0	1.2

TABLE 8.

Case 3. (J.C.) Partition of urinary nitrogen (mg./24hrs.).

Phase	Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hrs.	α-NH_2 N.		Ratio
							Bound	Free	Bound/Free
ACUTE	1	292	972	678	169	36	36.5	35.0	1.0
	3	153	1,637	1,028	174	33	17.3	20.6	0.8
	5	35	364	284	45	5.0	5.0	5.0	1.0
RECOVERY	13	470	1,936	1,120	158	28	19.6	21.2	0.9
	15	448	4,880	3,440	390	26	20.6	32.6	0.6
	17	408	2,340	1,520	178	35	21.5	17.7	1.2

TABLE 9.

Case 4. (F.B.) Partition of urinary nitrogen (mg./24hrs.).

Phase	Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine. mg./24hr.	α-NH_2 N.		Ratio Bound/Free
							Bound	Free	
ACUTE	1	785	2,410	1,580	390	182	72.6	18.4	4.0
	3	340	1,357	604	290	154	37.3	26.7	1.4
	5	998	2,409	1,893	303	200	44.7	73.1	0.6

TABLE 10.

Case 5. (T.I.) Partition of urinary nitrogen (mg./24hr.)

Phase	Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hr.	α-NH_2 N.		Ratio Bound/Free
							Bound	Free	
ACUTE	2	217	2,691	1,420	202	82	50.0	34.8	1.4
	4	261	1,670	1,020	503	85	26.7	22.4	1.2
	6	245	1,501	1,160	39	67	61.1	16.7	3.7
RECOVERY	22	467	3,190	2,690	197	161	28.8	30.0	1.0
	24	463	2,680	1,480	639	156	19.1	18.0	1.1
	26	424	3,434	1,630	922*	102	26.3	19.5	1.4

* This specimen appears to be infected.

TABLE 11.

Case 6. (S.G.) Partition of urinary nitrogen. (mg./24hr.)

Phase	Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hr.	α-NH_2 N. Bound	α-NH_2 N. Free	Ratio Bound/Free
ACUTE	2	180	1,172	595	80	42	23.5	18.2	1.3
	4	571	4,744	3,010	55	54	40.5	14.4	2.8
	6	318	4,045	3,560	183	81	75.5	56.5	1.3
RECOVERY	15	215	2,663	1,980	222	82	22.4	37.4	0.6
	16	306	2,038	1,610	198	61	23.4	28.4	0.8
	17	292	1,723	1,320	135	56	15.2	25.3	0.6

TABLE 12.

Case 7. (P.J.) Partition of urinary nitrogen (mg./24hr.)
(Acute phase only.)

Day	Vol. ml.	Total N	Urea N	NH_3 N	Creatinine mg./24hr.	α-NH_2 N.		Ratio
						Bound	Free	Bound/Free
1	105	925	361	239	23	7.1	20.0	0.35

collection of urine. This patient died 8 days after admission (Table 12.)

Discussion.

It is obvious from the results of urinary N partition that not all cases have been observed through the same stages of the disease process, although all have been managed similarly. Usually diuresis was induced by protein feeding. This was observed in cases 1, 2 and 6. Case 3 developed oliguria whilst on treatment and diuresis occurred after the fifth day. Case 4 had diuresis within the acute five-day period, but urine volumes were maximal on the 1st and 5th day. Case 5 had remarkably constant urinary output for the first 6 days (ca. 250 ml.) which was about half that of the recovery phase (ca. 450 ml.). Oliguria and diuresis dictate the absolute excretion of all urinary constituents and may affect the partition of these due to differential renal tubular re-absorption. This appears to be particularly so in case 1.

Table 13.

Case 1. H.L. Concentration of N (mg./100ml.)

Day.	Vol. ml.	Total N.	Urea N.	NH_3 N.	α - NH_2 N Bound	α - NH_2 N Free.
1	23.5	1,045	392	417	63.3	25.3
3	239	588	247	108	35.4	19.9
5	887	474	243	49	19.7	12.7
15	557	611	331	177	12.8	10.0
17	370	699	238	296	21.9	6.5
19	438	700	143	503	7.4	4.6

Table shows the concentration of nitrogenous substances

in urine of H.L. (Case 1). As expected the concentration of all of these was highest in the smallest 24-hour volume (23.5 ml. on day 1) but the concentrations of nitrogenous compounds during diuresis on day 5 did not fall in proportion to urinary output since, on a 24-hourly basis, there was an absolute increase in the excretion of the nitrogenous compounds which were measured. Other cases showed similar coincidence between periods of diuresis and maximum daily excretion. Although some material retained during times of oliguria was certainly present in the urine produced during diuresis, it is obvious from the relatively high concentration of nitrogen in this latter phase that there was an absolute rise in the rate and formation of urinary nitrogen towards the end of the acute phase, i.e. during protein repletion. These findings are in accordance with those of other workers, both in man[200] and animals[201] which show that urinary nitrogen is related to protein intake, but that during periods of low protein intake there is still a considerable loss of urinary N which is of endogenous origin. Total nitrogen, urea and ammonia excretion were low during the acute phase and there was thus a lower rate of production of urea from protein breakdown at the time when patients were admitted to hospital. This is in accordance with recent determinations which show a very slow turnover rate of such patients' serum albumin [93] and a quickening of its catabolism during protein repletion. Most cases showed maximum excretion of free amino acids during the acute phase which was above normal for children of this age[202] and was greater than that during recovery. The

FIG. 7a. Urinary Excretion of α-NH_2 Nitrogen

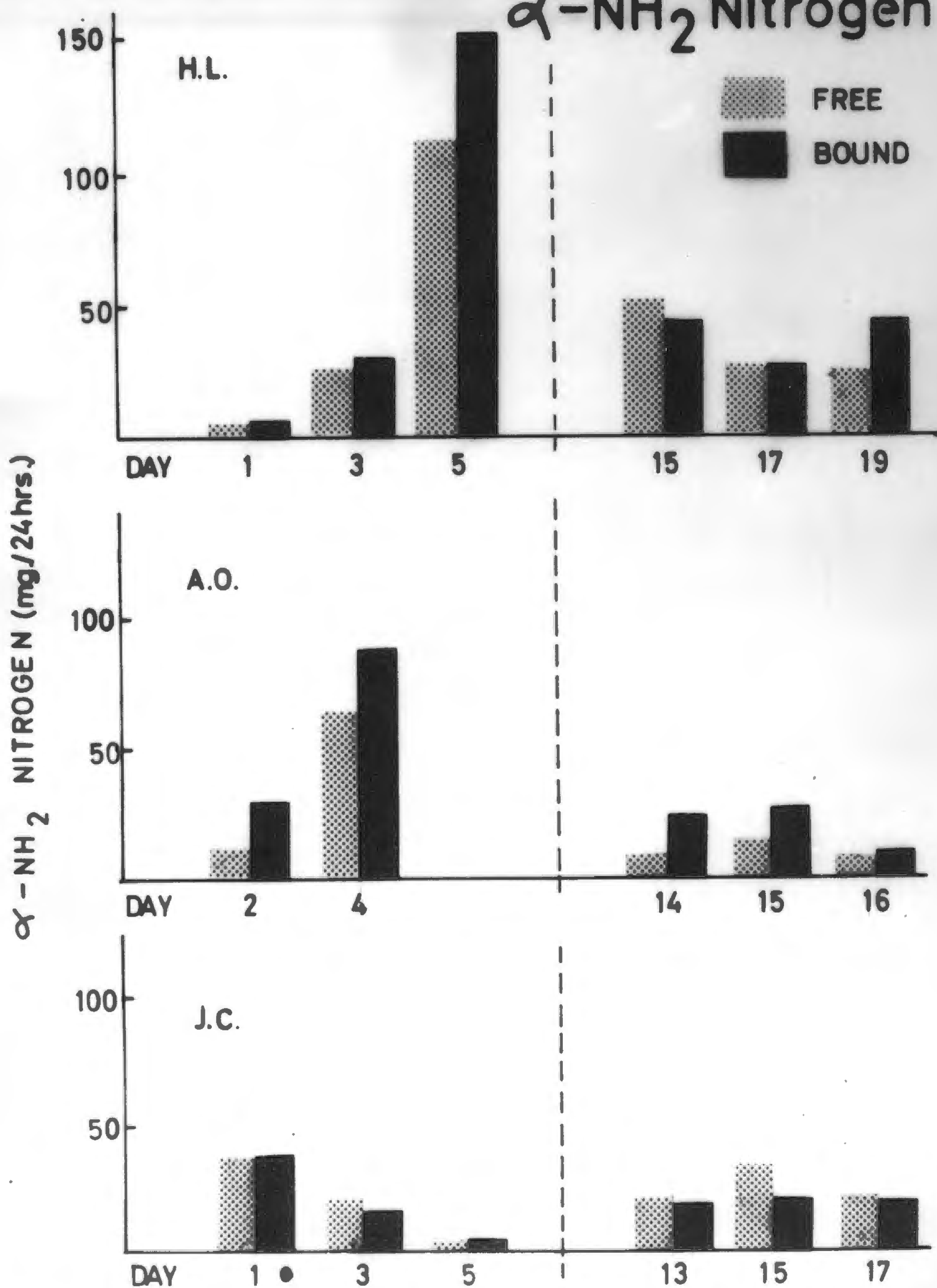
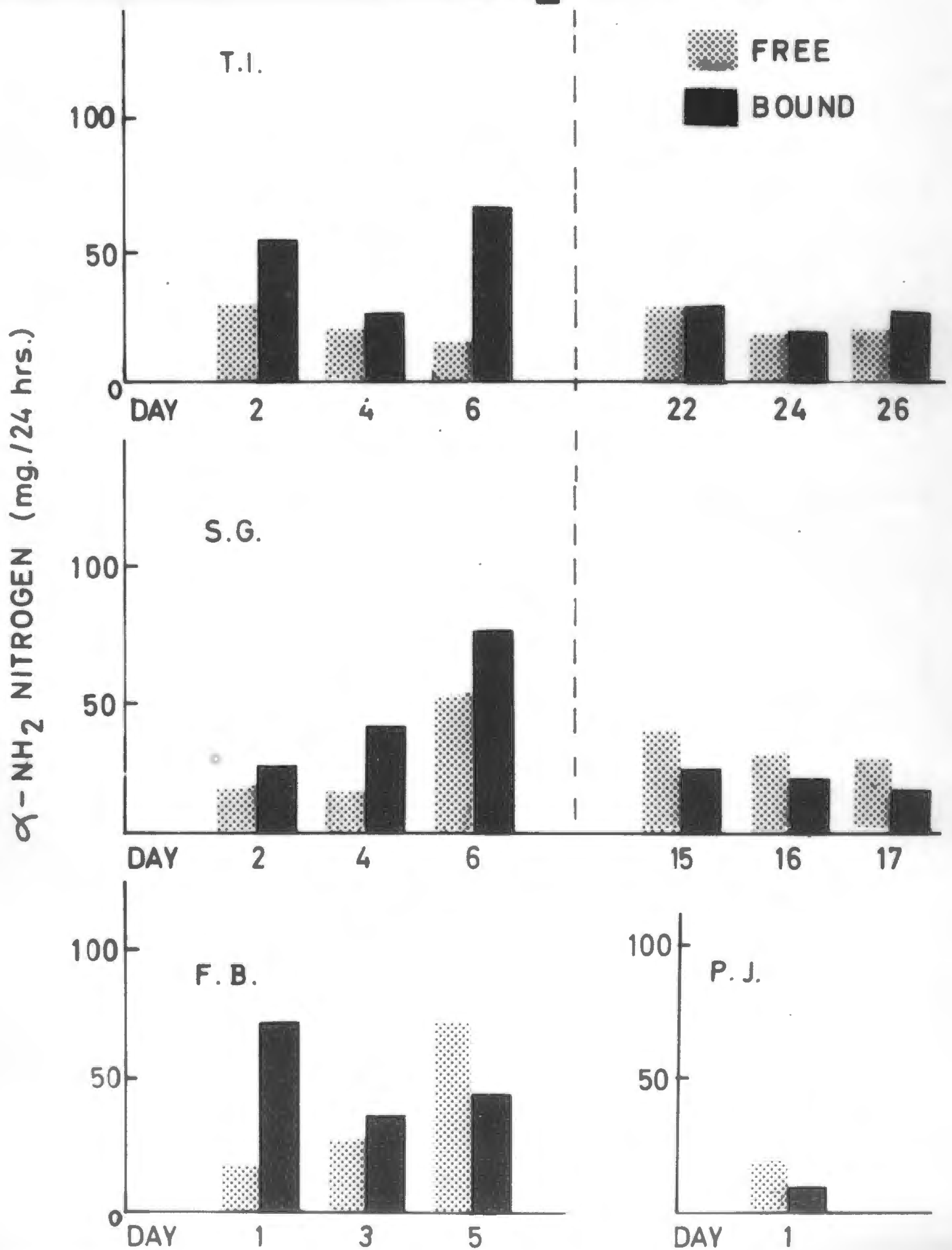


FIG. 7b. Urinary Excretion of α-NH_2 Nitrogen



picture of increased aminoaciduria was not entirely uniform. Case 6 had a maximum excretion on the 15th day. This finding confirms those of many other workers[203][204] and values obtained are comparative but depend upon the method used. Vasangadkar et al[205] found higher values in Indian children (40 tp 66 mg. of α-NH_2 N per 24 hours) by using the iodometric method of Albanese and Irbey[206] but severity of their cases may not be comparable.

Bound amino acids released on hydrolysis were of the same order of magnitude as that of free α-NH_2 N. The excretion of bound α-amino nitrogen showed changes which were far more impressive than those of free forms, having a peak value in the acute phase, (figs. 7a&7b) both quantitatively and in correlation with clinical stage of the disease. The ratio of bound to free forms was also significantly higher in the acute phase ($p < 0.05$).

In 5 out of 6 cases maximum excretion of bound forms was attained between days 4 and 6 and in one case, on day 1, but the latter case was anomalous, in that oliguria developed.

The ratio of bound to free amino Nitrogen varied widely with a range of 0.35 to 4.00. During recovery this value was reasonably constant - near unity. This was the value found in normal children.[202] There was thus a significant increase of this ratio in the acute phase and this coincided with diuresis.

Re-absorption of free amino acids by renal tubular cells has been well established.[207] This absorption is both active

and selective, since the pattern of free amino acids in plasma and urine is dissimilar. Several investigations have failed to demonstrate re-absorption of peptides[208(209)]. Alterations in urinary volume and tubular flow rate might, therefore, be expected to alter the ratio of free to bound forms and to be responsible for the observed changes. When the ratios were matched against urinary volume, no correlation between these two parameters was observed, (fig. 8) either in the acute or the recovery phase. Both showed greater scatter in the more dynamic acute phases of protein depletion and repletion. Renal factors did not, therefore, play a significant part in altering the bound/free ratio, and others were sought.

The findings were that there was an increased excretion of bound α-amino nitrogen which coincided with the beginning of protein repletion, and excretion rate fell during recovery.

Case 7, who died, is of considerable interest. This showed the lowest ratio of bound to free α-amino nitrogen (0.35) but unfortunately no further specimens could be obtained.

The ninhydrin reaction, by means of which free and bound α-amino nitrogen was measured, is not specific. Substances in urine which interfere include ammonia, urea, taurine, β and γ-amino carboxylic acids, ethanolamine and creatinine. Many of these occur in high concentration but their colour yield in the ninhydrin reaction is low. Taurine occurs in moderate amounts and has a colour yield equal to that of free amino acids.*

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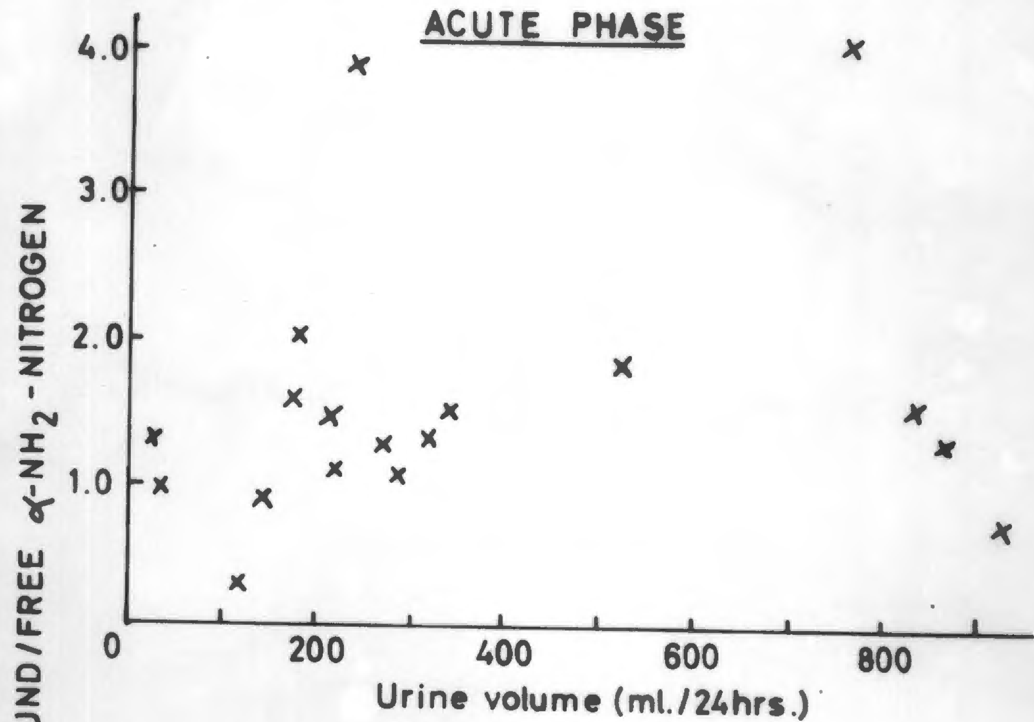
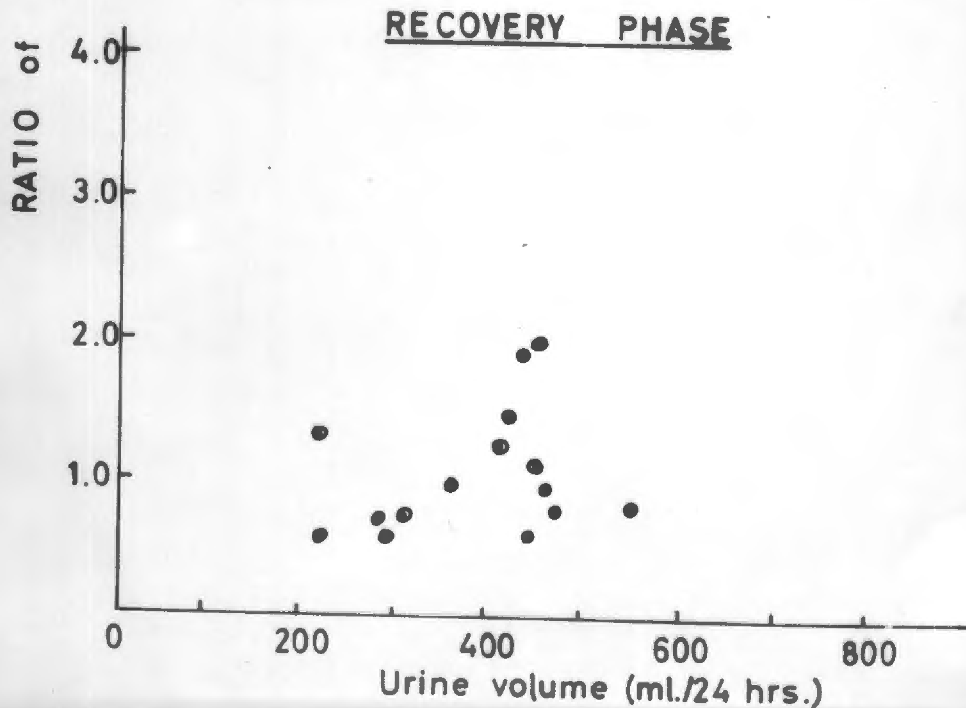
* Exact colour yields of urinary ninhydrin-positive materials have been measured and are reported on more fully in Chap. 9.

FIG. 8 EFFECT OF URINE VOLUME ON AMINO ACID EXCRETION





Bound amino nitrogen cannot for similar reasons be equated with peptide nitrogen.

Before the significance of these findings could be discussed it was necessary to fractionate urinary α-amino nitrogen and to measure the contribution of amino acids to the total amount of ninhydrin-positive material. This prerequisite was the indication for the work reported in the following chapter.

CHAPTER 9.

URINARY EXCRETION OF FREE AND BOUND AMINO ACIDS.

Introduction:

This experimental section describes the methods used to identify and measure the ninhydrin positive material present in the specimens of urine, which was liberated by acid hydrolysis. This corresponds to the bound α-NH_2 nitrogen measured in Chapter 8.

Several procedures are available for measuring amino acids and related compounds in urine and other biological fluids. Microbiological methods are unsuitable since there is evidence that at least some bound forms are utilised in preference to the free.[19]* Paper chromatographic techniques have been well studied and have the merit of simplicity. With simple amino acid mixtures resolution is adequate, but in more complex mixtures found in urine, there is considerable overlap of spots and identification is difficult. Although the sensitivity of paper methods is high, this can be a disadvantage, since overloading reduces resolution. This is particularly likely to occur in urine where the ratio of concentration of glycine to other important imino acids e.g. proline, may be as high as 10^3 to 1.

Ion exchange colum chromatography was the method of choice for the problem. In principle, amino acids are bound to an insoluble ion exchange resin and displaced by buffers of varying pH and molarity. Moore and Stein[209][210] have

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* This objection is not valid for plasma amino acids, since in this fluid negligible bound forms are present.

investigated thoroughly the behaviour of amino acids on Dowex 50 resins of varying cross linkage. This is a sulphonated polystyrene resin with SO_3^- active sites available for cation exchange. Acidic amino acids e.g. taurine, aspartic and glutamic acids will be less retarded than the basic ones (lysine, histidine, arginine) and neutral α-amino acids have intermediate behaviour. Retardation is markedly affected by pH of buffers used for development. Aromatic non-ionic binding retards tyrosine, phenylalanine and tryptophan more than predicted. This effect is minimised at higher temperatures.

Moore and Stein have developed suitable programming of buffer and temperature which gives excellent resolution of amino acids and related compounds present in urine and they have established normal urinary patterns for their procedure.[210]

An improved system has been developed by Spackman Moore and Stein using specially selected chromatographic grade resin, Amberlite CG-120, which has good resolution at high flow rates.[211] This system has been developed into an automatic method by monitoring the effluent of the columns for ninhydrin positive material, which obviates the tedium of collecting fractions.[212] Since it was envisaged that many urine samples would be analysed, both before and after hydrolysis, it was deemed worth while to use this automatic method.

Instrumentation and Methodology.

The method used was that described as the Automatic amino acid analysis procedure of Spackman, Moore and Stein.[212] The basic principle is that of chromatography of amino acid

mixtures on columns of Amberlite cationic exchange resin. The eluate from the column was continuously monitored for ninhydrin reacting material by mixing with ninhydrin, heating and the resulting colour was measured in a flow cell. Results were presented on a strip chart recorded (Fig. 9).

The technique and appratus was reproduced as accurately as possible but certain modifications were necessary owing to expense of compounds.

The improved method of Moore, Spackman and Stein[211] is based on previous methods using Dowex 50 (x8)[209] and Dowex 50 (x4).[210] The improved method has greater resolution and allows faster flow rates, thus speeding up analysis considerably. Neutral and acidic components in urine were separated on a 150 cm column of Amberlite 1R - 120,* whilst basic compounds were eluted from a 50 cm.column. Two buffers, of pH 3.25 and pH 4.25 (0.2M) were used for the 150 cm.column at temperatures of 30°C. and 50°C. respectively. A single buffer pH 4.26 (0.38M), was used for the basic column, and the temperature programme was the same as that for the 150 cm.column.

Resolution of simpler mixtures of amino acids present in protein hydrolysates can be achieved at a single temperature of 50°C. Neutral and acidic amino acids were separated on the 150 cm, column with the same two buffers as for urine. Ammonia and the three basic amino acids, lysine, histidine and arginine were separated on a 15 cm.column of resin by a pH 5.28 (0.2M) buffer.

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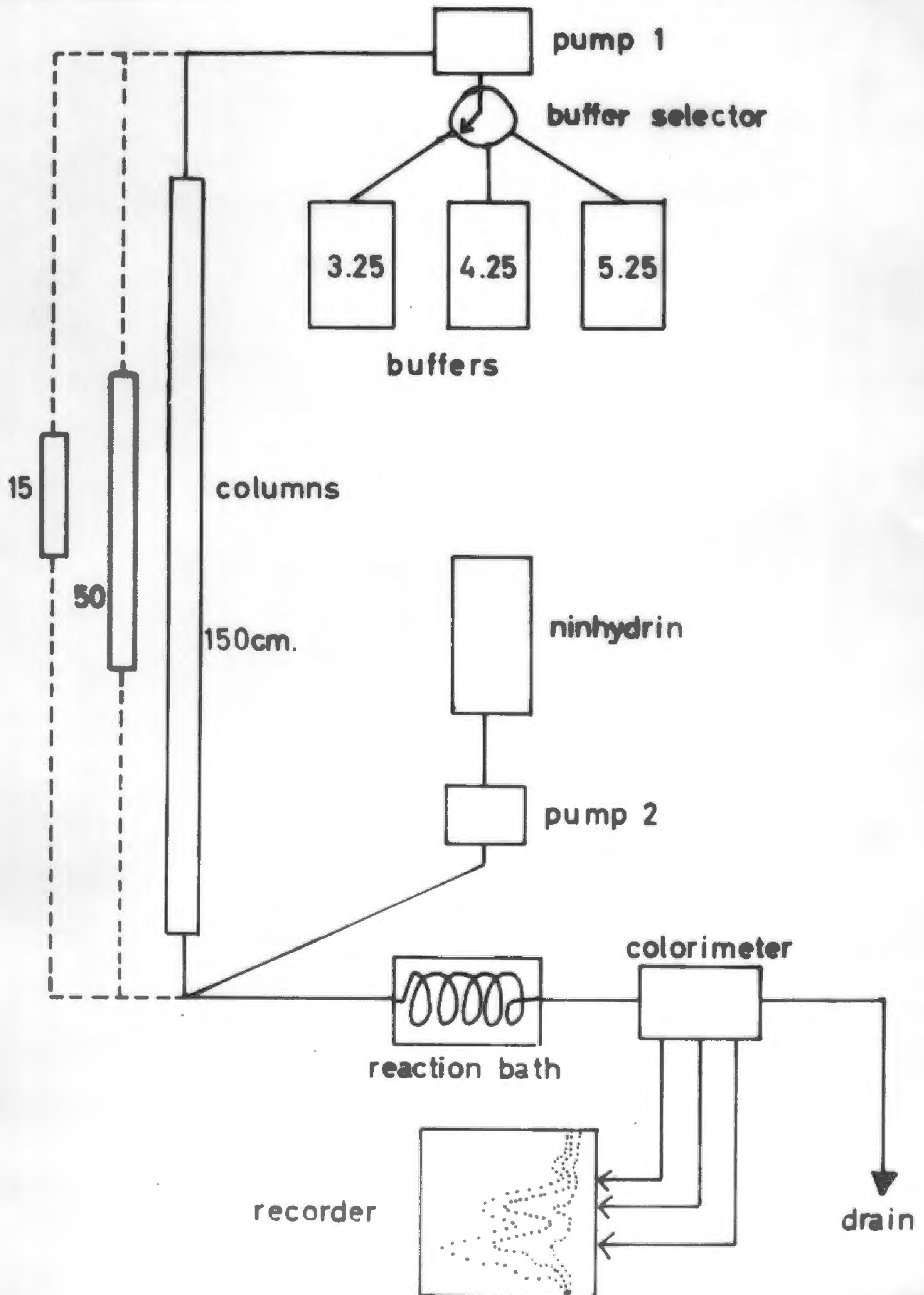
* Sulphonated polystyrene resin (RSO_3^-) cross linked with 8 - 10% divinyl benzene.

FIG.9 FLOW DIAGRAM OF THE AUTOMATIC AMINO ACID ANALYSER



Preparation of Resin and Reagents.

Resin: In order to obtain high flow rates with reasonable pressure, whilst maintaining high resolving power, particle size of the resin had to be carefully controlled.

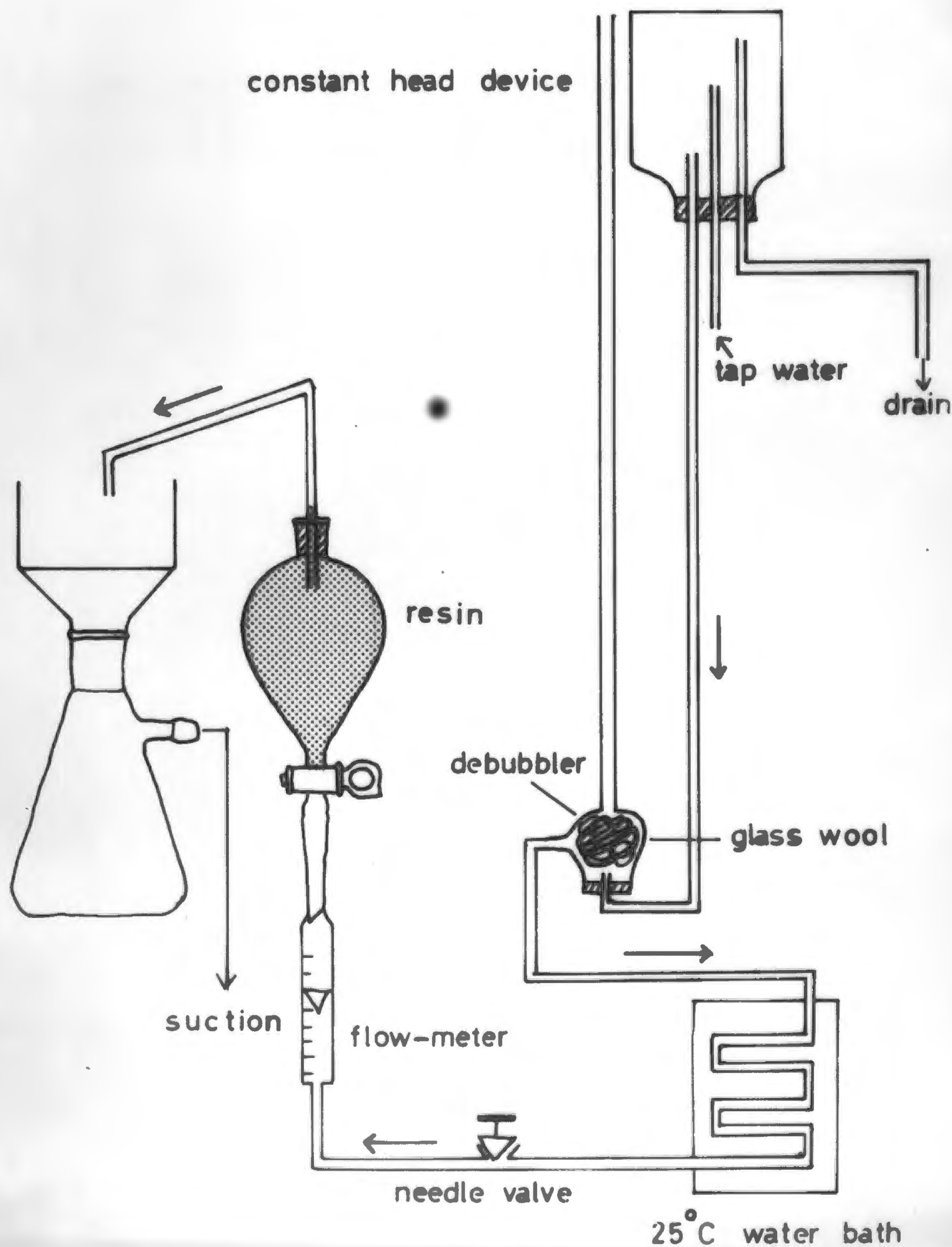
Amberlite 1R - 120 (Rohm and Haas Co.) was available in three grades. The finest grade - type III (400 - 600 mesh) yielded suitable material for packing columns.

One pound of Amberlite, 1R - 120 Type III (Batch No. 772567, Rohm and Haas, Philladelphia) was processed. The resin was transferred into a beaker and stirred into a slurry with 10 litres of distilled water. Resin trapped in foam on the surface was released with a few drops of acetone. The suspension was allowed to settle for 6 hours, after which the 'fines' remaining in suspension were removed by suction. After three such settlings, the remaining resin was treated on a Buchner funnel with 3 - 4 litres of 4N HCl. After washing until the effluent was neutral, the resin was suspended in 2 litres of 2N NaOH and heated to 80 - 90°C for 1 hour. The resin (now in the Na^+ form) was washed until neutral, and stored in 0.2N NaOH until required. Fractionation was achieved by the hydraulic method of Hamilton[213]. Particle diameter of 40 ± 7 u and 25 - 30 µ were used for the 150 and 50 cm. columns respectively. The resin, suspended in tap water, was placed in the 2 litre separatory funnel into the bottom of which flowed tap water at constant temperature. A uniform flow rate was achieved by a constant head device and needle valve, and the flow rate was read on a calibrated flow meter. The needle valve was opened fully until the separatory funnel was nearly full and then adjusted to give a flow of 56 ml./minute. A cork and overflow tube were inserted in the funnel and the stream, containing particles less than 25 µ in diameter was allowed to run to waste. The flow was continued until the waste stream and liquid in the funnel above the resin was clear (± 2 hours). The flow rate was then increased to 110 ml. per minute and the resin in the effluent stream, of particle size 25 - 30 µ, filtered off on a Buchner funnel. This fraction (labelled 50 - 110) was used for the 50 and 15 cm. columns. When the effluent was clear, the flow rate was increased to 280 ml./min. This 110 - 280 fraction, (± 40 µ diameter particles) was used to pour the 150 cm. columns. The resin remaining in the funnel could be used for columns in the manual method. The 50 - 110 and 110 - 280 fractions were refractionated at the appropriate flow rates to ensure uniformity of particle size. The resin was stored at 4°C in 0.2N NaOH.

Preparation of Ion exchange columns.

Glass chromatographic clumns were manufactured according to specification by the Scientific Glass Blowing Co., Cape Town. These were of 0.9 cm. internal diameter pyrex glass tubing and 160 cm, 60 and 25 cm. in length, from the lower

FIG. 10 HAMILTON'S HYDRAULIC FRACTIONATOR FOR RESINS



sintered glass disc to an upper B12/S cup joint. The glass water-circulating jackets were sealed on to the columns and extended from immediately below the sintered discs to ± 7 cm below the cup joints. Below the sintered discs, the columns were narrowed and sealed to a capillary B12/S ball joint. Before use, the columns were cleaned with alcoholic KOH and rinsed with distilled water until the film on the tube sides was even.

The fractionated resin, in 0.2N NaOH, was filtered on a Buchner funnel, washed with water and recycled with 4N HCL and 2N NaOH, to remove any metal ions which had been absorbed onto the resin from tap water. After removing the excess alkali by washing, the resin was equilibrated with pH 4.25 (0.2N) buffer (Table 14) - (containing no BRIJ or Thiodiglycol).

The resin was suspended in 6 - 8 volumes of buffer and settled 3 - 4 times, or until the supernatant was clear after 20 minutes settling, to remove fine particles which were invariable generated during manipulations subsequent to hydraulic fractionation.

Suspensions of resin in 2 volumes of this buffer per volume of settled resin were used to pour the columns. One hundred ml. of settled resin was sufficient for the 150 cm. column and proportionately less for the 50 and 15 cm.columns.

The long (150 cm) column was poured in five sections (20 ml. settled resin suspended in 40 ml. buffer). The glass column was clamped in the vertical position and checked with a spirit level. The first section was poured into the column with the outlet closed and allowed to settle under gravity, until a column, 2 - 4 cm in height had formed. The outlet was then opened and air pressure of 4 lbs. p.s.i. applied to pack the resin. Care was taken not to allow the column to run dry. When this did occur the column had to be repacked. The second section was poured on top of the first when all but 2 - 3 cm of buffer had passed through. Packing pressure for the second and subsequent sections was raised to 10 lbs. p.s.i. The column was packed initially to a height of 155 cm. to allow for subsidence with use. The 50 cm.column was packed similarly in 2 sections and the 15 cm.column in one section, in 0.38M pH 4.26 and 0.35M, pH 5.28 buffers respectively.

Buffers:

The sodium citrate buffers were prepared with the composition shown in table 14. Solutions were made up in 20 litre batches in 25 litre 'polythene' aspirator bottles with 'polythene' and rubber caps. These buffers were allowed to stand for several days before the pH was finally adjusted either with 50% NaOH or Conc. HCl (41% w/v).

Carbon dioxide free, sodium hydroxide solution, was

prepared by boiling 2 litres of distilled water containing 1.0 ml. of conc. HCl, cooling rapidly, and then adding 23 ml. of saturated (70% w/v) sodium hydroxide solution. This was stored under nitrogen in an aspirator bottle.

Boiled pH 3.25 buffer was stored under liquid paraffin and this and the 0.2N NaOH solution were placed on a shelf near the top of the 150 cm column for use during regeneration (fig. 21).

Ninhydrin Reagent.

The 'Ninhydrin' reagent contained triketo-hydrindene hydrate (ninhydrin) and its reduced form, hydrindantin, in a solvent mixture of 75% v/v methyl cellosolve and 25% 4M pH 5.1 sodium acetate buffer. Hydrindantin was formed from ninhydrin by stannous chloride during preparation of the reagent. Special precautions were taken to ensure an oxygen-free solvent.

The buffer was prepared in 5L. batches by adding 500 ml. of glacial acetic acid to 2720 g. of sodium acetate ($CH_3COONa.3H_2O$) in 3 - 4 litres of water and making up to volume. The pH was checked, but never required correction.

Preparation: (2 litre batch)

Fifteen hundred ml. of peroxide-free* methoxy-ethanol (methyl-cellosolve)** and 250 ml. of buffer were mixed in a 2 litre aspirator bottle, the bottom outlet of which was connected to a nitrogen supply*** by means of a B10 male joint, via a concentrated acqueous citric acid trap to remove any ammonia. Nitrogen was allowed to bubble into the solvent vigorously, whilst stirring was accomplished with a teflon coated magnetic bar. After 20 minutes, 40 grams of Ninhydrin****

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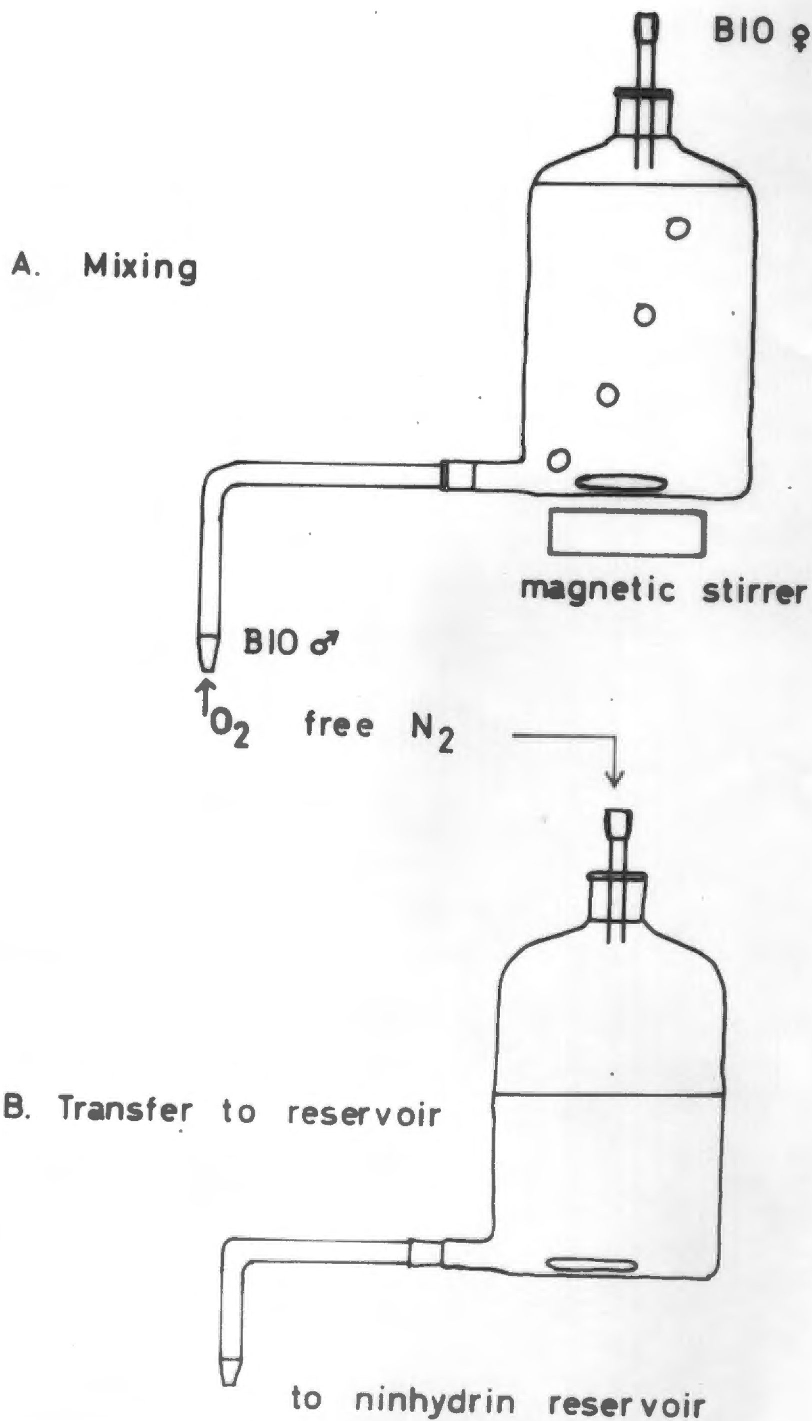
* Tested against KI solution.

** Although all brands of commercial cellosolve tested were peroxide free, many contained alarming amounts of ninhydrin positive material which varied amongst batches.

*** Commercial Nitrogen, (African Oxygen and Acetyline Co. Ltd.) was used.

**** B.D.H. reagent and Merck proved satisfactory. These gave slightly different colour yields, which, however, were checked for each batch.

FIG. 11 PREPARATION OF THE NINHYDRIN REAGENT



and 0.800 grams of stannous chloride, previously weighed out into tared vessels, were added separately and stirring and nitrogen flow maintained until the solids had dissolved. The reagent had a clear port-wine colour.

The nitrogen supply was then shut off and the aspirator bottle closed with a rubber bung containing a B10 female joint. The reagent could then be transferred to the ninhydrin reservoir via the B10 female joint to the inlet of the reservoir, opening the tap and applying slight positive nitrogen pressure to the reagent bottle. Transfer of reagent usually took 3 - 4 minutes.

The reagent was kept for up to 6 weeks in the reservoir bottle in a dark cupboard, without any fall-off in colour yield being detectable.

Buffer reservoirs consisted of 5 litre glass aspirator bottles for the 150 and 50 cm. columns, and a 2 litre bottle for the 15 cm column. The inlets were loosely covered with Petri dishes and the outlets were glass taps, connected by tygon tubing to the de-aerators. The reservoirs were placed 30 cm above the de-aerator flask to maintain a head of pressure against resistance in the glass wool filters in the latter.

Heating bath and de-aerator.

The de-aerators and coil for colour development were kept at 100°C in a 1 litre flask, covered by a glass lid with five B19 ground-glass sockets. Four of these housed the de-aerators whilst the fifth had a 40 cm. water jacketed condensor, (fig. 12). The reaction flask was heated in a heating mantle, and kept boiling gently by a hot wire type thermostat (electrothermal energy controller). The de-aerators (Scientific glass blowers) were made according to the design of Moore and Stein and were packed with 1 cm. of glass wool (fig. 13). With continued use this packing became clogged and was renewed every 3 - 6 months.

Buffer Selector and Change over Valve.

The outlets from the four buffer de-aerators were connected to the selector via 1/8" I.D. tygon tubing. Two three-way stop-cocks were used to effect buffer selection; either (a) pH 5.28, (b) pH 3.25/4.25 or (c) pH 4.26 for the 15 cm, 150 cm. and 50 cm. columns respectively, (fig. 15). Buffer change, when suing the 150 cm column, was accomplished by means of a solenoid operated valve (Automatic Switch Co., N.J., U.S.A.) connected to a preset timing device ('Gunfire' type GF2). The timer was also connected to change simultaneously the column temperature when the 30° - 50° system was used (Fig. 16 & 17). Some difficulty was experienced with the commercially available stainless steel solenoid valve. After approximately 12 months' use, corrosion affected the spring mechanism and the teflon

FIG. 12 HEATING BATH

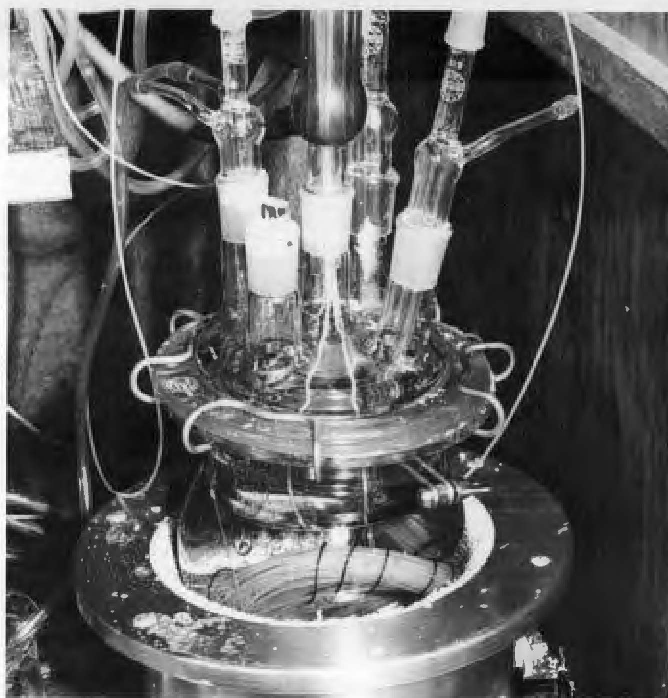


FIG. 13 BUFFER DEAERATOR

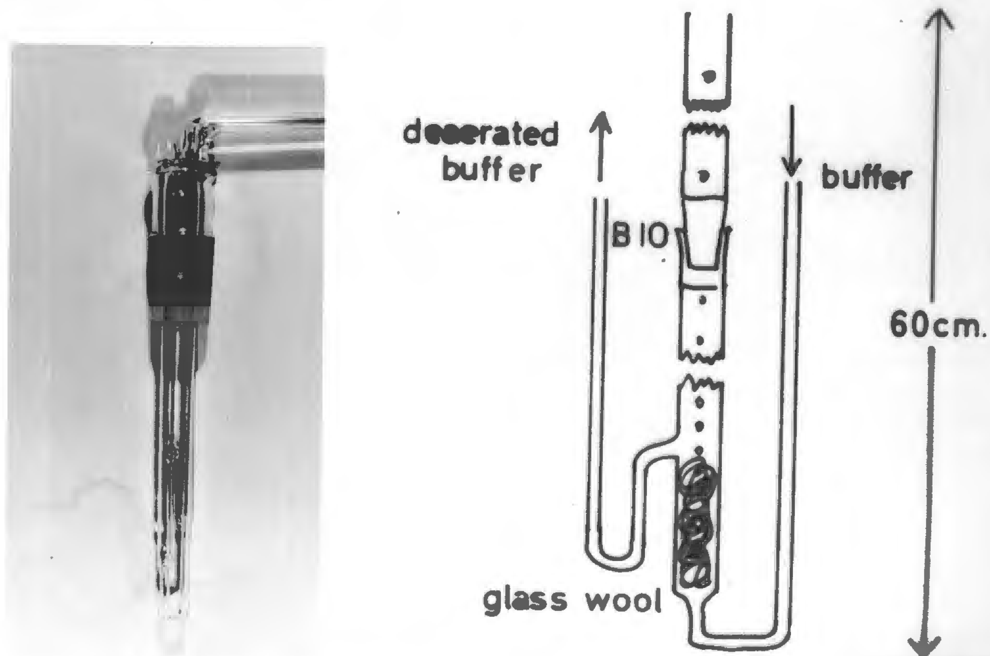


FIG. 14 BUFFER CHANGE OVER VALVE

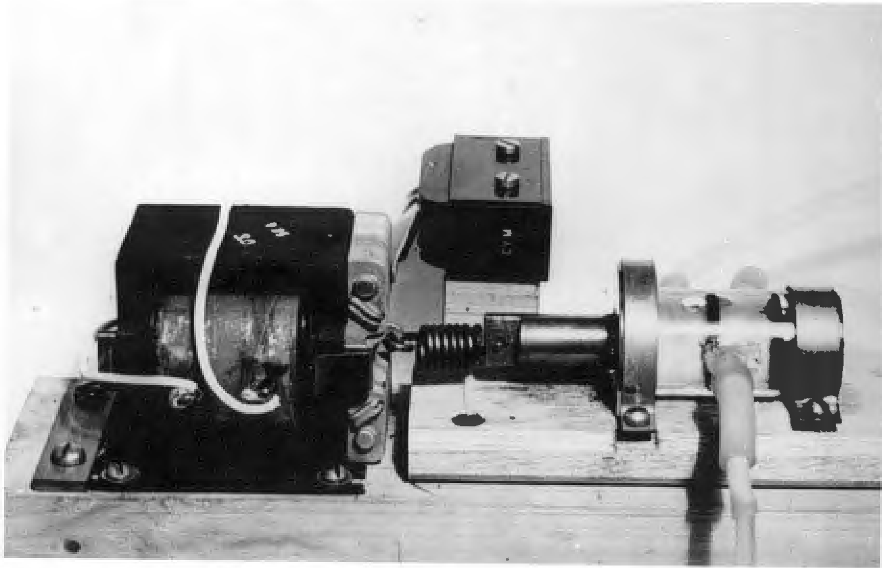
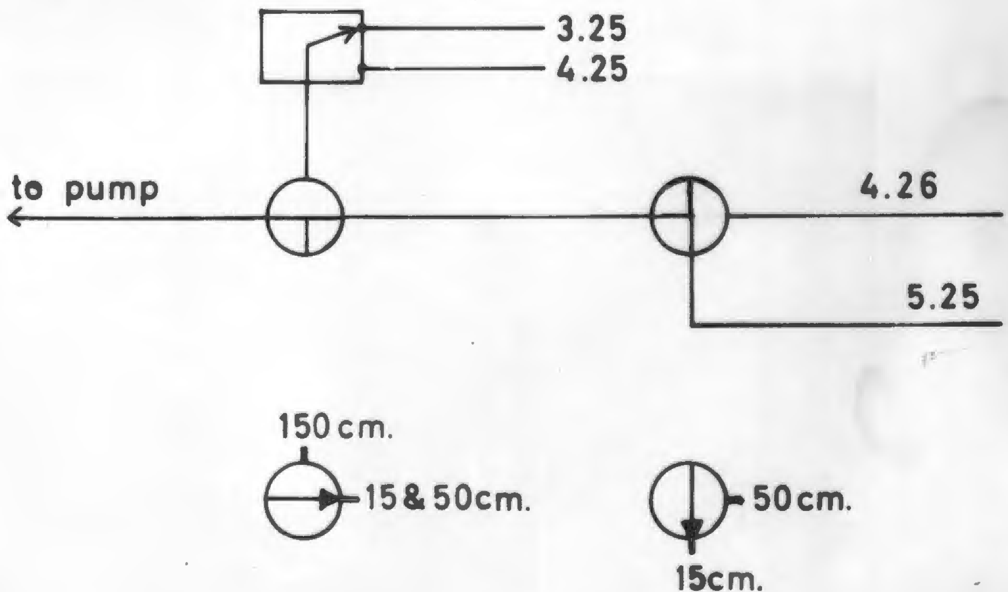


FIG. 15 SCHEMATIC DIAGRAM OF BUFFER SELECTOR MANIFOLD



needle valve became deformed, The presence of phenol in the acidic buffers may have been the cause of this. During later experiments a change over valve constructed in the departmental workshops from perspex with rubber gasket rings proved suitable (fig. 14).

Temperature control during Chromatography.

A five litre beaker containing a 500 watt immersion heater and two mercury contact thermometers set at 30°C and 50°C respectively, controlled column temperature. The thermometers, selected via the temperature programmer (fig. 17) were connected to the heater via a plunger type mercury relay. No water bath stirrer was used as agitation was sufficient from a centrifugal pump (Stuart-Turner Type 10) to maintain the temperature of the water jackets to within ± 0.5°C. The effluent from the circulating pump was connected to the bottom of each water jacket via a four-way glass cactus and rubber tubing. Return to the bath from the columns was by separate tubing leads. Pinch cocks at the inlet of the water jackets allowed circulation to be diverted to the column which was in use.

Ninhydrin Reservoir.

The ninhydrin solution was kept under nitrogen in a two litre aspirator bottle (no tap) which was sealed by a rubber bung with air inlet and tap, with a B10 female joint, for filling. Air was excluded by means of a water seal in two Winchester bottles (fig. 18).* The aspirator outlet was connected to the ninhydrin pump by teflon tubing, but short shoulder connections of tygon and rubber could not be avoided in this and in the delivery of ninhydrin solution from the pump to the manifold at the column outlet. **

Pumps.

A double-headed pump (one head each for the ninhydrin reagent and for the eluting buffers) was obtained from

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* In their original article Moore & Stein (1958) used liquid paraffin. The viscosity of the oil slows down the filling process. Oxygen is less soluble in water than in mineral oil.

** Teflon is insoluble in the 2 : 1 (v/v) cellosolve buffer mixture employed in the ninhydrin reagent. During stasis of the reagent, however, some material which was eluted or dissolved from tygon and rubber precipitated when the ninhydrin and buffer streams mixed. This caused an elevated baseline, but by reducing the surfaces of the connecting shoulders to a minimum, the baseline shift did not extend past the first 15 - 20 ml. of buffer on the elution (fig. 26).

FIG. 16 BUFFER AND TEMPERATURE PROGRAMMER

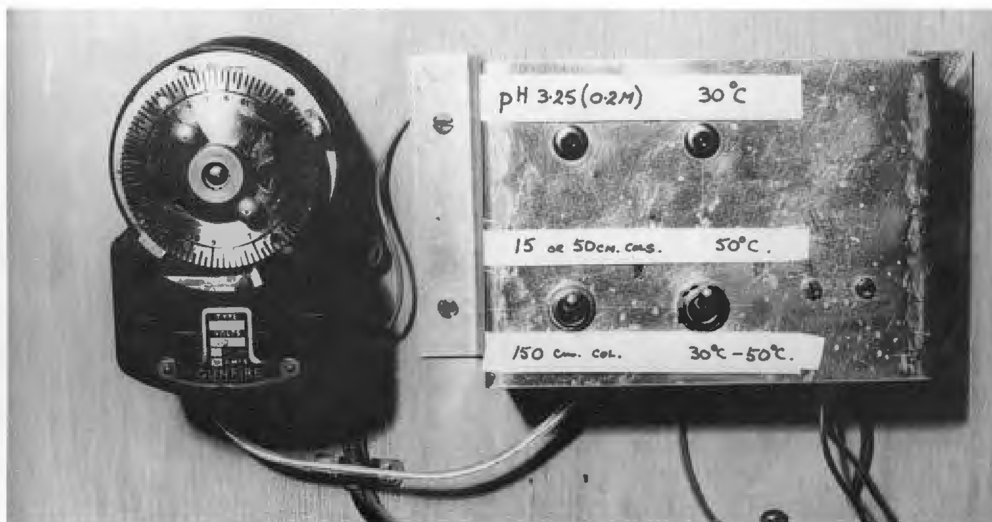
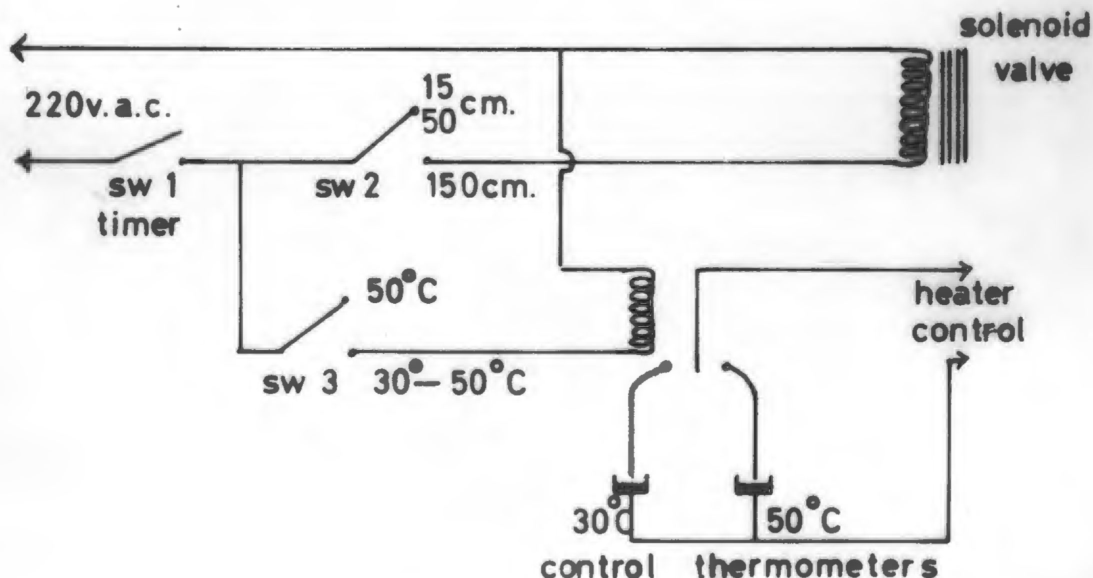


FIG. 17 CIRCUIT DIAGRAM



Distillers Corporation Ltd (Lewes, England). Two No. 1 heads (0 - 100 ml.) were used. This pump was driven by synchronous motor, and all metal parts were of stainless steel. Entrance and exit was by double non-return ball valves. Flow rate was adjustable by means of a micrometer screw, which limited travel of the piston. Since flow rate depends on back pressure, the scale 0 - 100 was arbitrary, and calibration was checked. Packing glands and stainless steel pistons were renewed every ± 3 and ± 6 months respectively.

Pressure monitoring.

Pressure gauges (0 - 100 lbs. p.s.i.) were connected by glass T-pieces to the buffer and ninhydrin feed lines. The connecting pieces were half filled with liquid paraffin at the gauge end, to prevent corrosion.

Heating Coil.

One hundred feet of AWG gauge 22 teflon tubing (nominal I.D. = 0.027 inch and O.D. = 0.051 inch) was obtained in a single piece. A length, calibrated by 15.0 ml. of diluted (1 : 10) ink, was scramble wound in a narrow pile around a 12 cm. former. The coil was then slipped off the former and kept in place by loosely wrapping with soft copper wire. Two feet of tubing was left at each end of the calibrated length to allow connections to be made. The coil was placed in the heating bath and the ends led out via the central condensor.

Flow Monitor.

Flow rate of effluent from the columns and ninhydrin stream was measured in a calibrated length of 2 mm (I.D.) capillary glass tubing. Two marks, 21.3 cm. apart were made on the tubing. A T-piece, with a rubber bulb on the side arm, connected to the proximal end was used to inject an air bubble into the stream, and its passage between the calibration marks was timed with a stop watch. During constant flow rate conditions, (± 45 ml/hr) flow through the tube was measured volumetrically over a period of 60.0 minutes, during which time 10 timings were made. The average of these (50.7 secs.) corresponded to a flow rate of 44.2 ml/hr. Corresponding readings for flow rates from 25.0 to 50.9 ml/hr were calculated and tabulated.

Flow Cell and Colorimeter.

The flow colorimeter was used to convert changes in optical density of the mixed buffer and ninhydrin streams into variations in electrical current, which could be conveniently recorded.

FIG. 18 NINHYDRIN RESERVOIR (oxygen free)

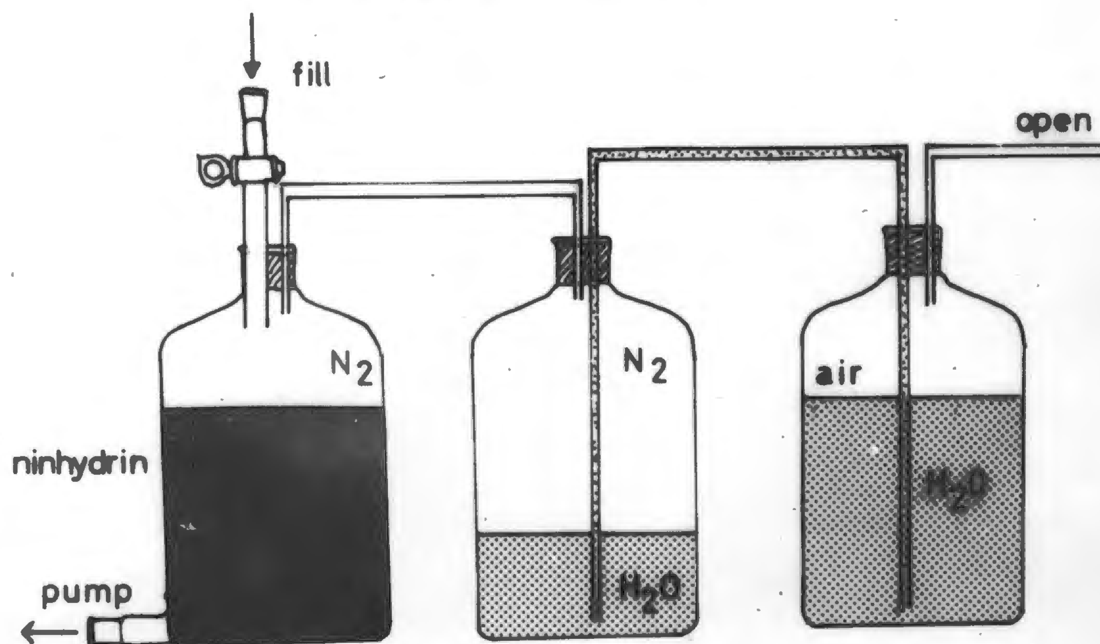
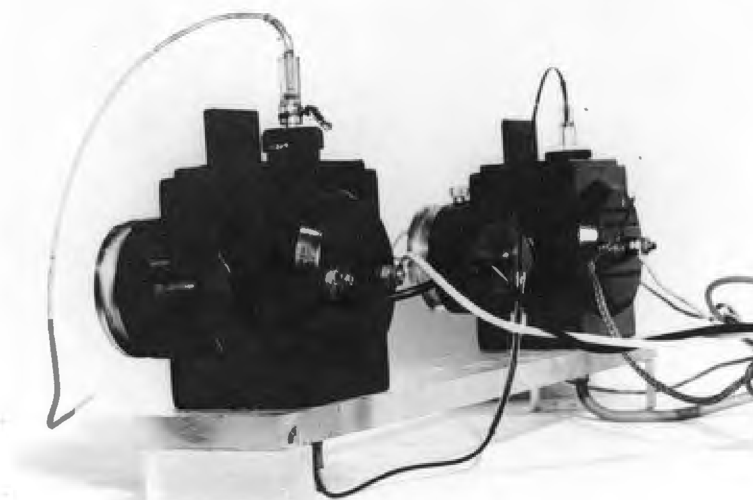


FIG. 19 FLOW COLORIMETER



Light source, slit, housing and selenium photocell were conveniently retrieved from an "EEL" photocolorimeter. The unit was removed from the instrument and mounted on a perspex base (fig. 19). A perspex adapter with a central 0.4 cm. diameter hole for the flow cell and a vertical slit was aligned with the light path and inserted into the test-tube aperture. Two such units were available and a third was constructed on similar lines. The flow cells were pieces of thin-walled glass tubing of 2.8 mm. internal diameter. These were centred in the light path. The three flow cells were connected in series and functioned as follows:

Cell no.	Wave length. mu	Filter.	Effective light path.
1	± 570	EEL No. OGR1	2.8 mm.
2	± 570	EEL No. OGR1	0.8 mm.
3	± 440	EEL No. 622	2.8 mm.

The effective light path of cell No. 2 was reduced by placing a short length of solid glass rod of 2.10 mm. external diameter inside the 2.8 mm. internal diameter tube and sealing it in place by heating the outer tube.

The light source for each unit was a 6-volt 0.3 amp. screw base bulb which was excited from the a.c. mains supply via a constant voltage transformer (Advance Instruments).

Recording and Measurement of signals from flow cells.

During the initial stages of development of the instrument, a single flow cell (570 mμ - 2.8 mm) was used. The output from this photocell was connected to a 10 m.v. potentiometric single channel strip chart recorder (VARIAN) via a 5,000 Ω potentiometer (fig. 20). Most of the observations were, however, made using three flow cells which fed a three-channel chopper bar galvanometric recorder (Jounes). With the filters in place and distilled water in the flow cells, the average output of the photocells was 8 μa. The recorder required 250 μa for full scale deflection. In order to achieve this current flow, a direct current transistorised chopper type amplifier was constructed. A 10 volt accumulator or 9 volt dry cell were tried initially to supply the amplifier, but a mains fed, transistor regulated power unit gave the most stable baseline (see Appendix).

Hydraulic Connections and Assembly.

Connections for all buffer feed lines was by 1/8" (i.d.) tygon tubing which was wired onto the glass and stainless steel nipples. The ground-glass cup and connection to the

FIG. 20 CONNECTION TO POTENTIOMETRIC RECORDER

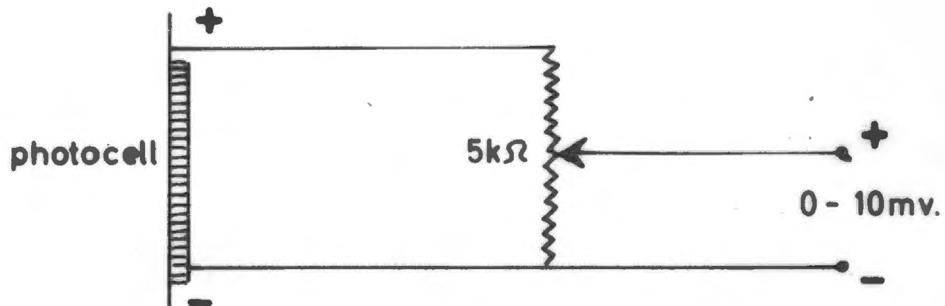
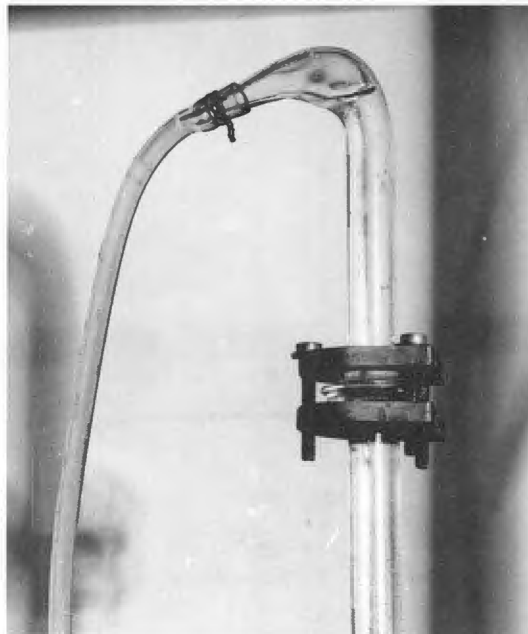


FIG. 21 HIGH PRESSURE CLAMP



top of the column was secured by two U-shaped brass plates which were secured with 4 BA bolts. A thin film of vaseline ensured a leak prood connection. The whole system was tested to 75 lbs. pressure (fig. 21).

The bottom ball and socket joint from the column was secured with a spring clip, and connection to the heating manifold was by tygon sleeves. Waste from the flow monitor was taken to a drain 80 cm. above the heating coil. This slight positive pressure prevented bubble formation in the coil and photometer.

The column were fixed to wall brackets and other parts of the instrument were conveniently located in cupboards below the bench (fig. 22).

A shelf at the level of the top of the 150 cm. column supported pH 3.25 buffer and 0.2N NaOH for regeneration of the 150 cm. column. The 3.25 buffer was boiled and stored under oil. The soda solution was CO_2 free. Both bottles were kept at 5 lbs. p.s.i. nitrogen pressure, and had outlet connections for attachment to the column.

Operating Procedure.

Table 15.

Material	Amino Acids	Column cm.	Buffer(s) pH	Temp.	Change at hrs.	ml.
Protein Hydrolysate	Acidic & Neutral	150	3.25/4.25	50	8.5	255
	Basic	15	5.28	50	-	-
Urine	Acidic & Neutral	150	3.25/4.25	30/50	10.5	315
	Basic	50	4.26	30/50	11.5	345

a. Protein Hydrolysates (commenced 9 a.m.)

The sample (pH 2.2) was applied carefully to the top of both 150 and 15 cm. columns with a Pasteur pipette, taking care not to disturb the resin surface. Nitrogen pressure was used to force the sample into the resin (± 10 minutes per ml. of sample). The walls of the tube were washed with 3 further 0.5

Fig.22. COMPOSITE PHOTOGRAPH OF THE AUTOMATIC AMINO ACID ANALYSER.

aliquots of pH 2.2 buffer.

During the application of samples the pH 5.25 buffer was pumped through the feed lines and then connected to the 15 cm column.

The flow rate was adjusted to 30.0 ml. per hour. At 50°C this flow rate produced a pressure of 10 lbs. After 40 minutes* the ninhydrin pump (15.0 ml. per hour) was started and 30 minutes later, the recorder was switched on and the base line for each of the three channels set at 90% transmission by means of slit adjustments. The pressure of the ninhydrin reagent was usually 3 - 4 lbs.

The 150 cm. column was closed off till used.

Arginine was eluted by 3.30 p.m. The ninhydrin pump and recorder were then switched off and after disconnecting the buffer line, this was washed out by pumping through pH 3.25 buffer for 20 minutes. At 5 p.m. the pH 3.25 buffer was connected to the 150 cm.column and the ninhydrin pump started immediately. The pressure of the buffer varied between 35 and 50 lbs. p.s.i. with the 150 cm.column. After 30 minutes the recorder was started and base lines adjusted if necessary. The buffer change over was set for 8.5 hours. Phenylalanine was eluted before 9 a.m. on the following morning. It was thus possible to do a complete analysis in 24 hours.

Urine.

The general procedure was similar but programming of buffer and temperature changes were as in Table 13. During the basic run on the 50 cm. colum there was no delay in starting the ninhydrin pump. Each column required 24 hours for completion, therefore single urine analysis took 48 hours.

Integration of the effluent curves.

The peaks on the effluent curve, corresponding to amino acids and other components were integrated by measuring the area under the curve, which was equivalent to the total amount of ninhydrin complex produced. The peaks were regarded as equilateral triangles of area ½ (base x height), or more conveniently, nett height x width at half height.

Nett height (H) was easily read off the scale as the difference between the baseline and peak height. A horizontal

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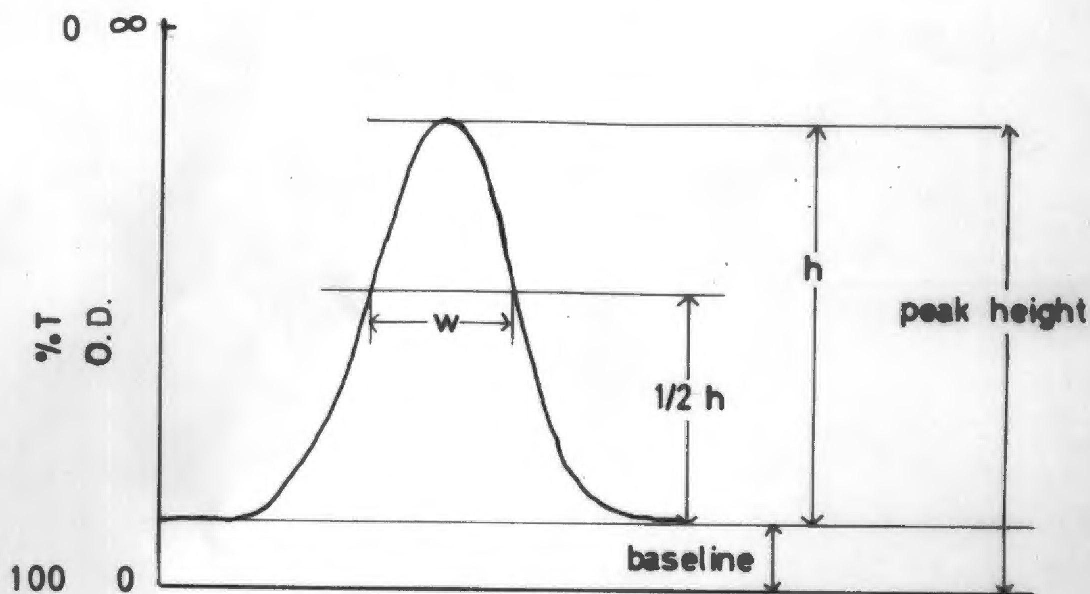
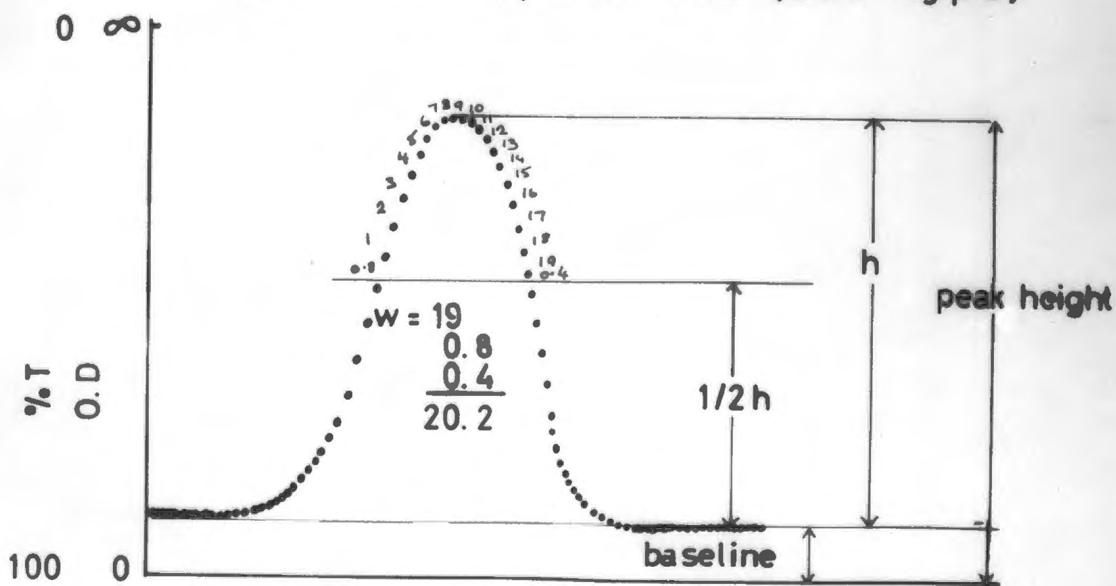
* If the ninhydrin pump is started immediately, the neutral and acidic components, which are eluted as a single large 'peak', precipitate the ninhydrin reagent in the heating coil.

FIG. 23 INTEGRATION METHODS

A. Continuous line



B. Interrupted line (dot type)



line was drawn through the peak at the calculated half height.* The width at half height was measured by either calipers (in m.m.) with the continuous line potentiometric recorder or the number of dots and fractions of a dot space were counted above half height in peaks produced by the chopper bar recorder (fig. 23 - interrupted line).

Unfortunately no chart paper for the recorders having a logarighmic scale was available and percentage transmission (0 - 100) had to be converted to optical density units. A series of mixtures of ninhydrin and glycine was made to cover the most used optical density range and were accurately measured at 570mμ in a Beckman DU spectrophotometer. The solutions were injected into the flow cell and the recorder reading corresponding to the known optical density at 570 mμ was noted. The results are shown in fig. 24. There was some deviation from the theoretical curve and this discrepancy included errors due to stray light refraction and non-linearity of the photocell, amplifier and recorder. A table derived from the observed readings was drawn up from the graph for convenience during calculations (see Appendix).

In order to give more manageable values for H and W, the width (dots or mm) was multiplied by 10. Integration was most conveniently performed on duplicated sheets (Appendix).

Standardisation:

Two sets of standards were prepared. One contained the 17 amino acids and NH_3, found in acid hydrolysates of protein (ca 0.5 μmoles of each/ml in 0.1% HCl) and the other was a composite standard which contained in addition urea, taurine, asparagine, β-alanine, etc., which are found in urine. The purity of standards was checked by means of ascending paper chromatography in butanol/acetic acid/water solvent (fig. 28).

Constants (C) for each standard substance were calculated and the results are given in tables 16 and 17 for both recorders, where $C = \frac{H \times W}{\mu \text{ moles}}$

The constants obtained by Moore and Stein are given for comparison. Changes in flow rate, length of heating coil and different batches of ninhydrin gave slightly different colour values. For each such change, constants were redetermined.
A typical standard curve is shown in
(a) in system for protein hydrolysis (fig. 25) and
(b) in system for urine (figs. 26 and 27).

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* Either base line + ½ nett height or ½ (base line + peak height).

FIG. 24 CALIBRATION OF FLOW CELL

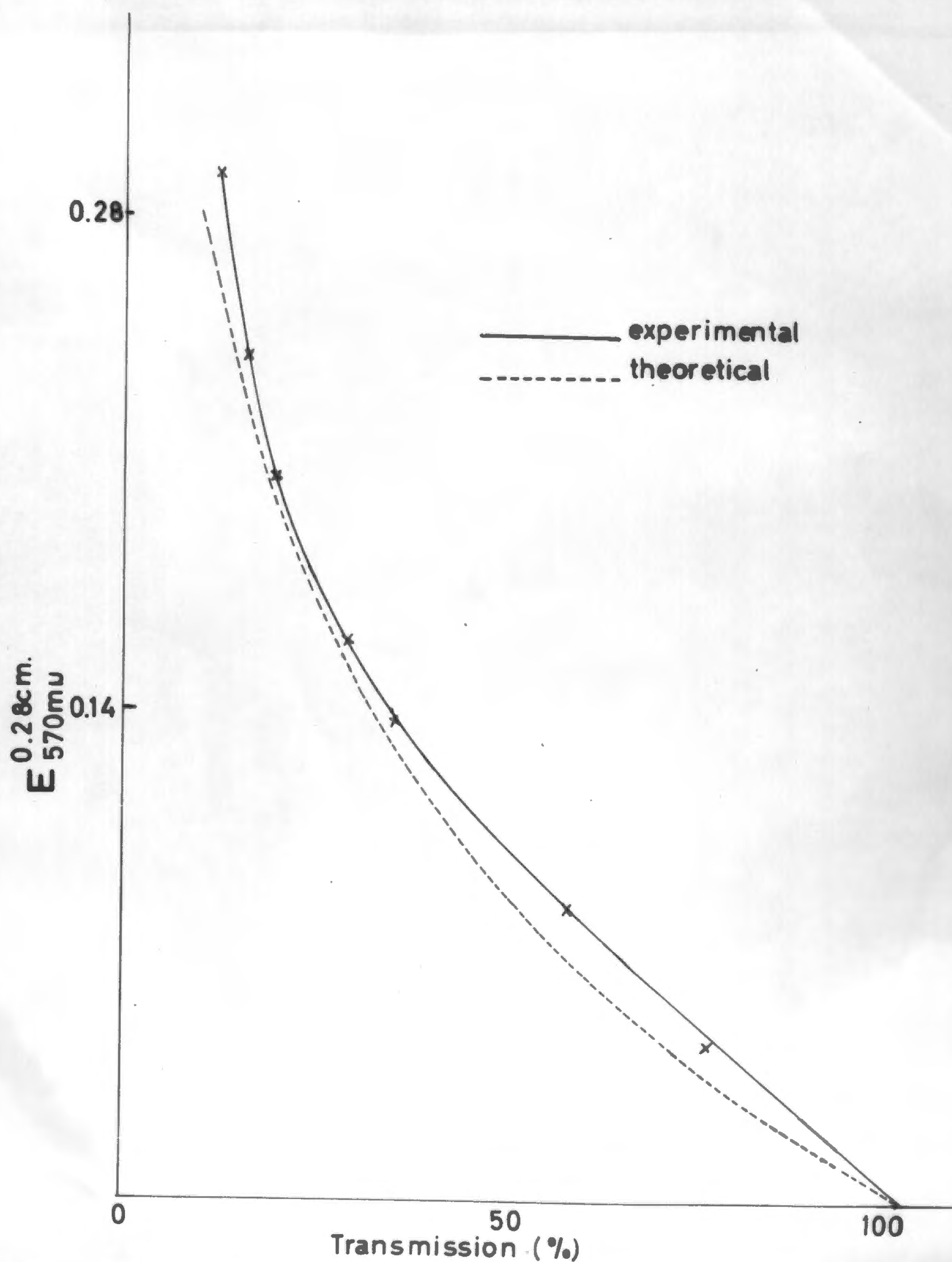


Table 16.

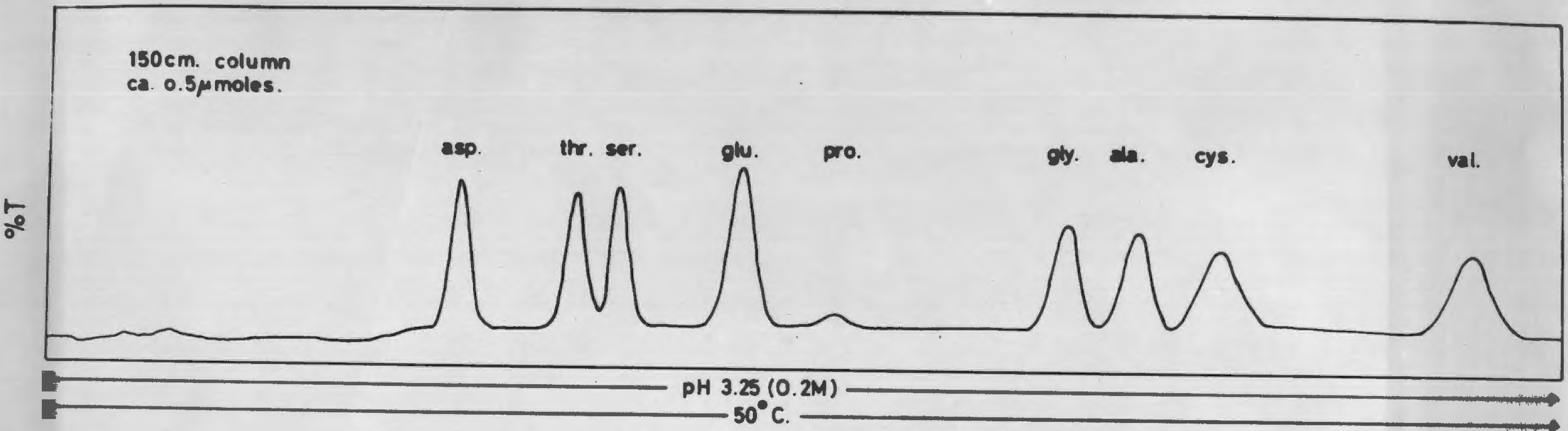
Constants for Protein Hydrolysate Standard*

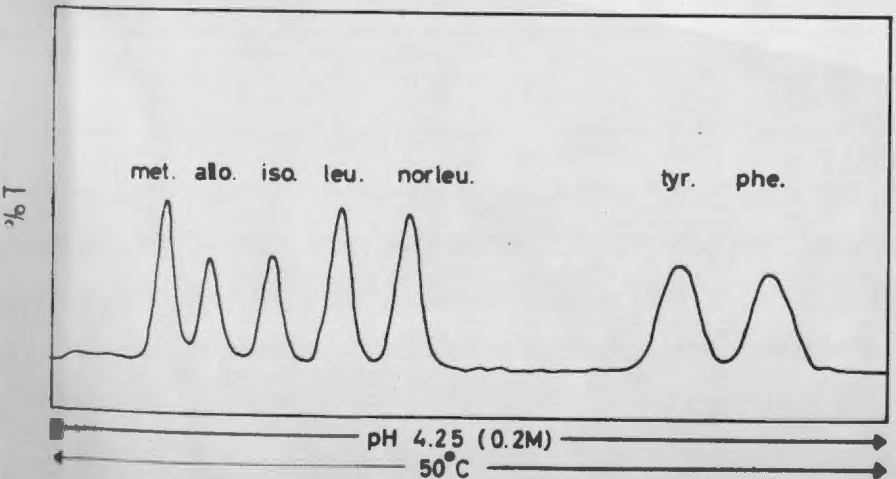
Amino Acid	Derived Constants (Galv.)	Constants(1) (M & S)	Ratio. **
Aspartic acid	49.6	25.2	1.96
Urea	51.8	25.4	2.04
Serine	53.8	26.1	2.06
Glutamic acid	51.6	26.3	1.96
Proline	8.5	6.37	1.36
Glycine	48.6	25.6	1.90
Alanine	50.8	26.0	1.96
Cystine	50.2	28.8	1.74
Valine	50.0	27.2	1.84
Methionine	45.2	25.6	1.76
Isolencine	56.0	27.3	2.06
Leucine	53.8	27.6	1.96
Tyrosine	52.8	27.0	1.96
Phenylalanine	51.0	26.7	1.92
Lysine	64.4	28.6	2.26
Histidine	60.4	26.5	2.28
Arginine	47.8	25.4	1.88

* As determined on Chopper bar galvonometer recorder.

** Different amino acids have slightly different maximum absorption. The simple filters used have much wider pass bands than the barrier type dielectric filters used by Spackman, Moore and Stein.

FIG. 25 STANDARD CURVES FOR PROTEIN HYDROLYSATES





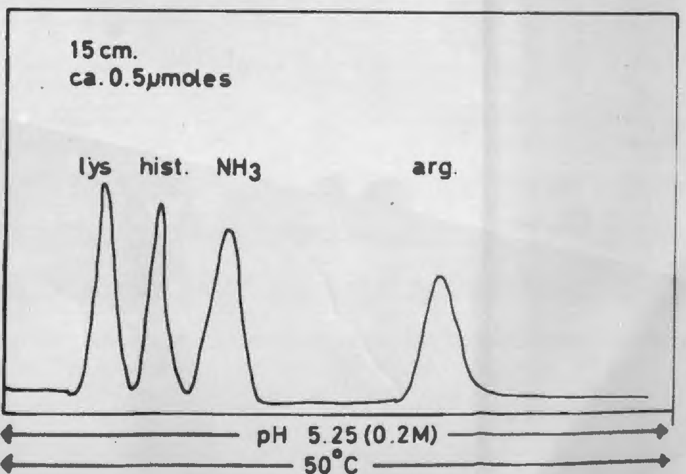
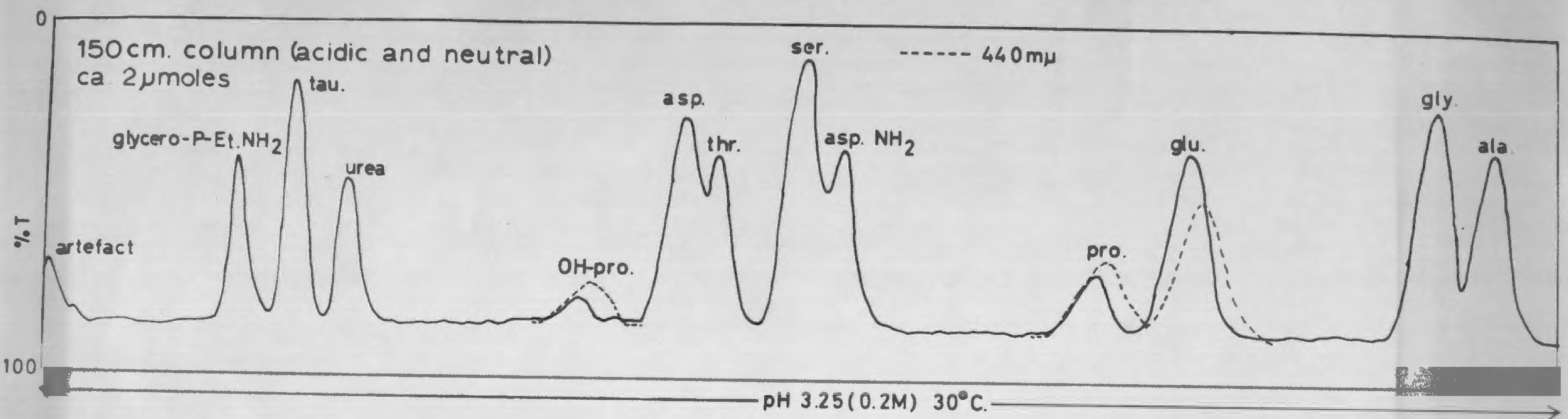


FIG. 26 STANDARD CURVE FOR BIOLOGICAL FLUIDS



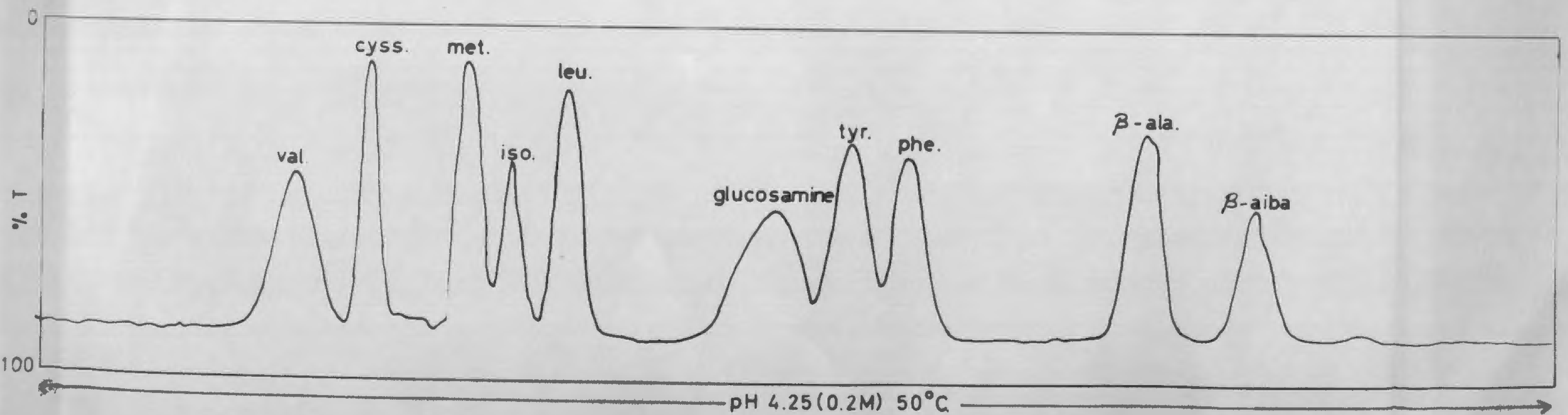
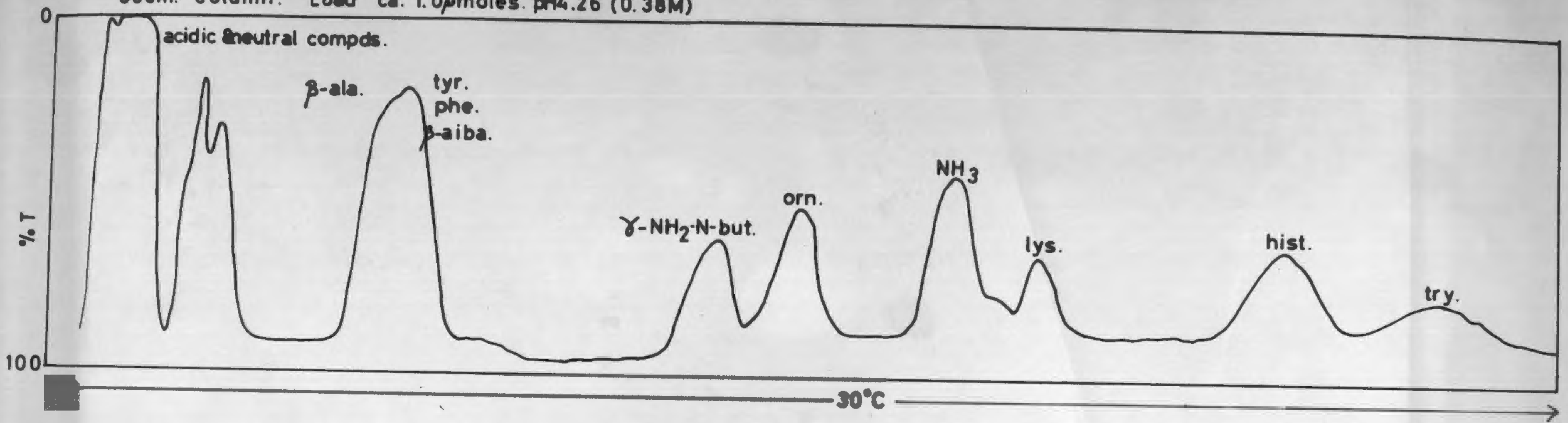


FIG. 27 STANDARD CURVE FOR BIOLOGICAL FLUIDS

50cm. column. Load ca. 1.0μmoles. pH4.26 (0.38M)



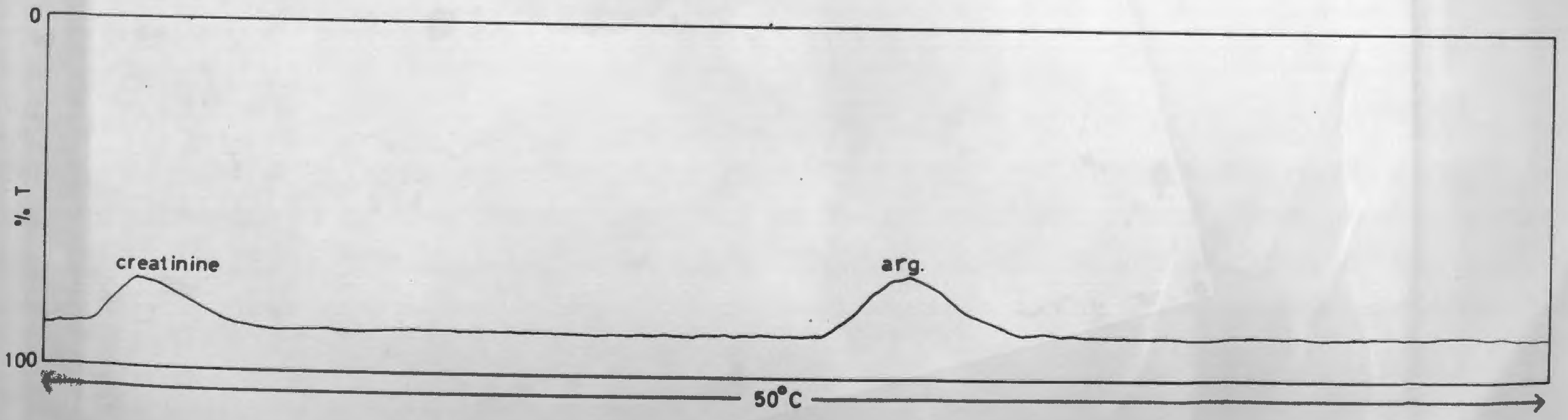


Table 17.

Constants for Composite Standard.

	Constants of M/S.+	Varian*	Ratio	Jounes*	Ratio
Phosphoethanolamine	15.7	26.2	1.48	17.2	1.10
Taurine	25.1	45.0	1.79	33.5	1.33
Hydroxyproline	2.54	1.93	0.76	2.58	1.02
Aspartic acid	25.2	47.2	1.87	42.1	1.67
Threonine	25.4	49.8	1.96	41.6	1.64
Serine	26.1	43.5	1.67	43.5	1.67
Asparagine	23.4	32.5	1.39	31.4	1.34
Proline	6.37	4.2**	0.66	9.73***	1.53
Glutamic acid.	26.3	50.3	1.91	39.1	1.49
Glycine	25.6	49.8	1.94	37.6	1.47
Alanine	26.0	47.9	1.84	41.4	1.59
Valine	27.2	46.7	1.72	42.0	1.54
Cystine	28.8	37.5	1.30	41.4	1.43
Methionine	25.6	55.2	2.16	39.5	1.54
Isoleucine	27.3	50.8	1.86	41.6	1.52
Leucine	27.6	42.4	1.54	42.2	1.53
Tyrosine	27.0	39.8	1.47	39.4	1.46
Phenylalanine	26.7	40.0	1.50	37.1	1.39
β-Alanine	11.4	17.8	1.56	18.4	1.61
β-Amino isobutyric acid	12.5	19.0	1.52	19.9	1.59
γ-Amino butyric acid	24.9	33.8	1.36	36.6	1.47
Ornithine	29.9	39.1	1.31	45.1	1.51
Lysine	28.6	38.6	1.35	44.6	1.56
Histidine	26.5	35.8	1.35	38.2	1.44
Tryptophan	18.5	24.8	1.34	27.1	1.46
Arginine	25.4	35.2	1.39	37.3	1.47
Urea	0.97	1.52	1.57	1.49	1.53

\+ Moore and Stein.

* It is fortuitous that width of peaks in m.m. on the potentiometric recorder coincided with the number of dots counted on the galvanometer chart. (Dots were 1 minute apart).

** 570 mμ.

*** 440 mμ.

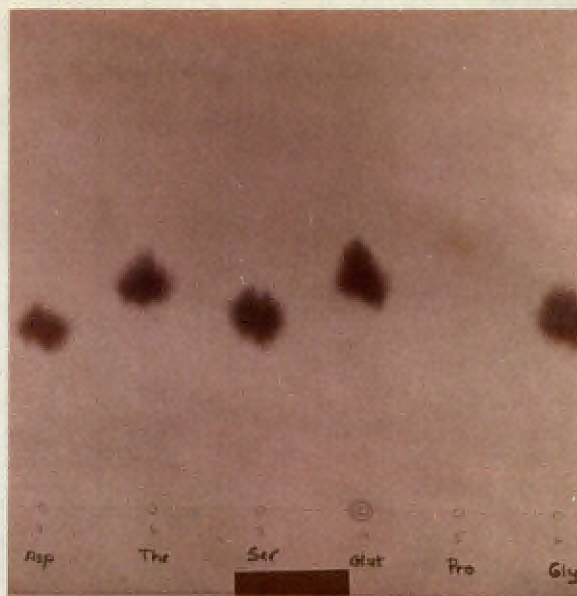


Fig. 28. One-way ascending paper chromatography of ca. 5 ug of amino acid standards in Bu/HAc/H_2O. No impurieis were detected (note yellow colour of pro. spot with ninhydrin).

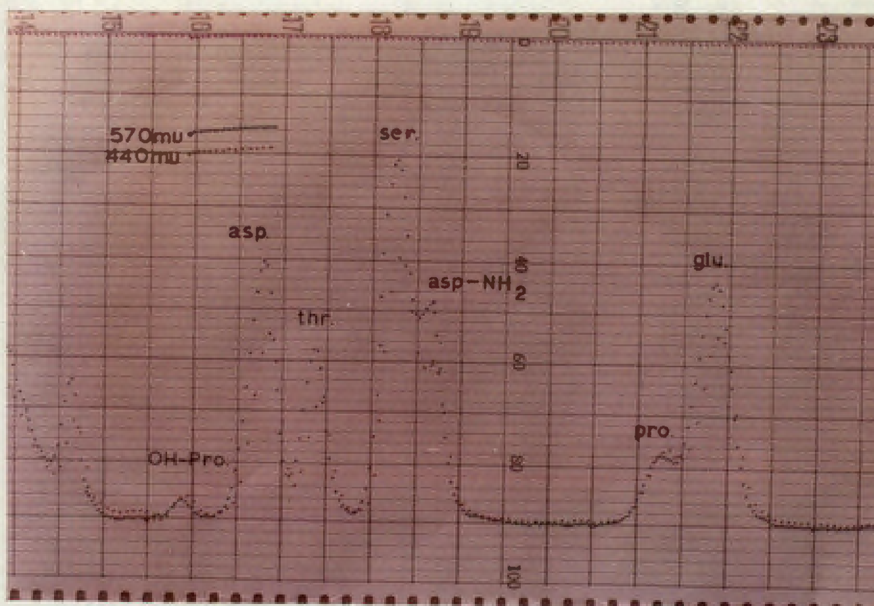


Fig. 29. Standard curve from Amino Acid Analyser. Increased absorption at 440 mu. of peaks of OH-pro. and pro. aids identification of these two amino acids in unknown mixtures

Many of the substances present in urine were not available as standards and constants could not be derived. For these, constants have been calculated based on those published by Spackman et al and the average of the ratios (1.49) in table 18.

Table 18.

Calculated Constants.

Substance	C of Spackman et al	C x 1.49
Cysteic Acid	25.5	37.9
Methionine Sulphoxide	25.0	37.2
Phosphoserine	ca. 27.2	40.5
Glycerophospho-ethanolamine	20.6	30.7
Sarcosine	6.48	9.7
Glutamine	ca. 21.8	32.5
Citrulline	26.5	39.5
δ-Amino adipic acid	23.7	35.3
Cystathione	31.9	47.5
Half-homocystine	25.4	37.8
Hydroxylysine	29.0	43.2
β-Amino N-butyric acid	27.3	40.7
Ethanolamine	20.5	30.5
1-Methyl Histidine	22.5	33.5
3-Methyl Histidine	ca. 22.5	33.5
Anserine	18.2	27.1
Carnosine	21.7	32.3

Linearity.

Linearity of the recorder mechanism and method of integration was tested on the 15 cm. column with varying loads of lysine, histidine, arginine and ammonia.

The results are shown in Table 19 and Fig. 30.

FIG.30 LINEARITY OF INTEGRATION METHOD

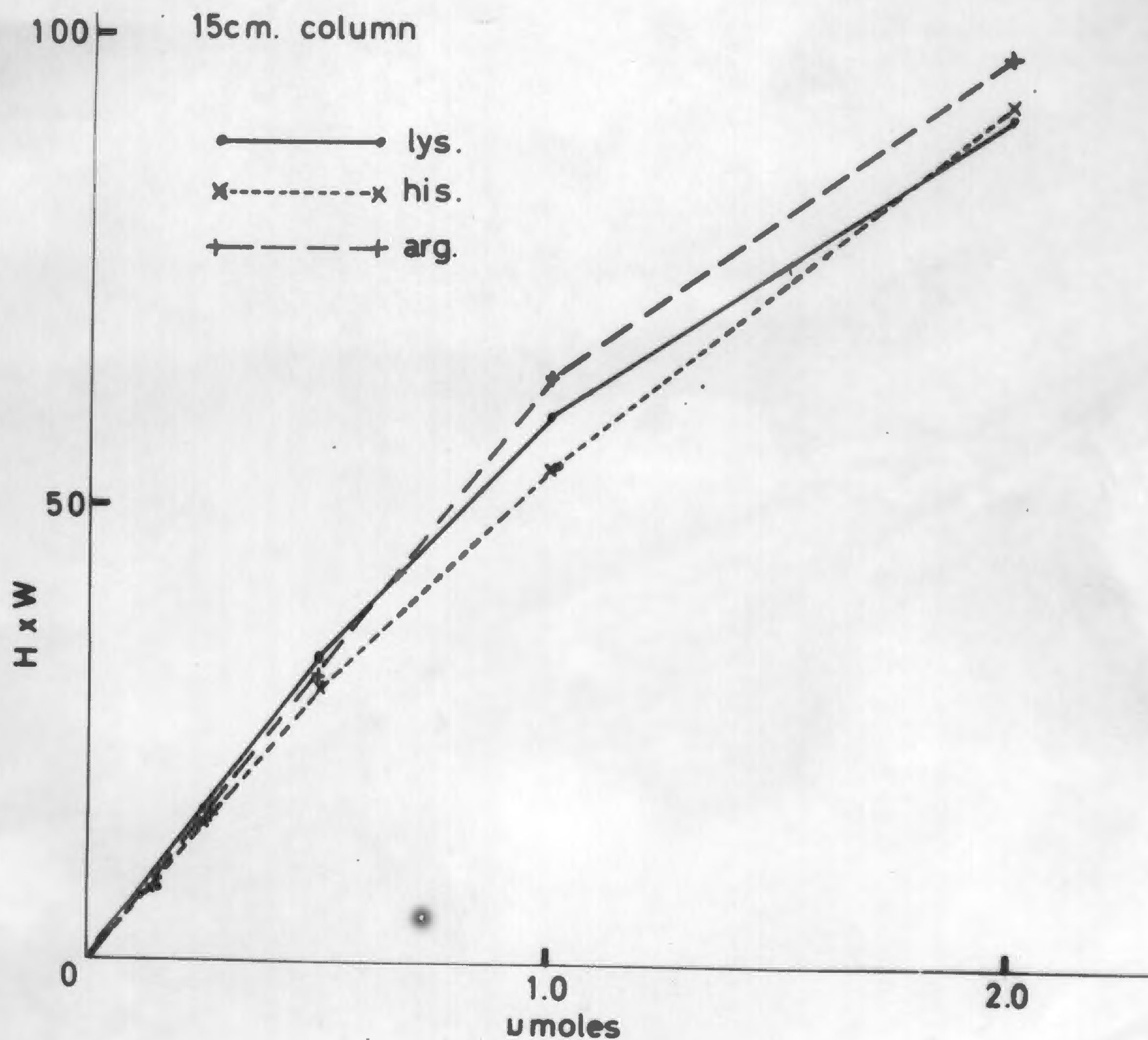


Table 19.

Linearity of Integration with varying loads.

Load	u moles*	0.125	0.25	0.5	1.0	2.0
Lysine	H x W	8.0	16.3	32.8	59.2	91.5
	C	61.1	61.9	62.3	56.3	43.5
Histidine	H x W	7.42	15.0	29.9	53.4	93.3
	C	56.4	57.5	57.3	51.2	44.7
Ammonia	H x W	8.64	14.3	30.8	58.9	82.0
	C	66.9	55.4	59.8	57.2	39.9
Arginine	H x W	8.01	16.0	31.0	63.4	98.0
	C	58.1	57.8	56.1	57.3	44.3

* To nearest simple fraction.

The ammonia values, especially at low loads CAUSED scatter due to contamination of the buffers, which could not be avoided.

The peaks of the amino acids off the column become broader and less sharp in sequence of elution and the optical density of peak height limited linearity. This explained why linearity for arginine was valid up to loads of 1.0 µ moles whilst the graph for lysine was linear up to 0.5 µ moles. With the present apparatus the method of integration was valid for peak heights up to 0.9 or 1.0 optical density units. Where peaks exceeded this limit, integration was made with a set of constants derived from the channel with reduced light path (0.8 mm. at 570 mµ.). This was only necessary for the peaks corresponding to asparagine glutamic acid, glycine and histidine during the experiments reported.

RECOVERY EXPERIMENT.

Free amino acids in 3 ml. of normal urine were measured before and after adding a known mixture of standard amino acids. Recovery of these is shown in Table 20.

TABLE 20.

RECOVERY OF AMINO ACIDS FROM NORMAL URINE.

AMINO ACID	URINE u-moles	TOTAL FOUND u-moles	ADDED u-moles	RECOVERED u-moles	RECOVERY %
Tau.	0.124	0.579	0.445	0.455	102
Thr.	0.058	0.497	0.319	0.349	109 ✓
Ser.	0.017	1.419	1.214	1.402	115
Asp. NH_2	0.411	0.920	0.508	0.509	100
Pro.	0.000	1.110	1.038	1.110	107
Glu.	0.228	1.041	0.727	0.813	112.
Gly.	1.544	2,376	1.105	0.832	112
Ala.	0.714	1.246	0.605	0.532	88
Val.	0.022	0.430	0.394	0.408	103
Cys.	0.011	0.414	0.436	0.403	93
Met.	0.017	0.410	0.414	0.393	95
Iso/leu.	0.035	0.293	0.293	0.258	85
Leu.	0.040	0.616	0.620	0.576	93
Tyr.	0.008	0.605	0.601	0.597	99
Ph. ala.	0.042	0.627	0.647	0.585	91
β-ala.	0.010	0.528	0.514	0.518	101
β-A.I.B.A	0.006	0.741	0.616	0.735	119

Free and Bound Amino Acids in Urine.

Free amino acids were measured on an aliquot of urine containing 0.2 to 0.5 mg. of α-amino nitrogen (usually 2 to 5 ml.). Bound amino acids were measured after hydrolysis of protein-free urine.

Ten ml. of urine was mixed with 1.0 ml. of 0.67N H_2SO_4 and 1.0 ml. of 10%w/v Sodium tungstate. The resulting precipitate was removed by centrifugation. One or two ml. of protein-free urine (0.2 to 0.5 mg. of total amino nitrogen) was then hydrolysed in a sealed tube at 105°C for 18 hours with an equal volume of Conc. HCl. The hydrolysate was filtered and washed through a glass wool plug to remove 'humin' and dried over KOH in vacuo.

Acidic and neutral amino acids were measured on the 150 cm column using the pH 3.25/4.25 and 30°/50°C system. The samples were transferred to the column in 0.2M, pH 2.2 sodium citrate buffer.

Basic compounds were assayed on the 50 cm.column using 0.38M, pH 4.26 buffer and 30°/50°C programming. The desiccated samples (free and total) were dissolved in 2 ml. water and adjusted to pH 8 with N NaOH solution. Ammonia was removed by evacuation with an oil pump for 15 minutes over conc. H_2SO_4. The samples were then adjusted to pH 2.2 with conc. HCl and applied to the basic (50 cm.) column.

Bound amino acids were the difference between total and free forms.

The excretion of free and bound forms of amino aicds were expressed as μ-moles per 24 hours.

Recovery Experiment - Losses due to Hydrolysis.

Five ml. of a composite amino acid standard were hydrolysed in a sealed ampoule under the same conditions as for urine. After desiccation and reconstitution in pH 2.2 citrate buffer, an aliquot containing ± 1.0 μ-moles of each amino acid was analysed on the 150 and 15 cm.columns for neutral and acids and for basic amino acids and ammonia respectively. The colour yields before and after hydrolysis and calculated percentage recoveries are shown in table 21.

TABLE 21.

Recovery of standard amino acids after hydrolysis.

Amino Acid	COLOUR VALUES H X W. Before	After	RECOVERY %
Glycero-P-Ethnanolamine	16.8	0.28	1.7
Asp.	26.7	26.6	99.6
Thr.	25.8	24.2	93.7
Ser.	27.0	22.6	83.6
Hydroxy. Pro.	3.20	3.62	113
Pro.	8.2	8.5	104
Glu.	41.5	39.1	94.2
Gly.	38.6	37.0	95.8
Ala.	23.8	25.3	106.3
Cys.	25.2	20.5	81.4
Nal.	25.9	22.9	88.3
Meth. a) As Meth.	23.3	3.3	15.1
b) Encl. Sulfoxide	14.0	14.7	105.0
Allo isoleu.	13.4	13.1	97.7
Leu.	27.1	27.5	101.4
Nor. Leu.	27.4	27.4	100.0
Tyr.	27.0	23.3	86.3
Ph.ala.	25.6	25.0	97.7
Lys.	33.9	32.3	95.3
Hist.	31.5	30.5	96.8
NH_3	36.2	50.6	139.8
Arg.	26.4	27.1	102.7

Results.

It was possible to examine three cases fully, i.e. before and during repletion, and on recovery. A fourth case was investigated during the acute phase only.

Results obtained on the urine of these cases are shown in tables 22 to 25. Amounts of individual amino acids have been expressed as µ-moles per 24 hours. Where no amino acid was found, the limit of detectability has been calculated and this depended upon colour value, average width of the hypothetical peak and volume of sample which was applied to the colum. Limits for trace amounts have also been calculated on the same basis.

No correction has been applied for losses due to hydrolysis. Most amino acids showed increases after hydrolysis. This represented bound forms. When hydrolysis diminished the apparent amount of amino acid in the sample, the difference was recorded as a negative value. The implication of these negative values will be discussed.

In order to detect and measure amino acids which were present in small quantities, a large sample had to be analysed. This caused overloading of more concentrated substances e.g. urea, glutamine/asparagine, glycine, alanine and histidine, which resulted in overlapping of these peaks. The optimal load varied between 1 ml. (oliguric specimens) to 5 ml. for more diluted urine. Most peaks were measured from the 570 mµ (2.8 mm.curve). When the peak height exceeded 1.0 optical density unit (10% transmission), integration was performed on the 570 mµ.(0.8 mm) curve. Proline and hydroxyproline were read

Table 22.

Case 1 (H.L.) Urinary excretion of free and bound Amino acids u moles/24 hours.

Day	1		3		5		15		17		19	
Volume (ml.)	23.5		239		887		557		370		438	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Tau.	58	<0.3	167	24	163	38	1.0	29	24	7+	2.6	15+
OH Pro.	<1.4	79	14-42	141+	<53+	635	<33	225+	<21	53+	<25	169+
Asp.	6*	35	32*	170+	5.3	462	8	326		66+		225+
Thr.	-*	19+	-*	119+	-*	391+	13	123	7.1	36	9.8	65
Ser.	-	30+	-	253+	-*	682+	3	187	2.1	59	6.0	94
Asp. NH_2	4	-	481	-	869	-	45	-	50.7	-	13.0	-
Sar.	1	<0.3	<1.4	3-8	<5	71	3.1	20	2.1	0		0
Pro.	<1.4	51	14	292	53	238	33	82	<21	101	<25	122
Glu.	2	104	35	504	89	2,321	43	776	28	389	6.3	472
Cit.	0.1-0.4	0.5	1.4	3-8	5-16	0	3-10	6	1.5	0		0
Gly.	54**	110**	656	223	1,438	772	236	1,152	190	270	139	572
Ala.			126	54	686	443	57	333	88	45	44	173
α-NH_2 Adipic	0.6	0.3	3.2	16	80	11	4	11	4.7	-0.3	3.3	9.3
Val.	0.1-0.4	7.5+	1.4-4.2	39+	5.3-16	100+	<3.3	60	2.7	12	2.1	7.3
Cys.	0.1	23	1.4-4.2	51	46	92	<3.3	15.2	1.4	13	1.0	39
Cystath.	0.6	1.0	21	0+	5-16	14+	<3.3	6.5-20+	<4	0	<4.5	0
Meth.	1.7	3.3	3-8.5	29+	5-16	11+	7	35+	2.1	-0.8	1.2	2.8
Iso.	1.9	3.0	3-8.5	24+	5-16	85+	3-10	44+	4.3	13	4.2	21
Leu.	0.5	12.5	11	58	5-16	226+	3-10	111+	49	0	8.8	49
Tyr.	<0.1	5.6+	3.7	47	5-16	146+	3-10	131+	1.0	20	3.1	30
Ph. al.	<0.1	6.0	4.2	43	5-16	139	3-10	145+	5.2	16	2.8	18
β-A.I.B.A.	11	12	12	28	5-16	87+	3-10	0	<4	0	4.5	0
γ-A.B.A.	2	0++	17	0	83	-3	21	0	<3	30	16	-0.7
Lys.	-++	-++	2.3	0.3	-++	-++	<3	26	98	17	170	34
Orn.	<0.1	0	1	0	5	0	<3	0	6	13	5.3	8.0
His.	15	-0.3	602	169	1030	-40	217	10	212	139	292	15
3-CH_3-Hist.	2	0	-++	-++	-++	-++	304	45	5.0	0	<2	9.4

0 = No significant quantities of bound forms.

* = Pattern obscured.

** = Overloading effect with confluent peaks.

+ = Total.

++ = Overlapped by ammonia peak.

Table 25.

Case 2. (A.O.) Urinary Excretion of Free and Bound Amino Acids (u moles/24 hours).

Day	2		4		14		15	
Volume (ml.)	190		846		460		281	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound
P. Ser.	20	188	<2	112**	<1	5.8**	<1*	23**
Tau.	16	101	32	14*	-*	8.0**	-	17**
Met. SO_3	14	-5	40	-	13	8.7	55	-40
OH -Pro.	8	177	<20	20-40	<12	12-24	<10	18**
Asp.	28	13	148	362	9.5	37	<3	35**
Thr.	16	-7.7	<4*	141	24	19	86	21
Ser.	<1	7.9	-*	1,005**	4.2	16	32	13
Asp. + Glut. $-NH_2$	32	-	1,573	-	70	-	51	-
Pro.	<10	93**	25	25-50	<20	<20**	<14	14-20
Glu.	86	274	168	1,912	20	179	8.5	96
Cit.	<1	1.4**	<4	<4	0.9	1-2**	1	1**
Gly.	169	319	695	2,448	681	670	25.4	412
Ala.	68	86	373	730	782	138	306	164
α-NH_2-N-but.	<2	2.9**	<6	<6**	2.8	2-4**	<1	2.1**
Val.	13	-9.3	6-12	53**	<3	4-8	28	19**
Cys.	11	6.2	6-12	12-24**	<3	4-8**	2-4	2-4**
Cystath.	13	-13	31	4-8**	<1	<1**	8	4-8
Met.	4.4	-1.0	6.8	4-8**	<1	1.7**	3.5	4.9
Iso.	7.1	2.0	8.5	8-16**	<2	3.6**	7.7	9.7
Leu	5.2	35	37	52	2.3	5-4	12	9.5
Tyr.	4.1	19	69	43	12	12	13	21
Phe.	1.8	25**	56	90**	8.8	3.8*	6.2	4.4**
β-ala.	2.4	26***	8-16	98	2-4	4-8	<2	7.0
β-A.I.B.A.	43	-**	508	317**	10	18	38	41
γ NH_2-N-but.	<3	19**	<6	<6**	32	-5	<3	<3**
Et. NH_2	248	50	6-12	6-12**	6.9	4-8**	3-6	3-6**
Lys.	29	71	511	62	20	55	38	95
His.	288	81	1,358	134	241	118	459	42

* Pattern obscured.

** Total after hydrolysis.

*** Result lost.

Table 24.

Case 3 (J.C.) Urinary excretion of free and bound Amino acids u moles/24 hours.

Day	1		3		5		13		15		17	
Volume (ml.)	292		153		35		470		448		408	
	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
P-Ser.	7.9	-7.2*	13	8.9	21*	11*	-*	68**	<1	9.3**	-*	21**
Tau.	54	-*	24	14	-*	22**	44	-23	80	-*	58	-41
Met. SO_3	<1	-	36	-13**	-	12**	44	-25**	119	-	30	-21**
OH Pro.	<10	13.6**	<8	138**	<1.7	36**	<20	40**	<78	18-36	<15	27**
Asp.	63	50	3.7	5-10**	1.6	48	2-4	206**	< 2	31	< 2	122
Thr.	16	84	80	245	128	-44	< 2	81**	28	288	2-4	16
Ser.	192	-171	42	59	28	41	9.9	165	34	205	8.4	47
Glut Asp.NH_2	193	-	145	-	27	-	101	-	397	-	89	-
Ser.	< 2	<2**	23	104**	3.4	71.3**	21	-4.8	32		19	-5.3*
Pro.	<15	15-30	12-24	61	2	9.2**	<20	20-40**	<20	85**	<20	20-40**
Glu.	255	239	65	713	3.5	114	<3	486**	<3	533**	15	361
Cit.	2-4	2	11	-3.0	0.3	0.8	< 3	3-6	<3	3	< 3	< 3
Gly.	767	352	317	656	57	206**	79	707	267	428	65	173
Ala.	162	72	82	63	12		44	54	173	23	26	27**
α-NH_2-N-but	54	9.9	62	7.4	1.3	17	< 4	10.2**	29	-21	< 4	4**
Val.	31	-23	11	4.5	2.4	17	4-8	13**	27	14	11	9**
Cys.	2-4	9.2**	43	13	0.7	11.1**	4-8	6.6**	4-8	4.5**	4-8	15**
Cystath.	5.2	-5.2**	19	29	<0.1	0.1**	2	-**	< 2	<2**	< 2	< 2**
Met.	2.4	1.2	18	5.4	0.1-0.2	2.8**	2-4	5.9**	<2	<2	1.8	1.9
Iso.	10	19	29	36	0.9	11.6	4.9	18	2-4	34	6.1	19
Leu.	10.3	19.4	29.2	35.6	0.9	11.4	4.9	17.7	4-8	17.2	6.1	18.7
Phe.	1.6	8.5	9.3	48	2.3	14	10	4.5	<2	35	15	5.3
Tyr.	4.7	14.4**	15.6	68.7	1.8	21.8**	17.0	10.2**	< 2	65.1	19.4	13.4
β-Ala.	2-4	3.5	1-2	31**	0.7	9.2	<3	12**	<2	74	2	30
β-A.I.B.A.	101	37*	204	24	15	68*	3-6	31*	<2	25**	2	19*
γ-NH_2but.	22	-	41		12	-	3	-	<3	22	8.9	-**
Eth. NH_2	54	4.2*	30	4.1	20	2.1	10.8	-2.0**	19	-1.5	4-8	4-8*
Lys.	192	-	32	23	16	4.6	3-6	< 6**	39	29	21	-
His.	441	92*	176	52**	19	-11	330	120**	756**	371**	141	122
3-Meth.His/ans.	227	-	19-20	20**	27	-27	<6	<12**		68**	4-8	4

* Pattern obscured.

** Total.

Table 23.

Case 4. (F.B.) **Urinary Excretion of Free and Bound Amino Acids (u moles/24 hours).**

Day	1		3		5	
Volume (ml.)	785		340		998	
	Free	Bound	Free	Bound	Free	Bound
P-Ser.	-*	59**	-	87*	-	66
Tau.	71	-26	101	-*	39	-1.2
Meth SO_3	51	47**	94	-	78	-*
OH Pro.	< 15	< 30**	< 8	< 15**	< 20	95**
Asp.	4-7	51**	< 1	149**	11	194
Thr.	8.5	3.3	11-4	85**	188	-38
Ser.	69	-10	68	88	416	-222
Glut. NH_2+ Asp. NH_2	67	-	405	-	881	-
Sar.	16		15		41	-1
Pro.	< 15	15-30**	< 8	17**	< 20	51**
Glu.	8	1,122**	8	394**	20	573**
Cit.	< 4	2-4**	< 1	< 1**	< 3	< 6**
Gly.	176	633	503	962**	2,478	115
Ala.	59	4		110**		
NH_2-N. but.	4-8	< 8	2-4	< 4**	29	2
Val.	5	-1	15	1	28	-5
Cys.	28	-18**	27	-10	38	3
Cystath.	4.5	2-4**	3.1	18	7.2	0
Met.	7.9	19	12	-7	13	-8
Iso.	12	2	8.8	41	29	-15
Leu.	8	7**	15	13	29	-14
Tyr.	4-7	39**	26	6	98	56
Phe.	4-7	11**	15	6**	16	5.6
-ala.	4-7	55**	2-4	24**	3-6	3-6**
A.I.B.A.	61	-18*	11	8	31	2.4
Et. $-NH_2$	4-8	-	36*	122**	84	-
Lys.	48	9	-	29**	34	7
His.	77	56	23	-0.5	731	-13

* Pattern obscured.

** Total.

at 440 mµ. Occasionally high, incompletely resolved peaks e.g. serine, asparagine/glutamine and glycine/alanine interfered with width (at half height) measurement. In these instances the width was taken as twice that of the number of dots between half height on the free side of the peak, and peak height.

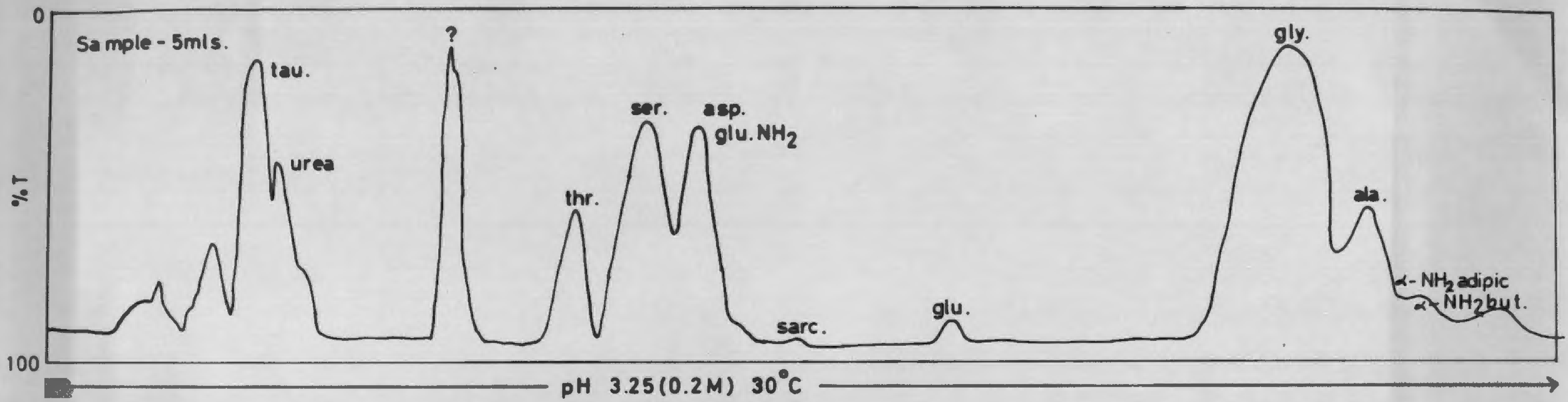
Observations on the patterns of the Chromatograms.

The elution pattern on whole urine of children with kwashiorkor closely resembled that of adult urine and by comparison with the standard curve there was no difficulty in correlating the elution volume of peaks from urine with those of known free amino acids (fig. 31 and 32). In the case of proline ahd hydroxyproline, the higher absorption at 440 mµ was an aid to identification. It must be stressed that this was the sole method of identification and two possibilities of error exist viz:

1. that the peak corresponded to another ninhydrin-positive substance having similar chromatographic behaviour or that
2. the peak represented more than one compound. In the manual column method, paper chromatography of desalted fractions is a confirmative procedure, but this was obviously impossible with the automatic method.

The elution pattern of the urine of normal adults and children has been well established and peaks unambiguously identified. It was unlikely that any peak was incorrectly labelled but it is possible that peaks did contain more than one component, especially since the urinary specimens examined

FIG. 31. NORMAL ADULT URINE -ACIDIC AND NEUTRAL



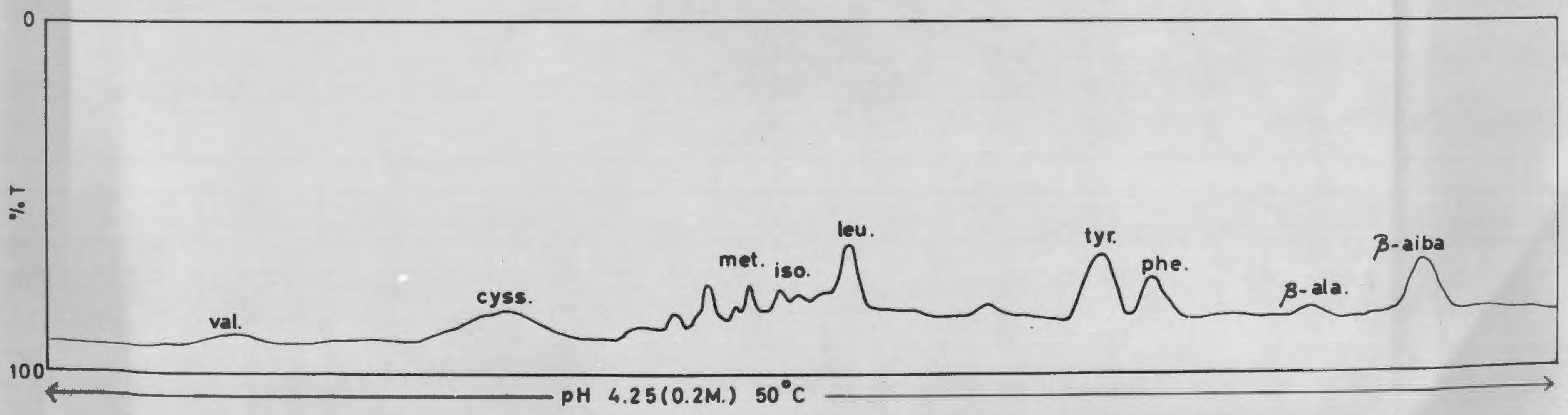
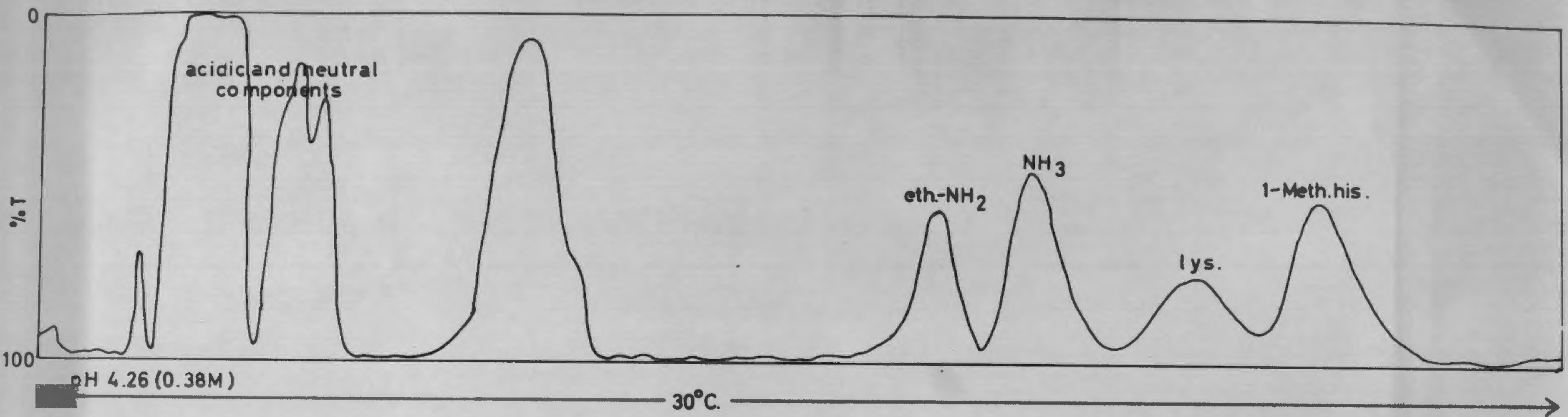
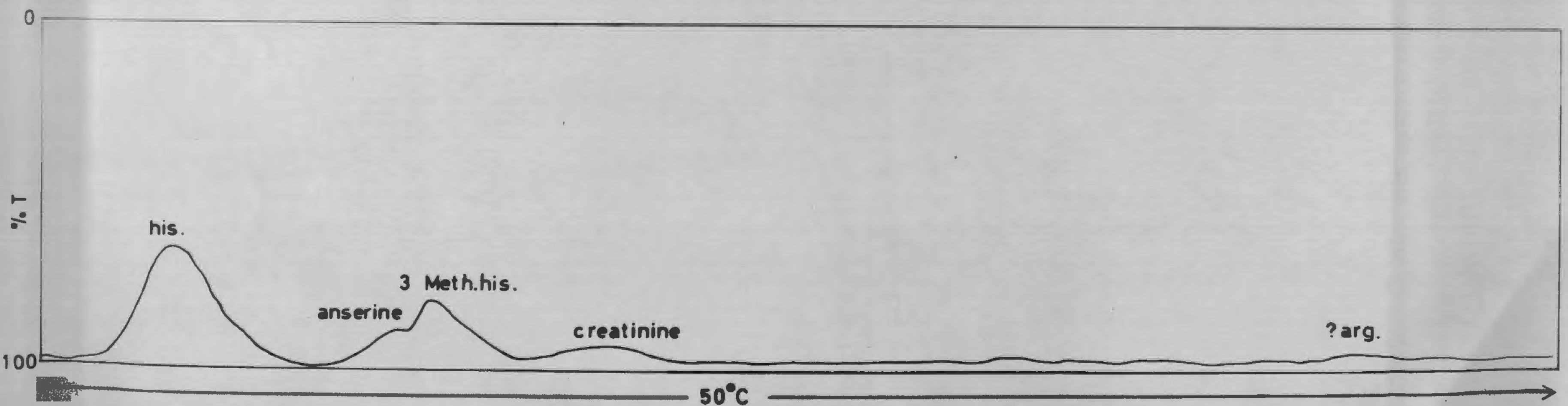


FIG. 32 NORMAL ADULT URINE — BASIC





were abnormal. In similar circumstances an error has been made in the recognition of methionine in the condition of maple-syrup-urine disease. This peak was later found to be due to alloisoleucine[214]. Evidence will be presented in later chapters that many of the peaks from which free and bound amino acids have been calculated contain appreciable quantities of a contaminating substance. This applies to the present data as well as that derived by other authors.

Notwithstanding the limitations implied by the above considerations, some general conclusions may be derived from the results. Certain amino acids were absent or present in only minute amounts. No free hydroxyproline was found in any specimen and aspartic acid, proline, methionine, lysine and arginine occurred in traces only. Small amounts of glutamic acid, valine cystine, leucine, isoleucine, tyrosine and phenylalanine were readily detectable, whilst serine, glycine, alanine and histidine were present in the largest amounts.

In addition to the usual amino acids found in protein, the following, not usually present in hydrolysates were detected: β-alanine, β-amino isobutyric acid, γ-amino N-butyric acid. Taurine was present in appreciable amounts.

In this analytic system cysteic acid and phosphoserine emerge from the column together. Integration of this peak was reported as phosphoserine since there is no cysteic acid in normal urine and under the conditions of hydrolysis employed, none was formed from cystine. Asparagine and glutamine could also not be separated. In Moore and Stein's original technique which utilised

pH 2.2 as one of the eluting buffers, glutamine was almost completely destroyed and none was present in the asparagine peak. At pH 3.25 (present system) glutamine is more stable and the peak was expected to represent both amides. α-Amino adipic and α-amino N-butyric acids, which were eluted following alanine, were incompletely resolved and were likewise considered together. Ammonia was not completely removed by alkaline evacuation and in some, more concentrated specimens, partly overlapped the peak of lysine. Histidine similarly was not completely resolved from its 1 and 3-methyl derivatives. No free tryptophan was detected in any of the samples and acid hydrolysis destroyed any which may have been present in bound form. Thus no values for this amino acid have been reported.

The chromatogram on hydrolysed, compared to unhydrolysed, urine showed certain differences. To prevent overloading, an aliquot of hydrolysate, equivalent to one-half of the volume used to measure free forms, was applied. All amino acids except taurine, cystathionine and sarcosine were increased on hydrolysis. Comparatively large amounts of serine, threonine, glutamic acid, glycine and histidine were liberated and almost all aspartic acid and all the hydroxyproline was present in bound form. In this respect no gross qualitative differences in the pattern of excretion of amino acids in free and bound forms was noted between the acute and the recovery phases, and these are very similar to results obtained from normal adults and children[202].

When compared to the pattern obtained from hydrolysed urine, the chromatogram from whole urine was considerably more complex

(figs. 33 & 34). Many minor peaks were acid-labile. These acid-labile peaks were conveniently grouped as they appeared to be eluted together.

Group I contained 7 components and was eluted between Phosphoserine and urea. Group 2 consisted of a complex, lying between aspartic acid and serine and another complex, group 3 was eluted with the pH 4.25 buffer and appeared between methionine and leucine. The peaks of some of the acid-labile components were sharp, but others were broader, suggesting that these were larger molecules. Moore and Stein showed similar acid-labile peaks in their chromatograms of normal adult urine conforming to group I and group III but none was noted in the group 2 position and they suggested that these might be peptides. No basic acid-labile peaks were detected in the effluent from the 50 cm. column, which simplifies future discussion.

Since optimum conditions for separation of the amino acids on ion exchange resins have been empirically derived and all the physical processes for this separation are unknown, no rationale concerning the behaviour of the labile peaks could be attempted. However, the resin was not expected to retain large molecules and the broad peak ahead of phospheserine appeared to be a larger peptide rather than an acidic one. Generally, acidic peptides would be expected to be eluted soon (Groups I and 2) and basic peptides later, with the front of the pH 4.25 buffer (group 3). Deproteinisation of the urine sample did not affect the elution pattern, thus excluding large proteins molecules in any of the labile peaks.

No quantitation or detailed analysis of acid labile peaks

FIG. 33 ACID LABILE 'PEPTIDES' IN ACUTE PHASE URINE

CASE 1. (H.L.) Day 3 2ml. unhydrolysed urine

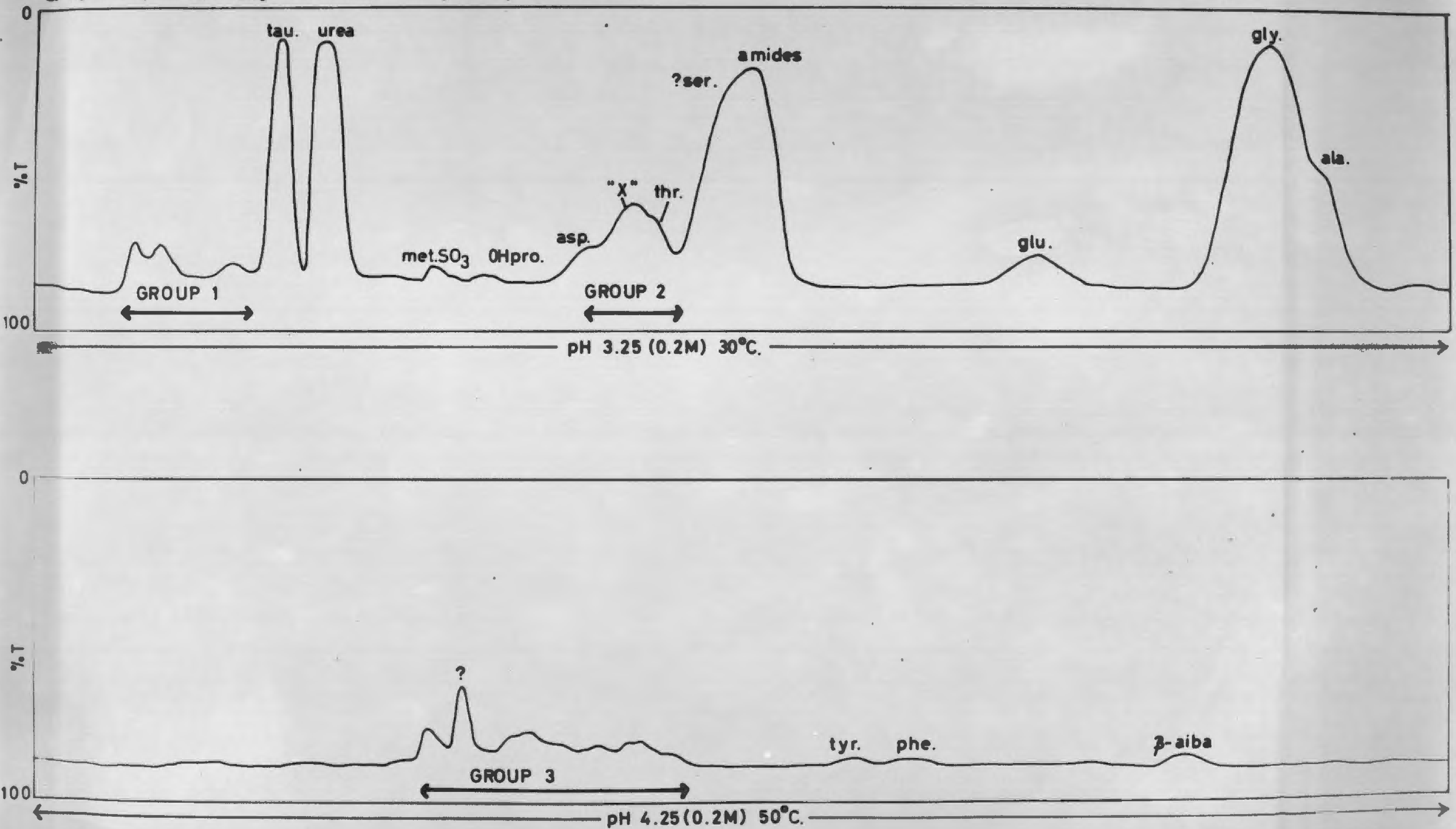
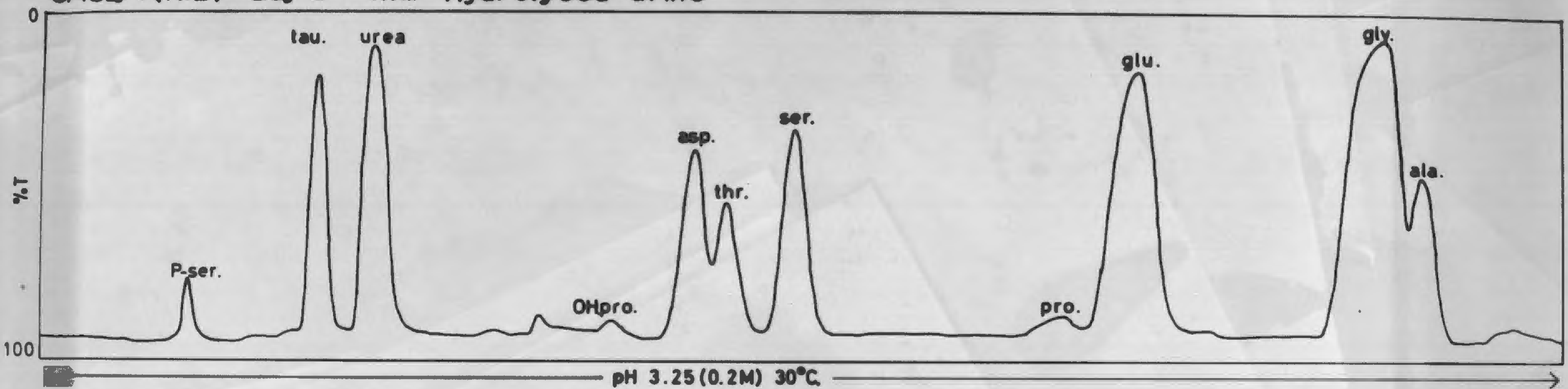
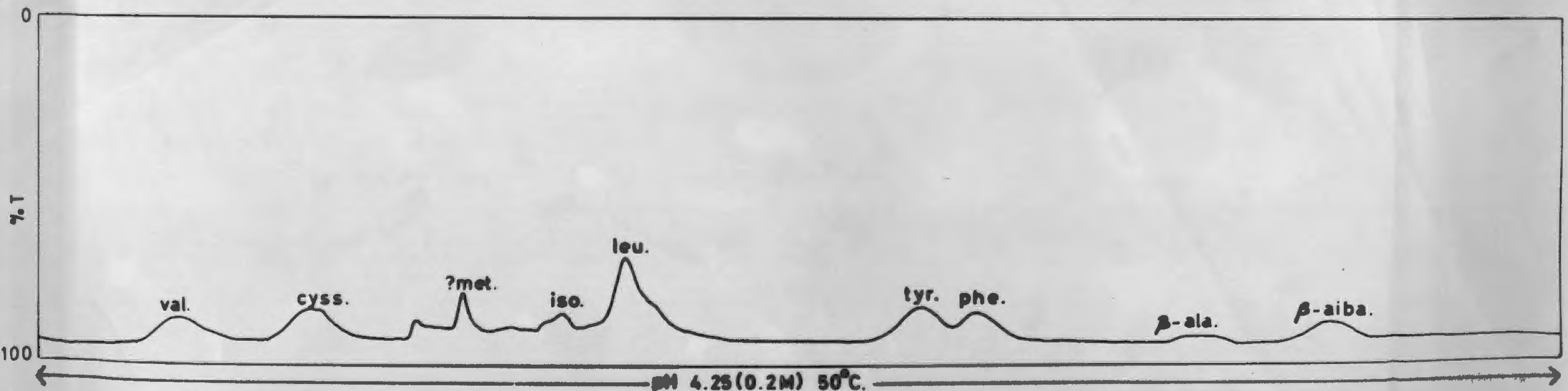


FIG. 34 HYDROLYSED ACUTE PHASE URINE

CASE 1 (H.L) Day 3 1ml. Hydrolysed urine





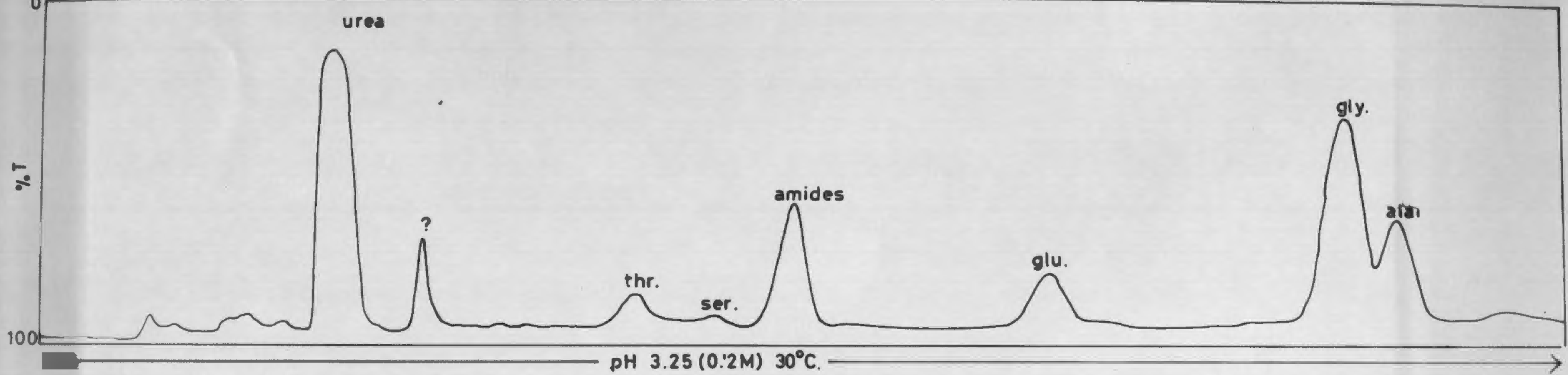
was attempted at this stage, owing to their variability from day to day and from case to case, and due to the many components detected. It was obvious that more and bigger acid-labile peaks were present in acute-phase urine, particularly in groups I and III peptides. The chromatogram from Case 1 in the recovery phase showed very few acid-labile peaks (fig. 35) when compared to the acute phase (fig. 33).

Case 1 showed very definite changes in the excretion of Group II material. This case was oliguric on admission and later developed diuresis. The chromatograms on days 1, 3 and 5 show increasing concentrations of a broad peak at the site of serine/threonine and was the major ninhydrin-positive component in this portion of the effluent. Hydrolysis accentuated the different chromatographic (ionic binding) behaviour of the labile component from that of the free amino acids which were expected to be eluted at this site (fig. 36). When allowance was made for the increased urinary volume from day 1 (23.5 ml.) to day 5 (887 ml.) it was apparent that the excretion of this component had increased several hundred fold. Reduced glutathione has similar chromatographic behaviour to the unknown, but has never been detected in urine or other extra-cellular body fluids.

The baseline between amino acid peaks from unhydrolysed urine showed many irregularities with shifts of up to 4% on the transmission scale. By contrast the baseline in hydrolysed urine chromatograms was much more stable. This was such a constant finding that instrumental error was eliminated as a

FIG. 35 RECOVERY PHASE URINE

CASE 1 (H.L.) Day 17 2ml. unhydrolysed urine



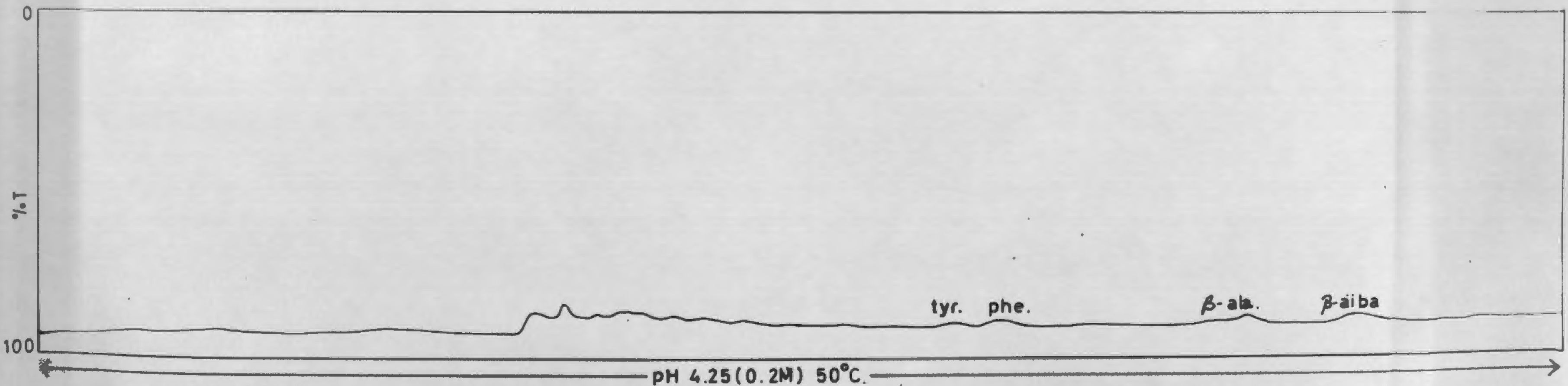
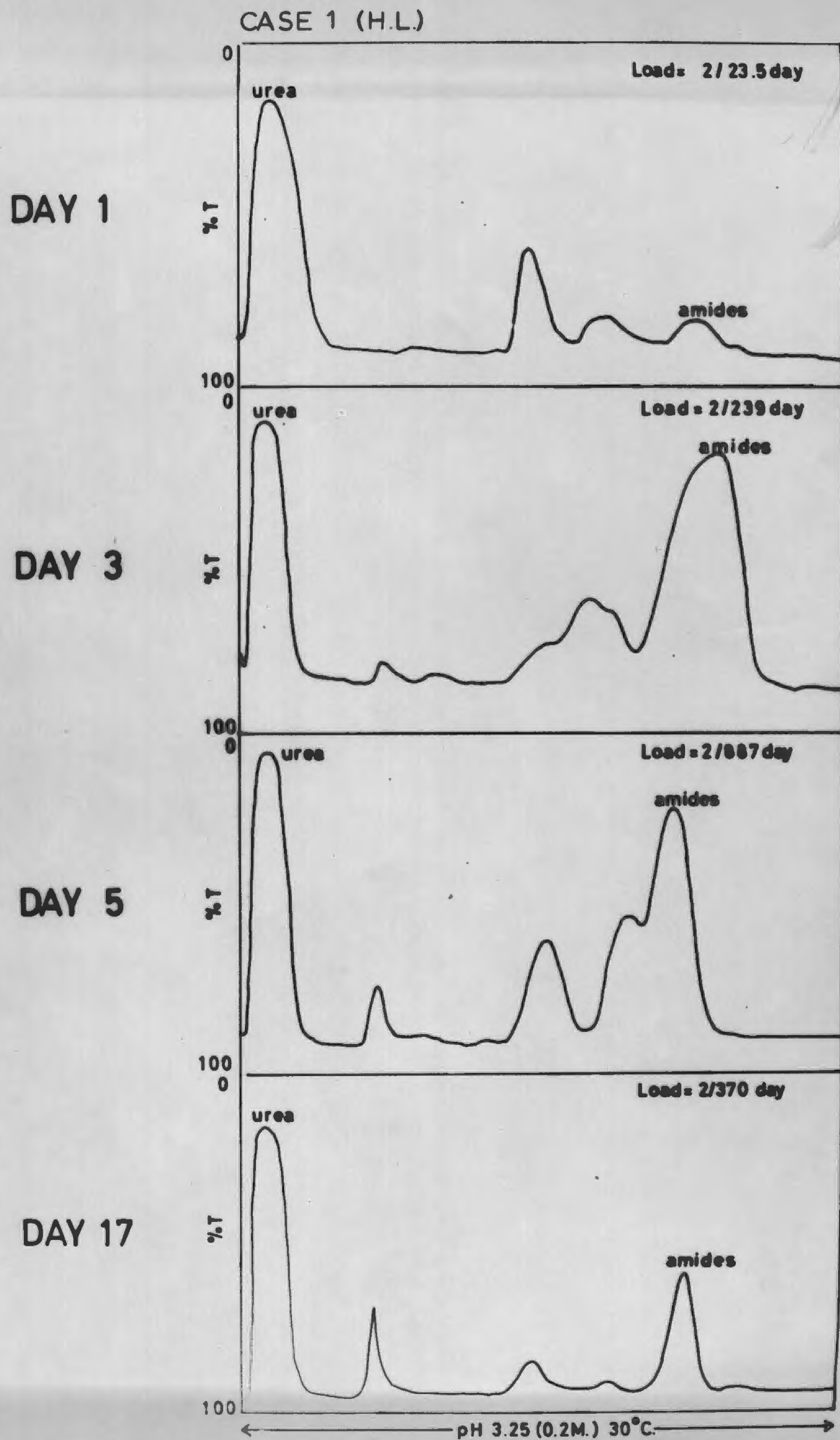


FIG. 36 EXCRETION OF GROUP 2 'PEPTIDES'



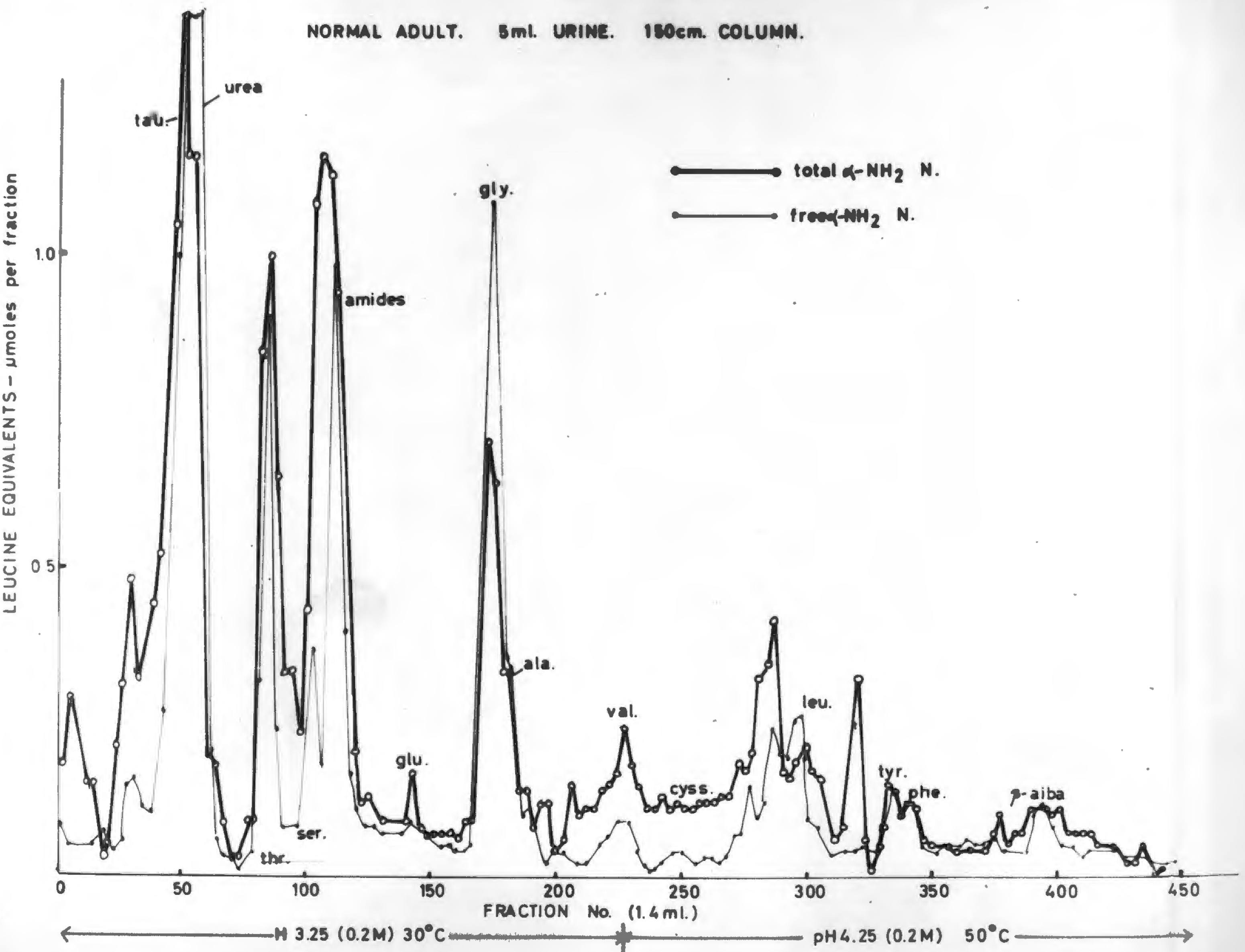
cause of this difference. As a check, a blank was performed, in which no sample was added to the column. The resulting baseline did not fluctuate by more than 1%. In addition to superimposed fluctuations, the baseline from phosphoserine to glycine was elevated by several % above that occurring before or after this regioh. The inference from these observations is that there might have been very great numbers of components which overlap on the chromatogram, and, because of either low concentration or low colour yield, were not detectable as discrete peaks. Lability in acid also suggested that they might be a spectrum of medium-sized peptides.

In order to check the above findings, 5.0 ml. of normal urine was chromatographed as before and the effluent collected in 1.8 ml. fractions. Each of these was analysed for ninhydrin positive material before and after alkaline hydrolysis. The results (fig. 37) confirm previous observations. Virtually all tubes contained some bound ninhydrin reacting material but largest amounts of this occurred in the sites occupied by Groups 1, II and III. The effluent of the 50 cm.column (?.38M pH 4.26 buffer) did not show similar fluctuations in baseline nor, when fractions were collected, were there significant quantities of bound ninhydrin reacting material. It appeared therefore that the bulk of the bound forms of ninhydrin reacting material was eluted from the 150 cm.column, and behaved either as neutral or acidic material and none was detected which was more basic than leucine.

Quantitative changes in excretion of bound urinary amino acids.

Excretion of individual amino acids both in free and bound

FIG. 37 BOUND NINHYDRIN POSITIVE MATERIAL IN NORMAL URINE



form showed the same type of changes as that of free and bound α-NH_2 Nitrogen. There was also maximal excretion of both these forms on the 4th to 6th day and increase in bound forms was generally greqter than that of the free.

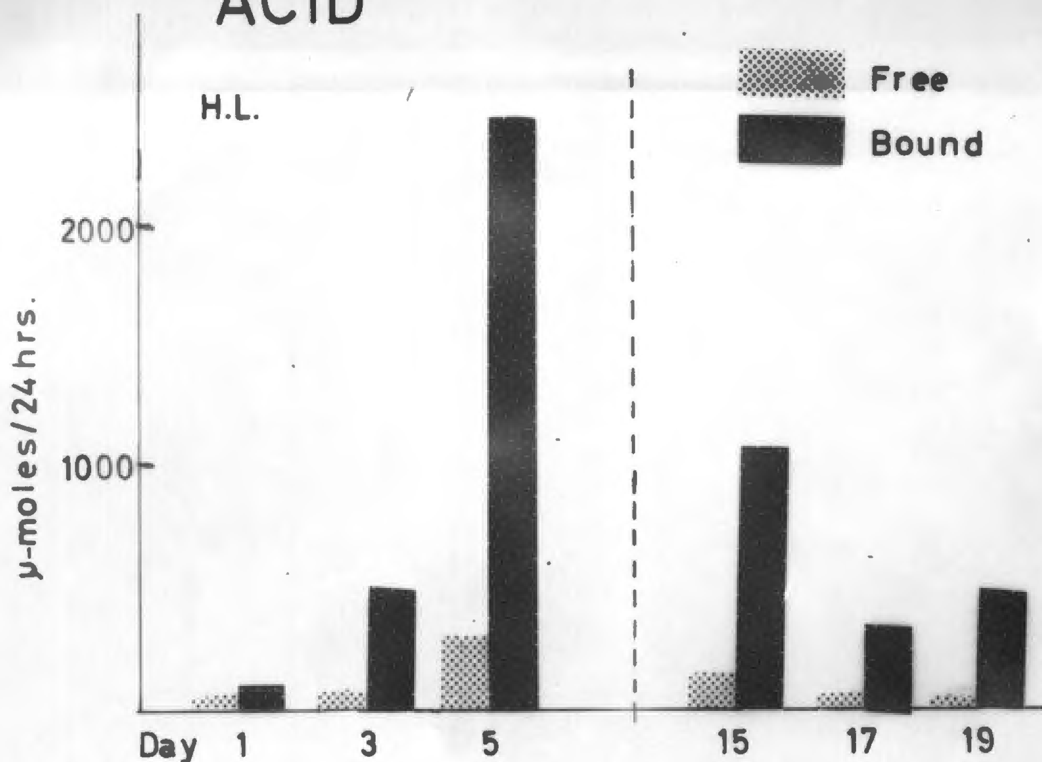
In order to examine the contributions to the total from individual amino acids, they were considered in two groups essential and non-essential, and a third group of those amino acids not usually found in proteins.

Non-essential dicarboxylic acids occurred mainly in bound forms. Free aspartic acid was virtually absent. It is also probable that the free glutamic acid resulted from glutaminase activity, since there is no free glutamic acid in blood[215]. Excrrtion of bound forms of other non-essential acids, i.e. serine, glycine, alanine, tyrosine and cystine, coincided with the beginning of protein repletion (4th to 6th day). Values fluctuated but generally were higher in the acute phase.

In the acute phase, prior to protein feeding (days 1 and 2) although relatively small amounts of essential amino acids were being excreted in free form, bound forms were being lost. The ratio of bound to free forms was significantly higher than for non-essential amino acids. Only a small proportion of histidine was bound. This difference in excretory pattern was most obvious when compared graphically (figs. 38 and 39).

Three out of the four cases had low values of bound valine in the acute phase and in a few specimens negative results were recorded. This suggested that at least part of the valine peak was acid-labile (?peptide). Later experiments (Chapter 11) showed

FIG. 38 EXCRETION OF GLUTAMIC ACID



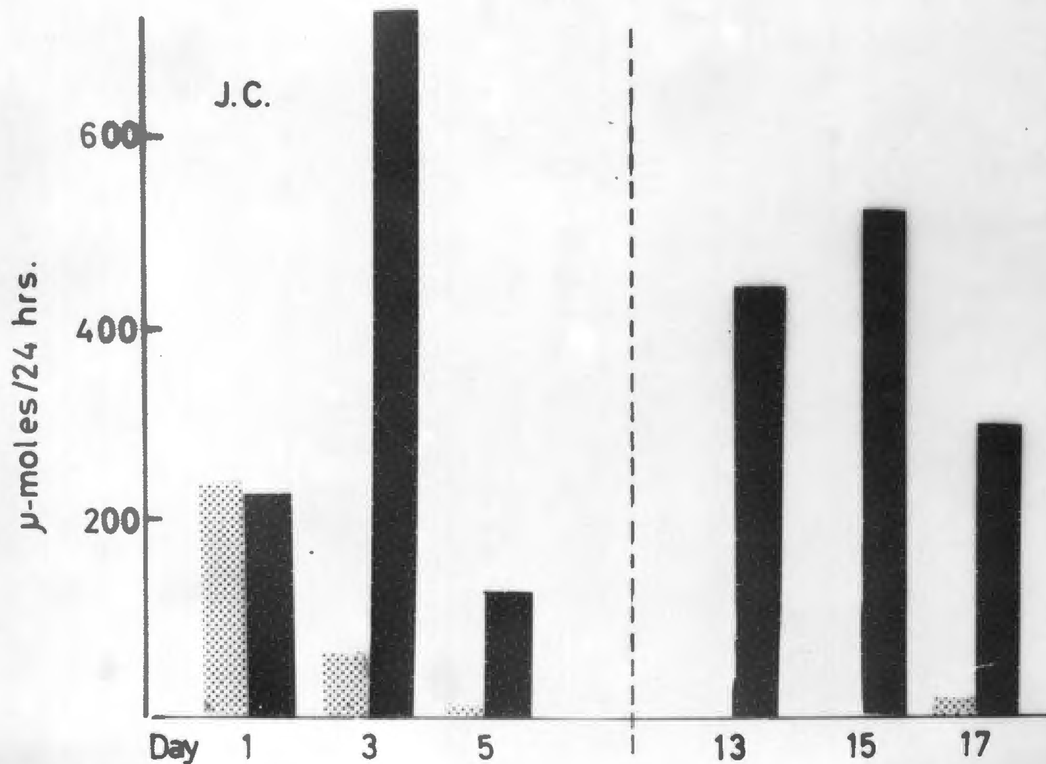
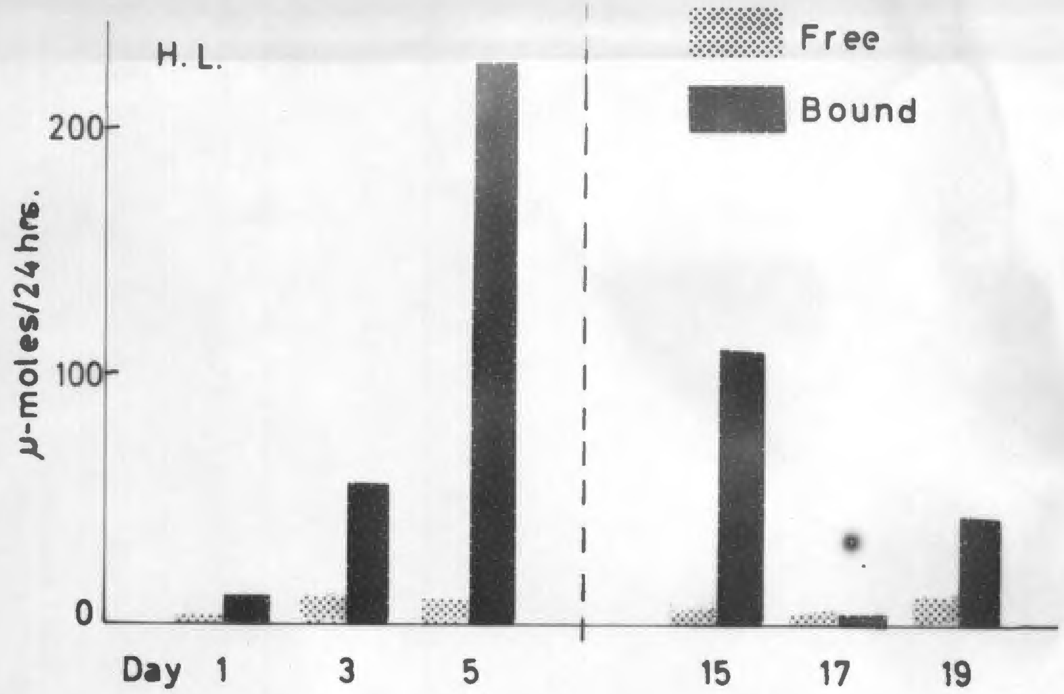
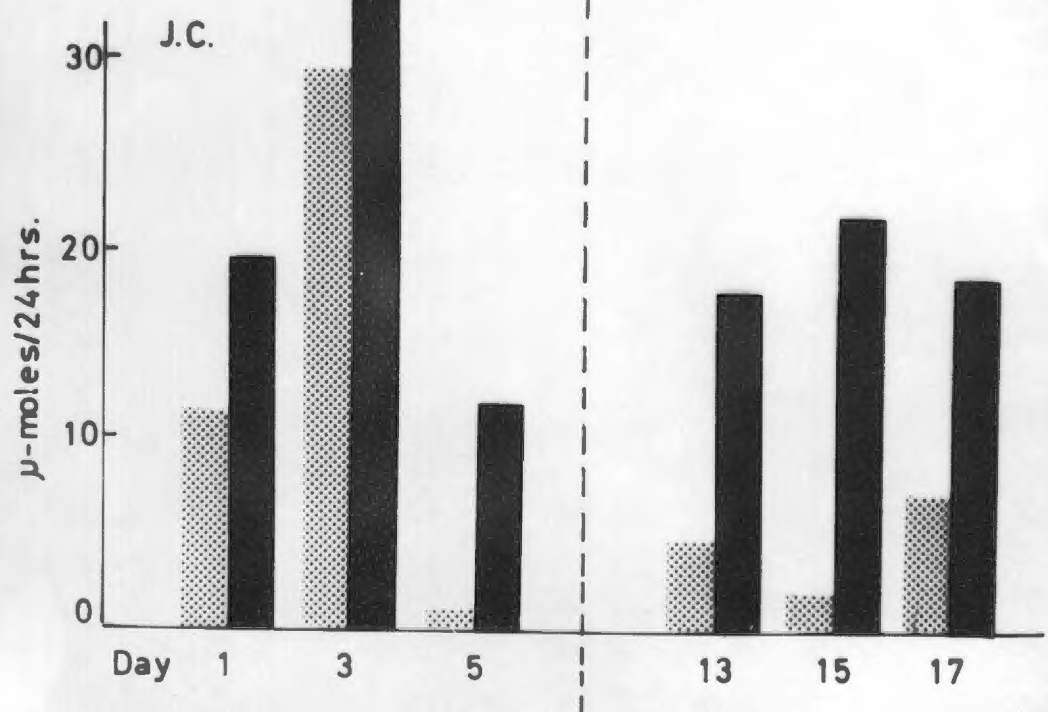


FIG. 39 EXCRETION OF LEUCINE



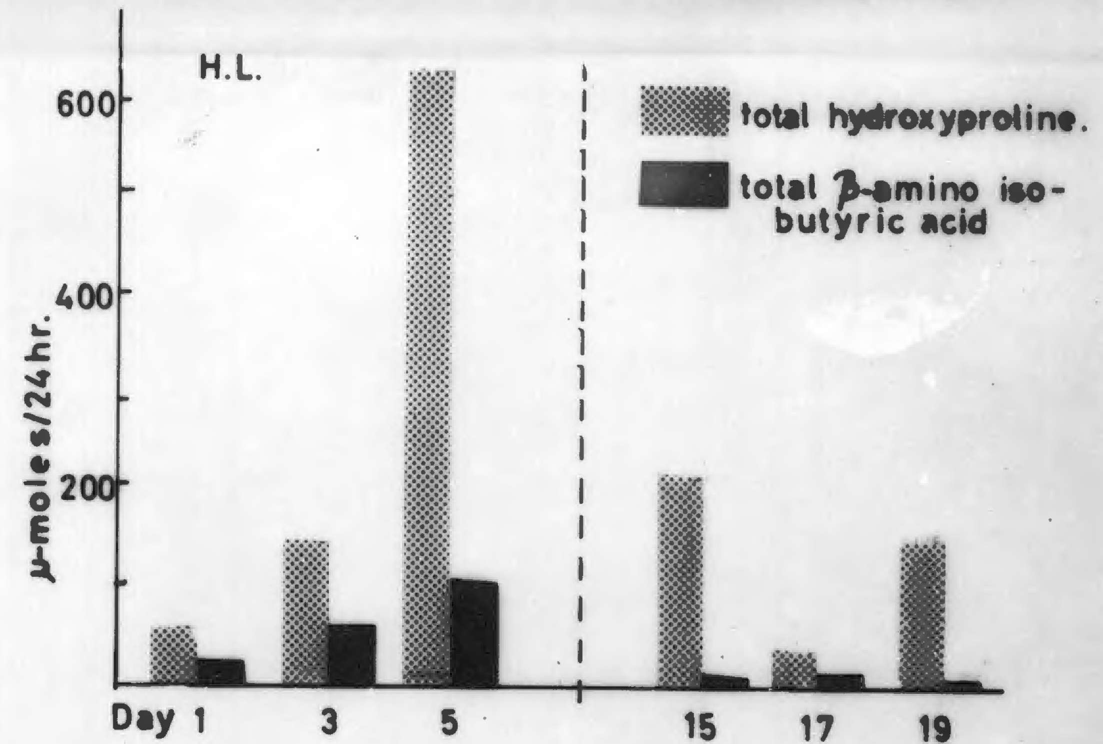


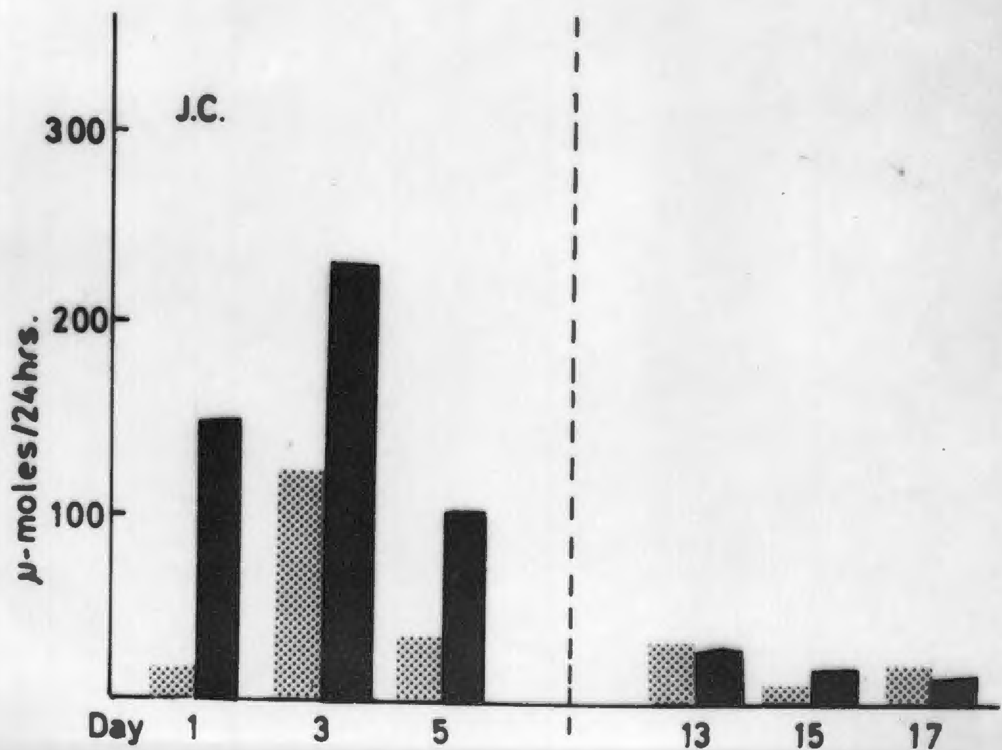
that this was indeed so.

In contrast to the amino acids involved in ordinary protein metabolism, those derived from tissue breakdown were maximally excreted prior to protein feeding, and decreased on feeding. This pattern was noted for hydroxyproline from collagen breakdown, β-amino isobutyrate derived from breakdown of pyrimidines and bound β-alanine from the dipeptides (anserine and carnosine) of muscle (fig. 40).

The results of estimations of free and bound amino acids obtained on specimens during recovery served as controls for those of the acute phase. The only comparable figures available on normals are those of Jonxis and Huisman who studied two normal children as controls during measurement of the urinary excretion of amino acids in rickets[216] and in scurvy[217], by means of chromatography on Dowex 50 cation exchange resin. These were in general agreement with the results repsented above but they found no bound aspartic acid or histidine. In both rickets and scurvy there was an increase in excretion of free serine, threonine, glycine/alanine complex and histidine. The amino aciduria of scurvy was characterised by large amounts of free tyrosine and moderate excess of phenylalanine. The excretion of bound amino acids showed no striking differences from normal except that bound glutamic acid was much increased. In one case there was some increase in some bound amino acids, which Jonxis and Huisman explained as being due to reabsorption of blood proteins from haematomata. The findings in scurvy and rickets are in contrast to those in

FIG. 40 EXCRETION OF CATABOLIC PRODUCTS.





kwashiorkor where increased excretion of bound amino acids was quantitatively more striking that that of free forms. In both rickets and scurvy some weeks of specific vitamin therapy was necessary before the amino aciduria returned to normal. Plasma amino acid levels were normal and it was therefore postulated that reduced renal tubular absorption was responsible for the findings[218].

Chapter 10.

INVESTIGATIONS ON THE SUITABILITY OF POSSIBLE METHODS FOR ISOLATING PEPTIDES FROM THE URINE OF CHILDREN.

Studies on the amino acids present in bound form in urine, reported in the previous chapter, suggested that at least part of the increase in the bound forms of amino acids was present in the form of peptides. It was obvious that the next step was to isolate and identify these peptides.

Review of the literature on methods and results of their application for measuring peptides in urine, both normal and pathological, showed (Chap. 5) that up to the time of writing no method existed whereby all the individual peptides present in urine may be identified and measured. Thus the peptide spectrum of normal urine is unknown.

If these limitations are acknowledged, the techniques of Westall appeared to be most promising, since by this method, over 40 peptides have been identified, and many more acid-labile but unidentified components were found. Westall's technique has been investigated and is described here. Although many peptides were found in normal urine, the volumes required for processing, even if the method could be scaled down precluded their use. Observations on this procedure suggested that peptides of the aromatic amino acids, tyrosine and phenylalanine, were not recovered.

For the above and other reasons, which will be discussed, some new techniques were developed and investigated. Ion-exchange chromatography is a method likely to have the resolution

required to separate complex peptide mixtures. Peptides released by tryptic digestion of oxidised ribonuclease, have been successfully resolved on columns of Dowex 50 (x2)[219]. When this technique was applied to urine[220] however, an extremely complex pattern resulted, due both to the number of peptides and to the presence of relatively large amounts of free amino acids and other ninhydrin-positive material. It seemed that if these interfering substances could be removed, the resulting 'peptide' fraction would be separable by ion-exchange chromatography.

Attempts were made to separate a peptide fraction by the technique of molecular sieving, but proved unsatisfactory owing to low capacity. Finally a method, which combined the advantages of ion-exchange, as well as molecular sieving, produced a neutral peptide fraction, which could be chromatographed by the automatic technique of Spackman et al.

This chapter will describe the experiments, which tested the above methods, and will discuss the theoretical considerations which prompted them.

SOME PROPERTIES OF PEPTIDES.

a. Structure and Composition:

The structure of peptides and proteins can be conveniently described by their primary, secondary and tertiary structures. The primary structure is the sequence of amino acids in the peptide chain. Since most (mammalian) peptides contain up to 18 different amino acids, the number of primary structures is given by N^{18}, where N is the number of residues per molecule.

The factorial could possibly be increased to 22, since the dicarboxylic acids can bind to an $-NH_2$ group via the $-COOH$, or they may be present in the chain as their γ-amides, glutamine and asparagine. It is therefore obvious that even small peptides, having the same amino acid composition and number of residues, may not have identical sequences. There appears to be no general preference for any particular dipeptide sequence. Of the possible 8,000 tripeptide sequences, 1,032 had already been encountered in protein structures by 1959[221]. It is of course impossible to exclude possible 'forbidden' tetrapeptide sequences from the theoretical total of 160,000.

The secondary structure of proteins and peptides describes the manner in which the linear peptide chain is folded in space. In the fibrous structural proteins, such as collagen, the chain is stretched, but most proteins belong to the globular type, in which the chain is folded. The most important forces maintaining secondary structure are disulphide bonds from cysteine residues and hydrogen bonding. The former may be either inter- or intrachain bonds. Tertiary structure is only possessed by proteins having more than one peptide chain, and described the spacial relationship of these chains.

b. <u>Dissociation Constants (pK')</u>:

Dissociation constants of the ionisable groups of peptides are relevent to methods of separating them by ion-exchange methods, since binding to the ion-exchange resin is determined by these values.

The dissociation constants of amino acids and peptides have been determined by potentiometric methods in concentration cells, or by means of hydrogen electrodes in liquid junction cells. These values have been compared and certain trends are evident[222].

Values for pK' (COOH) for mono-amino-monocarboxylic acids is ± 2.0 with a range of 1.83 (phenylalanine) to 2.55 (α-amino n-butyric acid). Proximity of the free $-NH_2$ group facilitated ionisation since when it is removed in peptide linkage, the average pK_1' (COOH) in the peptide rises to $\pm$ 3.0. Peptides are weaker acids and weaker bases than their constituent free amino acids. Similarly, pK_2' (NH_3^+) of the free acids are greater than 9.13 (phenylalanine) and average 9.6 whilst peptides of neutral free amino acids have pK_2' (NH_3^+) less than 8.42 (alanylalanine).

Table 26.

	pK_1' (COOH)	pK_2' (NH_3^+)
Glycine	2.34	9.60
Glycylclycine	3.06	8.13
Tetraglycine	3.26	7.91

More than one carboxylic or basic group has a corresponding effect on the pK values. The adjacent -COOH group of aspartylglycine increases pK_3' (NH_3^+) to 9.07. In general ionisable groups of non-substituted side chanis are unaffected by peptide linkages. Table 27 illustrates these points.

Table 27.

Ionisation constants of amino acids and peptides.

Substance	pK_1'	pK_2'	pK_3'	pK_4'
Glycine	2.34 (COOH)	9.60 (NH_3^+)	-	-
Glycylglycine	3.06 (COOH)	8.13 (NH_3^+)	-	-
Triglycine	3.26 (COOH)	7.91 (NH_3^+)	-	-
Tyrosine	2.20 (COOH)	9.11 (NH_3^+)	10.07 (OH)	-
Cystine	1.71 (COOH)	8.33 (NH_3^+)	10.78 (SH)	-
Aspartic acid	1.88 (COOH)	3.65 (COOH)	9.60 (NH_3^+)	-
Aspartylglycine	2.10 (COOH)	4.53 (COOH)	9.07 (NH_3^+)	-
Glutathione	2.12 (COOH)	3.53 (COOH)	8.66 (NH_3^+)	9.12 (SH)
Arginine	2.17 (COOH)	9.04 (NH_3^+)	12.48 (Guan.)	-
Phynylalanyl-arginine	2.66 (COOH)	7.57 (NH_3^+)	12.40 (Guan.)	-

These figures are of practical value when predicting the behaviour of these substances during ion-exchange chromatography.

1. PRELIMINARY INVESTIGATIONS UNDERTAKEN TO SELECT A SUITABLE METHOD OF IDENTIFYING URINARY PEPTIDES IN CHILDREN.

A. TECHNIQUE OF WESTALL[155].

This general method of examining urine for peptides and other ampholytes, was first described by Westall in 1956. The method (as discussed in Chapter 5) has the advantage of high capacity, thus yielding milligram quantities of individual peptides, sufficient for characterisation.

Westall has not examined more than one urine and has not pursued this work further. The method, with minor modifications was repeated on normal adult urine in order to assess its suitability for use in the project.

(i) Specimen:

All the urine passed by a young adult male laboratory worker, over a period of twelve days was collected in polythene bottles, and stored in a refrigerator at 4°C without preservative. The total volume of the specimen was 24.80 litres.

(ii) Concentration:

The urine specimen was concentrated in six batches; each of approximately 4 litres in volume. This procedure was performed in a 'Cyclone' evaporator under negative pressure, which was maintained by means of a waterpump (fig. 41). The temperature of the concentrate was kept below 45°C. The urine was fed into the evaporator continuously to balance evaporation and caprylic alcohol was added to prevent frothing. The total volume of the concentrate was 2,310 ml., which was increased to 2,510 ml. with

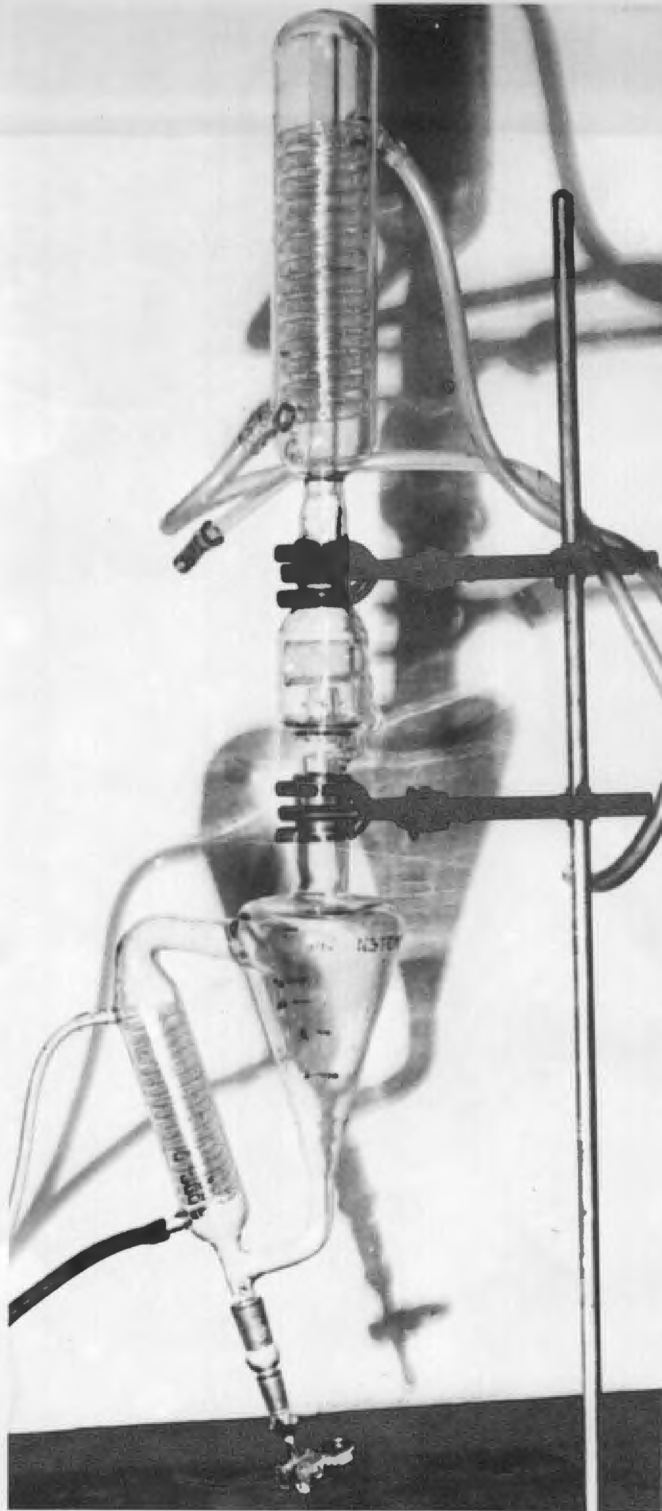


Fig.41. "CYCLONE"EVAPORATOR

washings and transfer.

(iii) Removal of Mucoproteins, excess salts and Urochrome pigments by means of ethanol precipitation and adsorption onto activated charcoal.

Aldehyde-free, absolute Ethanol (7.5 litres) was added to the concentrated urine resulting in a final concentration of the alcohol of 25% v/v. This mixture was stirred and allowed to stand overnight at 0°C. The mucoproteins and phosphates were precipitated, and after centrifugation, the supernatant solution was again concentrated on the cyclone evaporator to a syrupy consistency (approximately 1 litre) to remove the alcohol.

One hundred grams of finely powdered animal charcoal was activated by shaking for 1 hour with 5% v/v glacial acetic acid in a mechanical shaker. The charcoal suspension was filtered on a Buchner funnel and washed with distilled water until the effluent was neutral.

The syrupy concentrate was diluted to 3 litres and 100 g. of activated charcoal added, shaken in a mechanical shaker for 1 hour, filtered and washed with five 200 ml. aliquots of distilled water. The total volume (4 litres) of urinary concentrate was kept at 0°C until it was applied to the chromatography columns. This solution had a brown colour, which had not been appreciably diminished by charcoal adsorption. The charcoal was kept after filtration and when this was dried in a desiccator, highly refractile cystals were noted. One gram of the charcoal, suspended in 5 ml. of 0.1N, HCl and an aliquot (0.1 ml) of the supernatant was

subjected to one dimensional ascending paper chromatography in Butanol/Acetic acid/water. At least two intense ninhydrin-positive spots whose mobilities corresponded to those of tyrosine and phenylalanine were detected. Quantitation was not attempted. The rest of the charcoal was discarded.

Preparation of Chromatographic Columns:

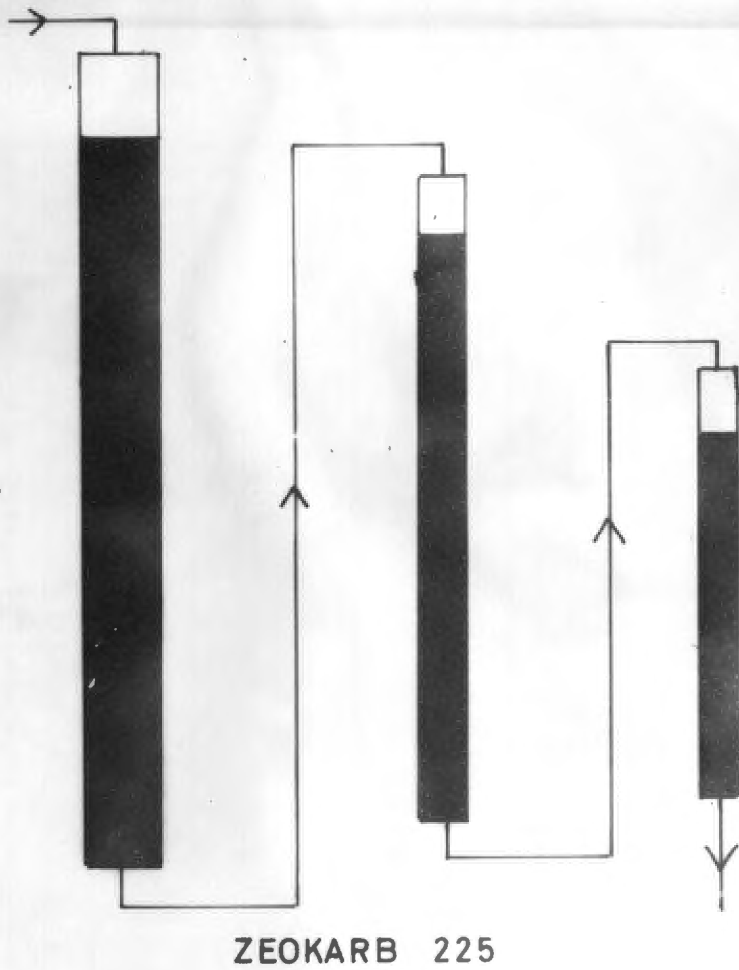
Two serial chromatographic procedures have been used: cationic and anionic exchange resins being employed respectively.

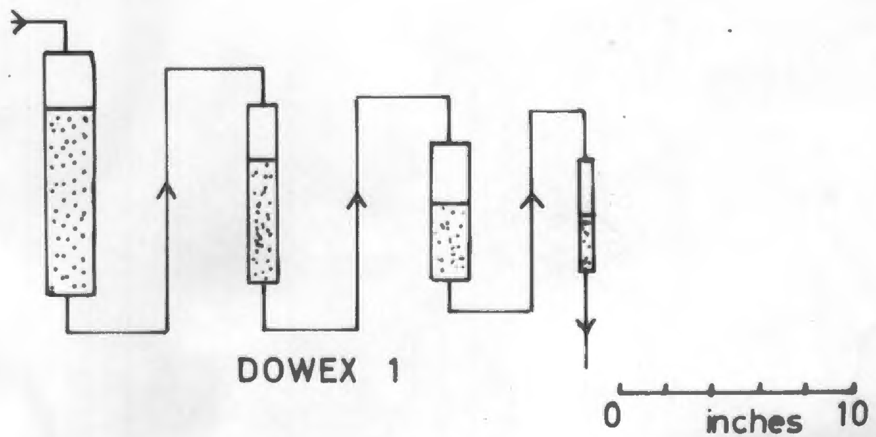
One kilogram of Zeokarb 225 (30 - 60 mesh) was fractionated by dry sieving. This fraction was treated with 1N HCl at room temperature overnight, then poured in a slurry to form three columns, 3"x 40", 2" x 24" and 1.5" x 15" respectively. These were then connected in series by means of narrow bore glass tubing (see fig. 42). Five litres of distilled water was used to wash the resin and then the resin converted to the NH_4^+ form with 10 litres of 1N aqueous ammonia at a rate of approximately 2 L. per hour. When the effluent became alkaline the columns were washed with 5 litres distilled water. Five litres of 0.2N HCl regenerated the resin to the H^+ form. Excess HCl was removed by washing with 8 litres of water till the effluent rose to pH 5. During the final washing the resin expanded and it was necessary to remove the excess from the top of the column.

Application of the Urinary Concentrate to the Columns.

The concentrate (4 litres) was diluted to 50 L., i.e. twice the original sample volume. This was then applied to the column system under slight positive pressure (1-2 lbs./ sq. inch).

FIG. 42 ION EXCHANGE COLUMNS (WESTALL)


ZEOKARB 225



DOWEX 1

The resin in the first column shrank slightly but no pigment was detected in the head of liquid over the second column, until approximately 35 litres had been applied. There was thus no possibility of overloading the ion exchange capacity of the resin bed. The resin was then well washed with 10 litres of water and the washings discarded.

Elution of Ampholytes.

This was accomplished with 0.2N NH_3, at a rate of 500-600 ml. per hour. The effluent was collected in 25 ml. fractions in an automatic fraction collector. Progress of chromatography was gauged by passage of a pigment band down the column and elution into the head overlying the succeeding column. The NH_3 front was also characterised by local heat production. Pigment appeared over the second column after 15 litres and was completely eluted after 20 litres. After 26 litres the free α-NH_2 Nitrogen content of the effluent rose sharply. This changed from a value of 4.8 mg. (α-NH_2 N) per ml. to greater than 50 mg/ml. within the space of 4 fractions. The fractions were numbered from the last tube having low α-NH_2 N content. Free ammonia was detected (by smell) in tube 67. Qualitative analysis of fractions was performed by spotting 0.1 ml. aliquots of even numbered fractions onto 18" x 22.5" Whatman No. 1 Filter paper sheets, and developing the papers by ascending partition chromatography overnight in Butanol/acetic acid/water (4 : 1 : 5 v/v/v mixture). The following morning the papers were dried in an air stream and dipped into 0.25% Ninhydrin in acetone which contained 1% Pyridine (v/v) and then developed at 90°C

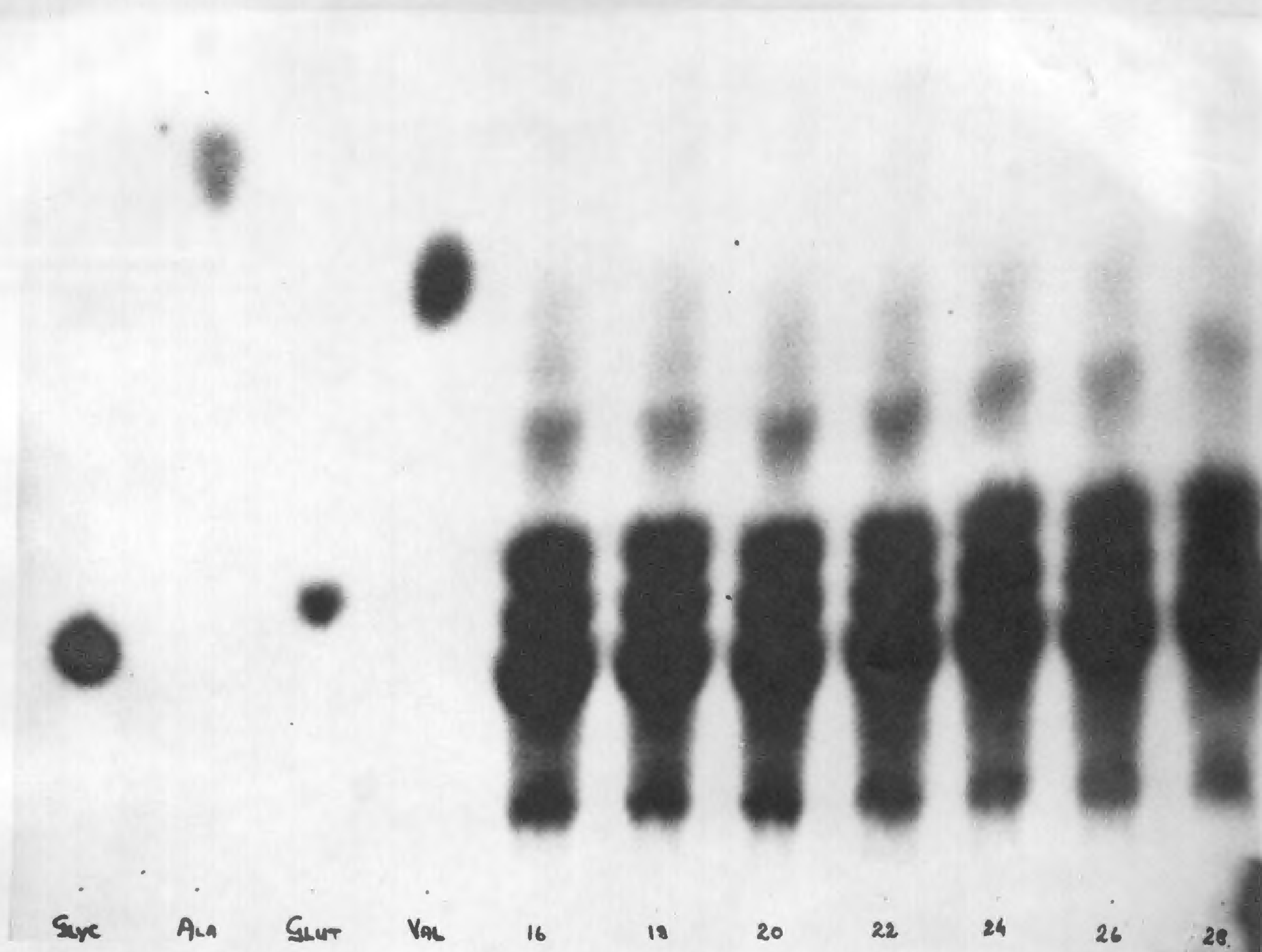


Fig. 43. Single dimension paper chromatography (Bu/HAc/H_2O) of fractions from Zeokarb 225 columns. Each fraction contains multiple components and adjacent fractions have similar composition.

in an oven, until the background started to show faint traces of colour. A separate set of chromatograms was stained by means of the Jaffe's reagent for creatinine. The papers were dipped in 1% Picric acid in 96% ethanol and then allowed to dry; then dipped in 5% NaOH in 80% ethanol, and dried.

Aliquots of solutions of known amino acids (1.5mg/100 ml) were chromatographed concomittantly.

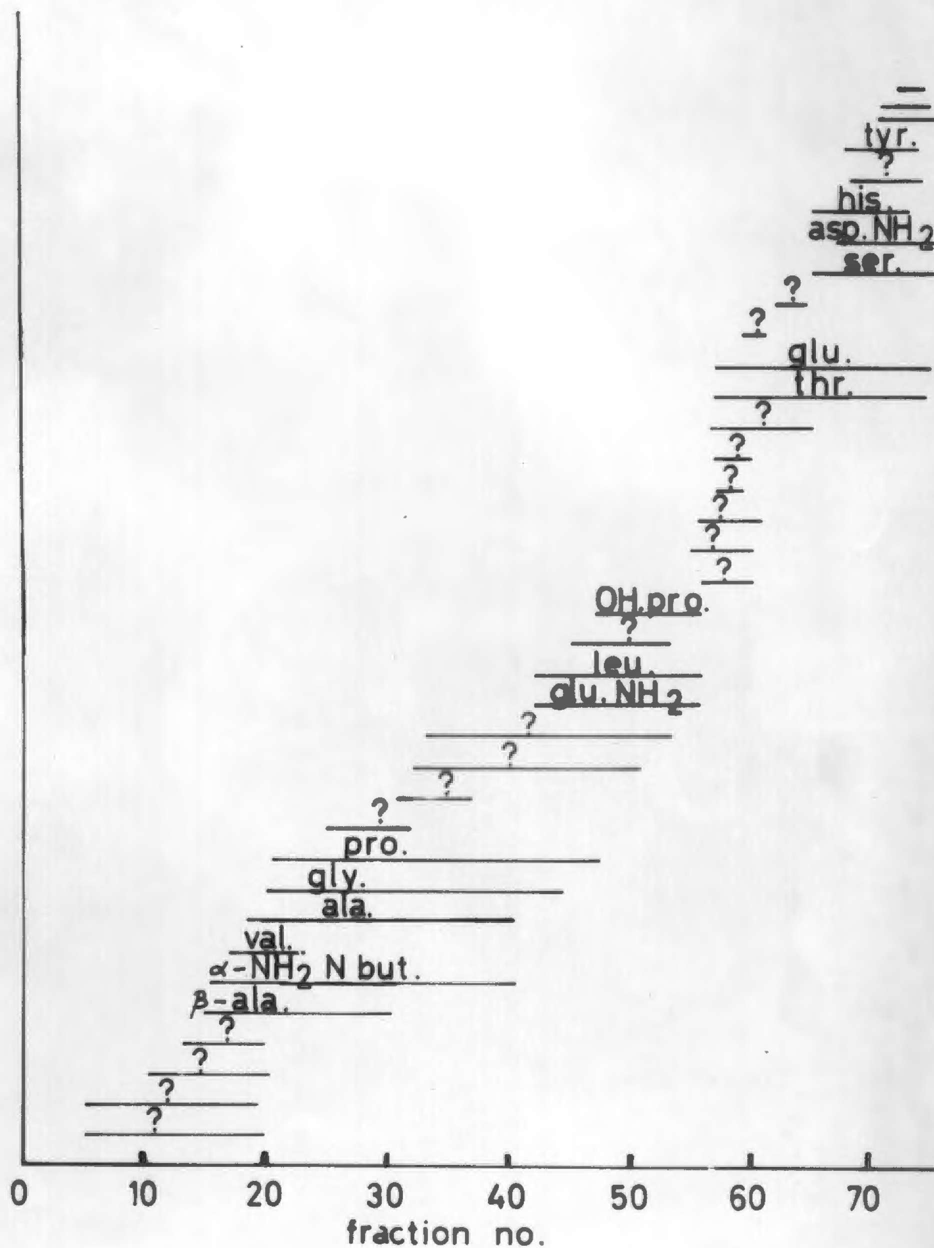
Ninhydrin-positive material was found in fractions 5 to 75 and in most fractions numerous spots were present on the paper (see fig. 43). Provisional identification of the commonly occurring amino acids was established, based on the Rf value in the solvent and their expected order of elution from the clumn. The distribution of commonly occurring amino acids and other unknown ninhydrin-positive compounds in the fractions collected is shown in figure 44.

Fractionation on Dowex 1, Anion Exchange Resin.

Four glass chromatography columns, 2" x 8", 1.25" x 4.5", 0.75" x 3" and 0.35" x 2.25" were connected serially with thin glass tubing as illustrated in Fig. 42. The largest column contained approximately 30 mesh Dowex 1 resin, the smallest 250-300 mesh, and the remaining two were filled with 60 - 90 mesh resin.

The resin was mixed into a slurry in N-HCl and then washed with water. After standing overnight in 2N-NaOH, it was washed well with CO_2-free water, poured as a slurry into the columns and again washed with CO_2-free distilled water until the effluent p^H fell to less than 8. This required 7.5 litres of water.

FIG.44 ELUTION OF URINARY AMPHOLYTES FROM ZEOKARB 225



Fractions 21 to 72 inclusive, obtained from the eluate of the cation exchange column were bulked and heated rapidly to 60°C under reduced pressure to remove dissolved CO_2. Storage under liquid paraffin prevented reabsorption of gas. The total volume of the sample was 1,300 ml. This was run into the column system at a rate of 500 ml/hr. The columns were washed with 1 litre of CO_2-free distilled water and then eluted with 0.1N HCl. Amino acids and other ampholytes caused expansion of the resin and their passage was followed by this and by the elution of pigment. Elution was performed at a rate of 100 - 150 ml/hr., and the eluate was collected in 25 ml. fractions as soon as swelling of the resin in the last column was noted. The fifth fraction contained ninhydrin-positive material (paper spot testing). A total of 83 fractions was collected, the last of which contained chloride ions. Each of these fractions was examined by single way ascending chromatography in Butanol/ Acetic Acid/Water solvent as before. The results are shown in table 28. The ninhydrin-positive spots have been numbered according to order of emergence of that ampholyte from the column (table 28) and from their Rf in the chromatographic solvent.

TABLE 28.

RESULTS OF ONE WAY CHROMATOGRAPHY OF EFFLUENT OF ANION EXCHANGE COLUMN DOWEX 1 (X8).

SPOT NO.	FRACTION No.	Rf. ≁	NINHYDRIN COLOUR.	REL. ≁≁ AMOUNT	PROVISIONAL IDENTIFICATION.
1	6-20	0.05	Violet	10	?
2	5-19	0.17	Violet	40	?
3	12-21	0.19	Blue-Violet	70	?
4	13-20	0.15	Blue-Violet	40	?
5	15-24	0.26	Purple	20	?
6	16-41	0.40	Purple	20	α-NH_2-N BUTYRIC
7	17-23	0.50	Purple	30	VALINE
8	18-41	0.32	Purple	70	β-ALANINE
9	20-44	0.22	Brown	50	GLYCINE
10	25-32	0.26	Purple	20	ALANINE
11	31-37	0.29	Purple	15	?
12	32-53	0.55	Purple	35	?
13	36-56	0.37	Purple	70	?
14	42-56	0.17	Blue-Purple	80	GLUTAMINE
15	42-56	0.67	Purple	40	LEUCINES
16	21-45	0.15	Pale Yellow Brown	10	PROLINE
17	45-53	0.13	Blue-Purple	20	?
18	47-56	0.18	Brown	75	HYDROXY-PROLINE
19	55-60	0.50	Purple	10	? TYROSINE
20	56-60	0.09	Purple	20	?
21	55-61	0.12	Purple	20	?
22	57-59	0.09	Purple	10	?
23	57-60	0.41	Purple	20	? TYROSINE
24	57-65	0.17	Purple	10	?
25	57-62	0.22	Purple	20	?
26	57-56	0.34	Purple	30	GLUTAMIC ACID
27	63-76	0.38	Purple	90	?
28	59-61	0.14	Pink	10	?
29	62-64	0.14	Blue-Purple	10	?
30	65-77	0.23	Blue-Purple	50	SERINE
31	67-77	0.17	Brown	50	ASPARAGINE
32	68-70	0.14	Blue	10	?
33	68-72	0.54	Purple	10	?
34	68-73	0.48	Purple	10	?
35	71-76	0.59	Yellow	40	?
36	74-76	0.25	Yellow-Brown	30	?
37	72-77	0.15	Purple	20	?
38	71-75	0.65	Purple	10	?

Subscript to Table: ≁ in Bu/HAc/H_2O.
≁≁ Arbitrary Scale 0-100 assessed visually.

ATTEMPTS AT IDENTIFICATION OF AMPHOLYTES PRESENT IN URINE, FROM THE ELUATE OF ANION EXCHANGE COLUMN.

Owing to the fact that adjacent fractions give similar patterns on examination by single way chromatography, they were bulked to yield a large fraction designated by the following letters:

Bulk fraction.	No. of 25 ml. fraction. (inclusive)
A	5-9
B	10-14
C	15-18
D	19-23
E	24-28
F	29-33
G	34-37
H	38-41
I	42-46
J	47-51
K	52-56
L	57-60
M	61-62
N	63-67
O	68-70
P	71-73

The bulked fractions were examined before and after acid hydrolysis.

After desiccation, an aliquot equivalent to 0.10 ml. was spotted onto a sheet of 18" x 22.5" Whatman No. 1 filter paper. This will be referred to as the free forms of ampholytes or the unhydrolysed sample.

Hydrolysis of 1.0 ml. of sample, with 1.0 ml. of conc. HCl was performed in sealed glass ampoules, overnight at 105°C (16 - 18 hrs). The hydrolysate was transferred quantitatively to a 15 ml. beaker via a glass wool filter to remove humin and dried over KOH pellets in a vacuum d siccator. After reconstitution

with water, an aliquot, equivalent to 0.10 ml. of original bulked fraction was spotted onto paper.

Chromatography on the 18.5" x 22" sheets was performed first in Butanol/Acetic Acid/Water (60:15:26) overnight by descending technique at a constant temperature of 25°C. After air drying the second solvent, Phenol:Water: Ammonia (400 : 100 : 50, v/v/v) was run at right angles, overnight. After drying in air, the paper sheets were dipped in 0.2% (w/v) ninhydrin in Acetone containing 1% (v/v) pyridine. Colour development was allowed to take place at room temperature overnight. The papers were then heated to 95°C in an oven for 4-5 minutes. Any further spots appearing were marked O.H. (on heating).

The papers were photographed and representative examples are reproduced in fig. 45.

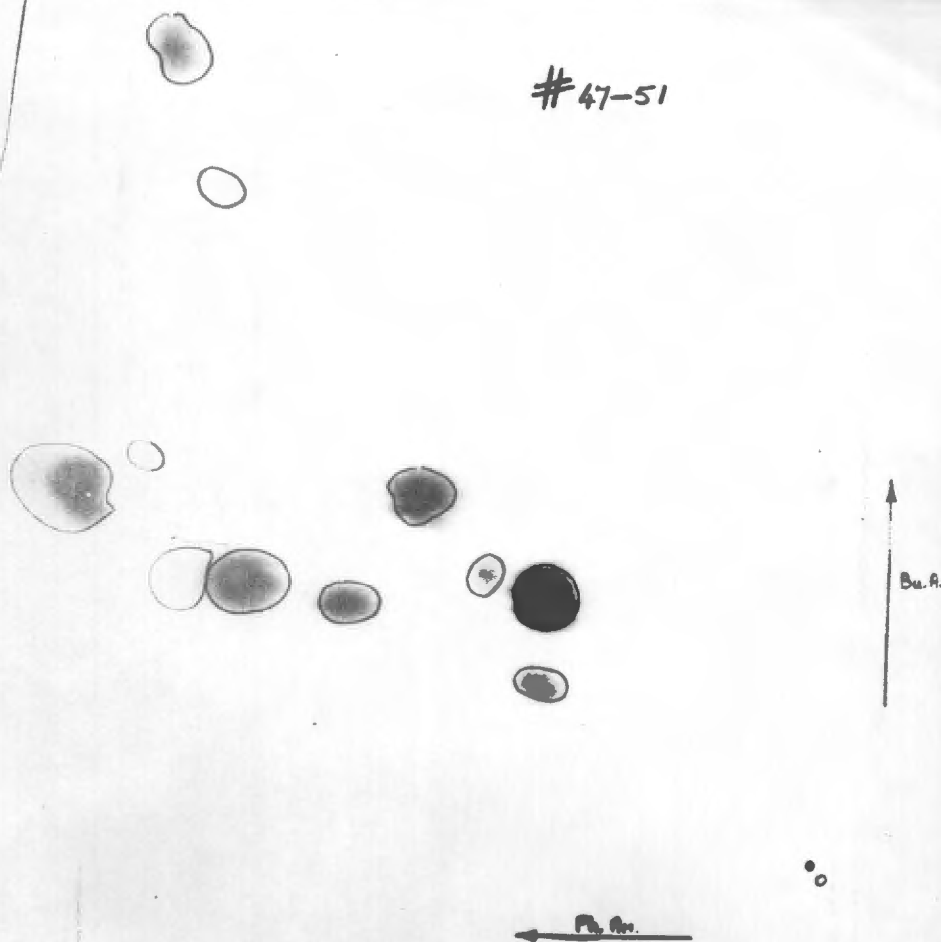


Fig. 45. Two dimensional paper chromatography of bulked fractions (47 - 51) from Dowex I columns.
1st solvent: Bu/HAc/H_2O
2nd solvent: Phenol/NH_3/H_2O.

RESULTS OF CHROMATOGRAPHY:*, **

(i) Bulk Fraction A (5 - 9)

Predominant spot (0.14, 0.68) corresponds to lysine and ornithine. Present in fractions B and C as well.

Spot No. 1: (1.0, 0.26) Ninhydrin - purple.
Appears in fractions A to E. Corresponds to position of ethanolamine. Disappears rapidly on heating at 100°C.

Spot No. 2: To right of Spot 1. Fractions A to E. Very faint; Resists acid hydrolysis.

Spot No. 3: (0.04, 0.41). Ninhydrin-purple. Ehrlich-red. Elongated in Ph. Am solvent. Disappears after acid hydrolysis.

Spot No. 4: (0.32, 0.93) In position occupied by proline, but purple spot with ninhydrin. Not fluorescent. Corresponds to Westall's compound 1. ? Picolinic acid.

Spot No. 5: (0.65, 0.67) Below lys/orn. Spot similar position to histidine and α,γ-diamino-n-butyric acid but is acid labile.

Spot No. 6: Fractions A to E (0.49, 0.75). Corresponds to valine.

(ii) Bulk Fraction B (10 - 14)

Contains spots 1, 2, 3 and 5 as above, and in addition:-

Spot No. 6: (B & C only) (0.07, 0.46). Ninhydrin purple. Resistant to acid hydrolysis.
Rf's correspond to (i) Asparagine which is acid labile, and gives a brown spot with ninhydrin;
(ii) Djenkolic acid (contains S) (0.09, 0.30) or hydroxylysine (0.11, 0.58).

Spot No.7: (0.15, 0.65). (Fractions B and C). Result of hydrolysis difficult to assess as it is close to spot of lys/orn and trailing would mask it is present. Possibilities include ornithine, histidine.

Spot No. 8: (0.60, 0.17) gives an immediate (in cold) purple colour with ninhydrin. Similar possibilities to spot 7. Most likely is ornithine.
After acid hydrolysis, additional spots appear in positions corresponding to glycine, serine and glutamic acids.
Spot 3 and possibly spots 5 and 7 are acid labile.

---

* Fractions in brackets are Rf's in 1st and 2nd solvents respectively.
** Described with origin of chromatogram in bottom left-hand corner.

Fraction C (15 - 18).

Amino acids present include:

Glycine, (0.23, 0.40) in fractions C to K.
Phenylalanine (0.54, 0.76) in fractions C to F.
Alanine (0.35, 0.60) in fractions C to I.

Spots 1, 2, 3, 6, 7 and 8 described above.

Spot No. 8a: (0.22, 0.81) Arg. is (0.15, 0.86) ninhydrin purple. Appears in cold. Acid stable. Probably arginine.

Spot No. 9: (0.26, 0.60) Ninhydrin purple on heating only. Disappears on acid hydrolysis. Seen in C only.

Cf: γ-Methylglutamine (0.26, 0.68)
and γ-Methylene glutamine (0.23, 0.69)

Probably a peptide or substituted amino acid.

β-Alanine (0.39, 0.67) Ninhydrin purple on heating only. In fractions C, D & E.

α-Amino-N-butyric acid (0.33, 0.79) ninhydrin purple (on heating) only).
Present in fractions C and D; not affected by acid hydrolysis.

Methionine sulphoxide (0.30, 0.80) Acid stable.

Spot No. 10: (0.58, 0.81) Above phenylalanine.
In fractions C and D. Appears on heating with ninhydrin only.
Possibilities are

α-amino-n-valeric acid (0.56, 0.77)
δ-amino-n-valeric acid (0.50, 0.84)
Ethionine (0.58, 0.84)

Spot No. 11: (0.44, 0.88) Ninhydrin purple. Appears in the cold. Destroyed on acid hydrolysis. In fractions C, D and E. Substances having similar Rf's include

Felinine (0.44, 0.85)
β-Amino-N-butyric acid (0.42, 0.84)
Phenylglycine (0.44, 0.93)

Spot No. 12: (0.48, 0.89) In fraction C only. Resistant to acid hydrolysis. Identity - similar possibilities to Spot No. 11.

Fraction C (15-18) contains four acid-labile spots (3, 9, 10 and 11)- probably peptides

Serine, glutamic acid and threonine appear, and Cysteic acid and aspartic acid spots are increased in intensity after acid hydrolysis. A large spotas intense as alanine, appears next to alanine at (0.33, 0.25). The purple colour with ninhydrin appears on heating only. No similar spots occurred in unhydrolysed fractions. Probable identity is β-alanine.

Fraction D (19 - 23).

The amino acid glycine, alanine, methionine, -alanine, hydroxyproline, γ-NH_2-N-butyric acid, phenylalanine and lysine and unknown spots 1, 2, 10 and 11.

Spot No. 12: (0.51, 0.52) Faint yellow colour with ninhydrin on heating only. Disappears on hydrolysis.

Spot No. 13: (0.16, 0.12) Ninhydrin purple on heating only - trace amount - cannot be distinguished after hydrolysis.

On hydrolysis, serine, glutamic acid, proline, aspartic acid and β-alanine appear.

Fraction E (24 - 28).

The amino acids glycine, alanine, phenylalanine, β-alanine and hydroxyproline, arginine and ornithine/lysine are present plus

Spot No. 14: (0.37, 0.76) - acid labile.

Spot No. 15: (0.39, 0.81) ninhydrin purple.
Possible identity

γ-Methyl-glutamine (0.26, 0.68)
5-hydroxypipicolinic acid (0.29, 0.71)

Not acid labile - present in F & G as well.

On hydrolysis of the whde fraction, aspartic and glutamic acids are liberate.

Fraction F (29 - 33).

Contains free amino acids, glycine, alanine, methionine, valine, phenylalanine and hydroxyproline, plus spots 11 and 15 and

Spot No. 17: (0.10, 0.47) Acid labile.

On hydrolysis aspartic acid and group of basic amino acids (histidine and lysine) are liberated.

Fraction G (34 - 37).

Free amino acids glycine, valine and leucine are present. On hydrolysis the basic amino acids are liberated.

Fraction H (38 - 41).

Contains free amino acids, glycine, serine, glutamine, threonine, alanine, valine, phenylalanine, leucine, N-Methyl-histidine and

Spot No. 18: (0.22, 0.72) Ninhydrin purple. Appears in cold Resists acid hydrolysis.

Spot No. 19: (0.26, 0.78) Ninhydrin purple. Appears in cold. Acid stable.

Spot No. 20: (0.35, 0.80) Ninhydrin purple. Appears on heating only. Destroyed on acid hydrolysis.

Spot No. 21: (0.13, 0.60) Ninhydrin purple. Appears in cold. Destroyed on acid hydrolysis.

Spot No. 22: (0.19, 0.58) - same characteristics as 21.

Spot No. 23: (0.21, 0.65) - ibid.

On hydrolysis of fraction H, spots 21, 22, 23, 19 and 20 disappear and glutamic acid and hydroxyproline appear.

Fraction I (42 - 46).

Contains free amino acids glycine, serine, glutamic acid, threonine, alanine, valine, phenylalanine, N-Methyl histidine and ? peptides 18, 19 and 21 plus:

Spot No. 24: (0.09, 0.37) Purple with ninhydrin in cold. Destroyed on acid hydrolysis.

Spot No. 25: (0.07, 0.28) - ibid.

On hydrolysis of fraction I, spots 18, 19, 21, 24 and 25 disappear and aspartic acid appears. The serine spot is increased in intensity and traces of proline and hydroxyproline are liberated.

Fraction J (47 - 51).

Free amino acids: serine, glycine, threonine, phenylalanine, leucine, histidine and glutamine.

Possible peptides 18, 19, 21 and 24.

On hydrolysis of the fraction, glutamine, disappears and aspartic acid, glutamic acid and hydroxyproline appear.

Fraction K (52 - 56).

Free amino acids: serine, threonine, histidine, N-methyl histidine, phenylalanine, leucine,glutamine and aspartic acid.

Possible peptides 18, 19, 21 and 24 and

Spot No. 26: (0.28, 0.69) Acid labile.

Spot No. 27: (0.16, 0.25) Acid labile.

Spot No. 28: (0.10, 0.25) Acid labile.

Spot No. 29: (0.18, 0.52) Acid stable.

On hydrolysis 21, 24, 26, 27 and 28 disappear whilst glutamic and aspartic acids and hydroxyproline appear.

Fraction L (57 - 60).

Free amino acids Serine, threonine, N-methyl histidine, tyrosine, asparagine and cystine, possible peptides 21, 24, 28 and 29 and

Spot No. 30: (0.10, 0.26) Acid stable.

Spot No. 31: (0.03, 0.21) Acid stable.

Spot No. 32: (0.06, 0.13) Acid labile.

Spot No. 33: (0.50, 0.72) Ninhydrin brown - disappears on acid hydrolysis.

On hydrolysis glutamic acid, aspartic acid, valine, alanine, β-alanine and lysine.

Fraction M (61 - 62).

Free amino acids are phenylalanine, glutamic and aspartic acids.

Major spot is

Spot No. 34: (0.38, 0.28) Ninhydrin complex is bright pink and appears in cold. Acid labile.

Spot No. 35: (0.39, 0.32)
Possibilities are α-amino adipic acid (0.30, 0.30)
α-amino pimelic acid (0.35, 0.35)
and γ-methyl glutamic acid (0.33, 0.31)

On hydrolysis, glutamic acid, aspartic acid, glycine, serine, threonine, alanine, tyrosine, phenylalanine, leucine and cystine derivatives appear.

Fractin N (63 - 67).

Free amino acids are glutamic and aspartic acids and:

Spot No. 26: (0.33, 0.51) Ninhydrin complex is yellow and develops in the cold. Acid labile.

Spont No. 37: (0.46, 0.38),

Spot No. 38: (0.50, 0.44),

Spot No. 39: (0.53, 0.53),

Spot No. 40: (0.22, 0.70),

Spot No. 41: (0.12, 0.55) and

Spot No. 42: (0.06, 0.57):

are all acid labile.

On hydrolysis aspartic acid, cysteic acid are increased and glycine, β-alanine, hydroxyproline and lysine/histidine complex are liberated.

Fraction O (68 - 70).

Glutamic acid is present as well as spots 35, 36, 37 and 38.

Several pigment spots which are poorly defined are destroyed on hydrolysis.

Apart from the ampholytes detailed above, a further 19 discrete spots were distributed throughout the fractions. These were acid-stable and give purple complexes on prolonged heating with ninhydrin. They were, however, barely visible against the background colour and were masked by neighbouring spots when attempts were made to overload the paper.

The distribution of the major free amino acids in the fractions is shown diagramatically in fig. 44.

Comments on the suitability of Westall's procedure.

The procedure was repeated and confirmed that a large number of ninhydrin positive compounds occur in normal urine. All the amino acids occurring in protein hydrolysates (excluding tryptophan) have been identified, as well as 42 additional compounds many of which were acid labile. The method with its high capacity appears to be ideal for investigating nitrogenous compounds which occur in very small quantities in urine. Several considerations, however, preclude its use in the present investigation.

The large volumes of urine required, even if the method was scaled down 10 fold, would require approximately 7 days' collection. The dynamic processes which are to be investigated have much shorter response periods.

Sequential resolving systems each add another dimension to the resolving power of the system, thus aiding identification. The losses are, however, accumulative but would not interfere with a semiquantitative study. During the charcoal adsorption process a large amount of tyrosine (and possibly other aromatic ampholytes) was removed. It is also probable that not insignificant amounts of material would be lost during precipitation of calcium phosphate and other salts in cold 75% ethanol.

Difficulties in identification of spots with closely related Rf's have been noted. The capacity of the final paper chromatographic steps is low and several papers would have to run concommitantly in order to yield sufficient material for confirmatory steps.

The investigation took 3 months to complete and is therefore not a practical procedure for serial investigations.

Experiments Designed to Investigate the usefulness of Molecular sieving, applied to Urine, as a method for isolating peptides from urine.

Urine is an extremely complex solution and it would appear that the number of compounds (N) which can be isolated, is directly related to the volume of urine that is processed (V); all positive values are possible if

$$N = \int_{V=0}^{V=\infty} \frac{dN}{dV}$$

This concept has limited practical value, since the methods used, have limited capacity and resolving power, and are sensitive to overloading.

The free amino acids, urea, creatinine and ammonia, form the bulk of ninhydrin reacting material in urine. Although there are by weight more amino acids present in the bound form than free, the colour yield of the former in the ninhydrin reaction is less by several orders of magnitude.

The problem could be resolved by a preliminary procedure whereby the free amino acids are removed from the bound forms, allowing greater volumes of urine containing only bound forms to be handled.

The technique of molecular sieving, by means of which the small molecules (urea, creatinine and amino acids) could be removed from larger ones (peptides) on a molecular weight basis, appeared to be an attractive avenue of investigation. Several experiments were performed to explore this possibility.

The technique of molecular sieving is based upon selective penetration of solute molecules into the matrices of swollen

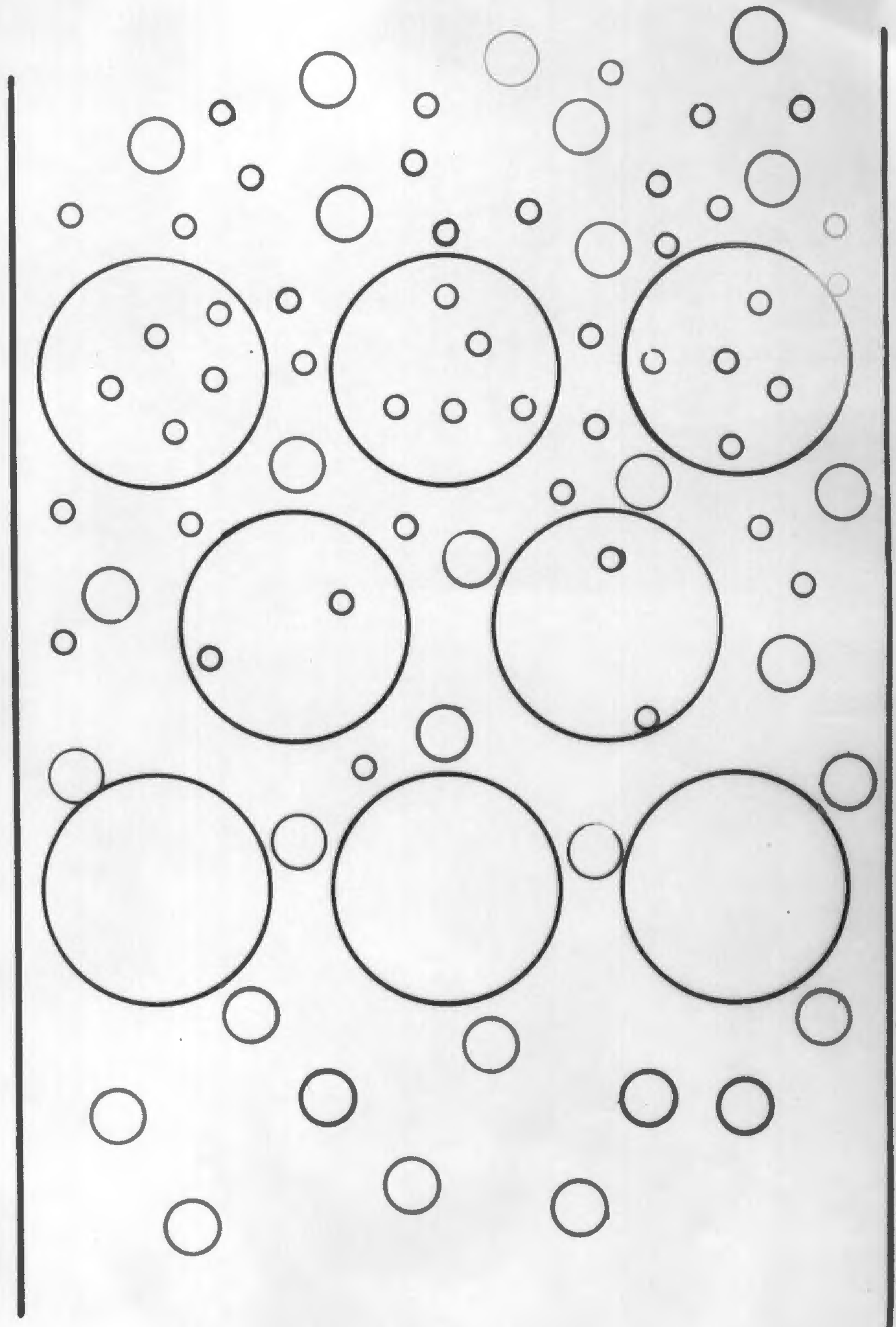
inert particles. Large molecules are completely excluded, whilst smaller ones penetrate at a rate which has an inverse relationship to molecular size (fig. 46). This technique has been used to measure diffusion constants and agar packed in columns[223] will fractionate high polymers[224].

Cross linked extran gels[225] have similar sieving properties, and are available with various degrees of cross linkages by ether bonds between polysaccharide chains. Sephadex (Pharmacia, Uppsala, Sweden) is a dextran gel, and is available as grades G-25, -50, -75, -100 and -200, in order of increasing cross-linkage. The numbers refer to the lower limit of complete exclusion (Mol. wt. x 1,000). G-25 is the exception to this rule, and has a lower exclusion limit of 5,000. This is the grade of resin which has been used in the experiments, which are reported below, since maximum resolution of compounds with molecular weights below 5,000 can be expected with this resin, and this is the range in which the urinary peptides are expected to fall.

Preparation of Columns of Sephadex G-25.

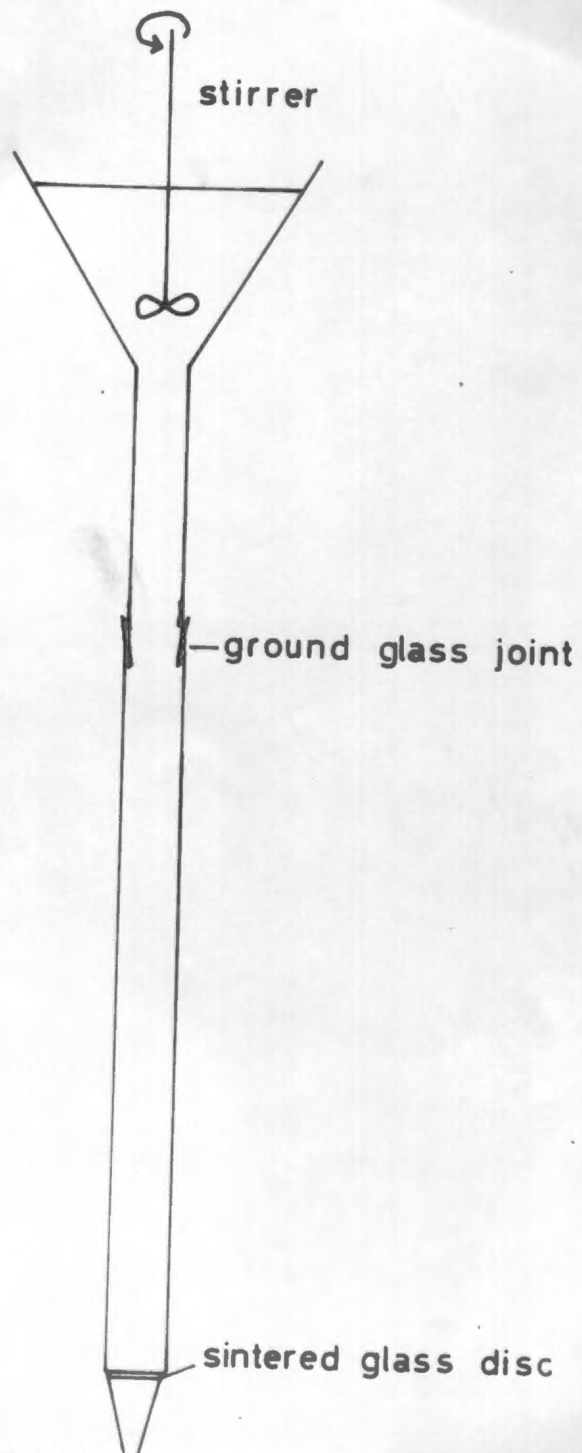
The dry resin was allowed to swell overnight in excess tap water or dilute buffer solution. The resin was then poured into the glass column, according to the technique recommended by Porath[226]. A funnel with a long stem (containing a stirrer) was arranged over the column (fig. 47). The total volume of the apparatus was filled with equilibrating buffer, and the resin added as a slurry into the funnel. Packing was allowed to proceed under gravity, whilst homogenecity was assured by continuous agita-

FIG. 46 MECHANISM OF GEL FILTRATION



tion with the stirrer. After 2 - 4 cm. of the column had been packed, the bottom outlet was opened and settling continued until the column of resin had reached the desired height.

FIG. 47 METHOD OF PACKING COLUMNS OF SEPHADEX



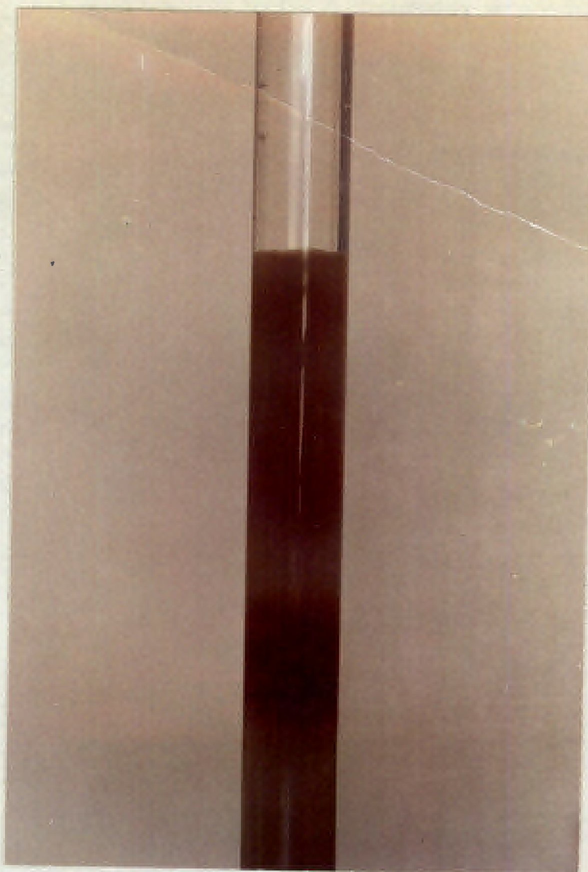


Fig. 48. Separation of haemoglobin, Vit. B12 and methyl-orange by molecular sieving on Sephadex G-25.

Experiment 1. Demonstration of molecular sieving.

A mixture of 1 ml. each of 1% (w/v) haemoglobin, 1% (w/v) methyl-orange and 1 mg. per ml. of cyanocobalamin (vit. B12), was added to a 3.5 x 40 cm. column of Sephadex G-25 and the mixture was developed with 1% (w/v) unbuffered saline. The separation of the three components was evident as soon as the mixture had travelled a few cm. into the gel bed (fig. 48).

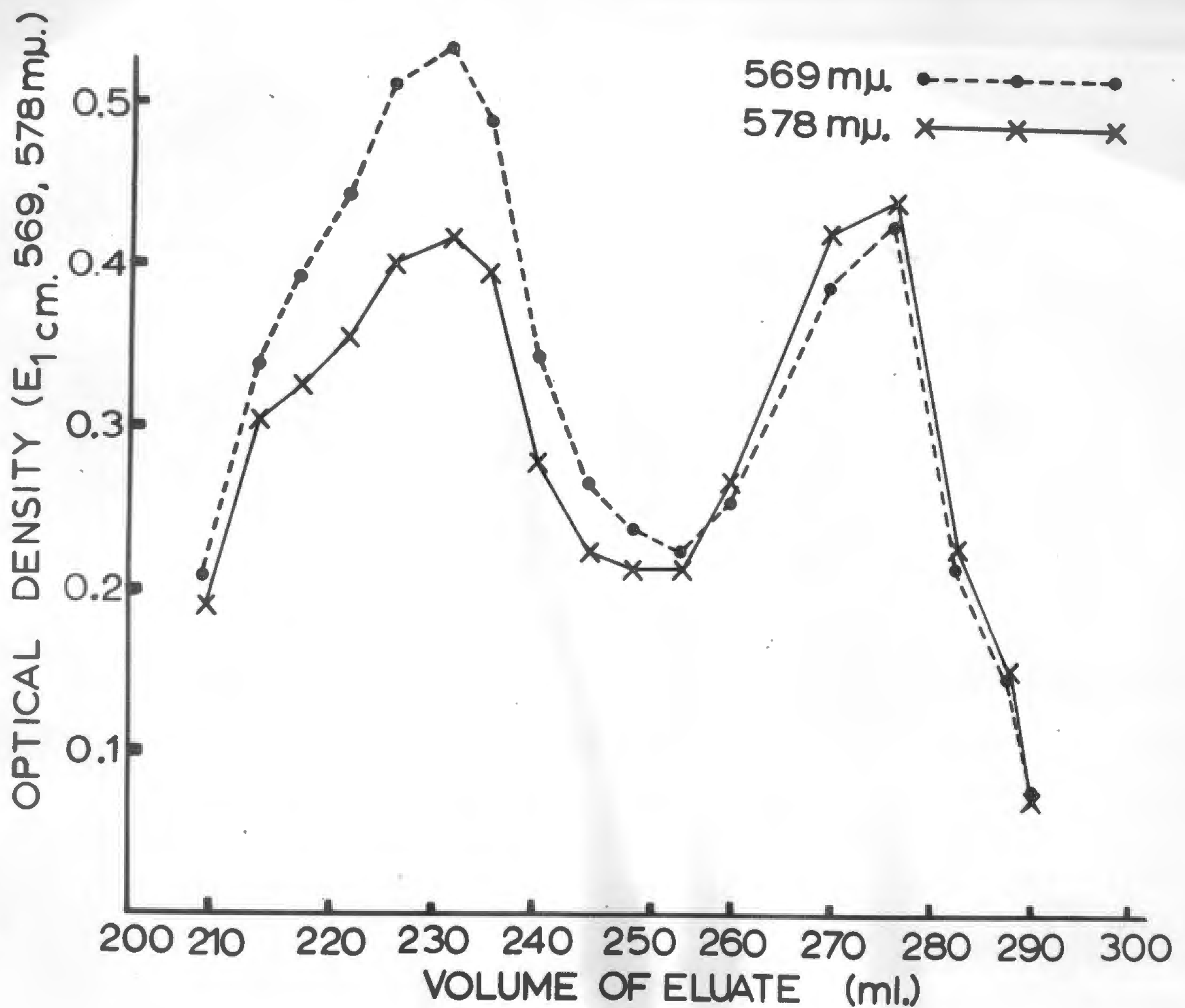


FIG. 49.

Experiment 2. Separation of haemoglobin and myoglobin.

A mixture of the two haem pigments was obtained from the crushed human psoas muscle, and the carboxyl derivatives chromatographed on a column of Sephadex G-75 with saline. This experiment formed the basis of a suggested method for the detection of myoglobin in human urine following crushing injuries to muscle.[227] (See attached reprint).

Experiment 3: Separation of a polypeptide from the DNP derivative of a free amino acid by gel filtration.

Bacitracin (Mol. wt = 1311) in 10 mg. and 2 : 4 DNP -DL α-alanine, 5 mg. were applied to a 3.5 x 40 cm column of G-25 Sephadex and eluted with 0.1M NaCl. The effluent was monitored for ninhydrin reacting material, by the automatic scanning method, as for amino acid analyses, and the pattern recorded on a potentiometric recorder. The graph has been reproduced in fig. 50.

Bacitracin emerged from the column, well separated from the yellow band of the DNP derivative of the free amino acid. The latter gave no colour with ninhydrin, and only minimal absorption at the recording wavelength (570 mu).

Experiment 4: Separation of Bacitracin and alanine.

Bacitracin (8.9 mg.) and DL α-alanine (0.83 mg) were applied to the same column, eluted and monitored as in experiment 3. The effluent graph is shown in fig. 51. The separation of the polypeptide from the free amino acid was not as complete as from the DNP derivative. The DNP compound was retarded more than expected on the column. In another experiment alanine was completely separated ahead of DNP-alanine. This result could not have been predicted on the basis of gel filtration alone, since on this basis the DNP-compound should have emerged first, since it has a higher molecular weight. This non-specific binding to the gel is shown by other aromatic compounds, and has also been reported recently by Porath[228].

Experiment 5: Gel filtration of Alanine, Bacitracin and Corticotrophin (ACTH).

In an experiment, similar in detail to the previous two, ACTH and Bacitracin emerged as a single broad peak, ahead of alanine (fig. 52). Since the first peak is broader than the second, it must have contained at least two components.*

The above five experiments demonstrate the feasibility of gel filtration as a method of separating peptides from free amino acids on a molecular weight basis.

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* Greater retardation on the column implies a larger bed volume, and hence broader peaks.

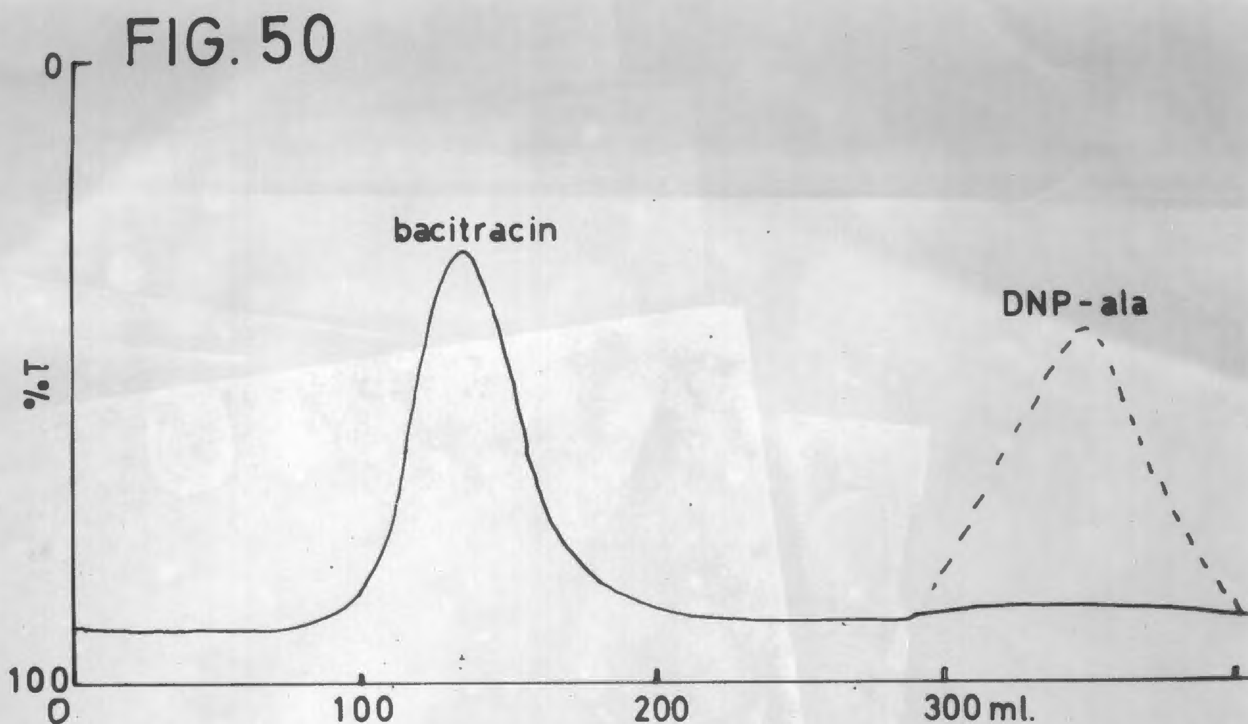


Fig. 50. Separation of Bacitracin from DNP-alanine on a column of Sephadex G-25 (3.5 x 4cm). Eluted with 0.1M NaCl (Experiment 3).

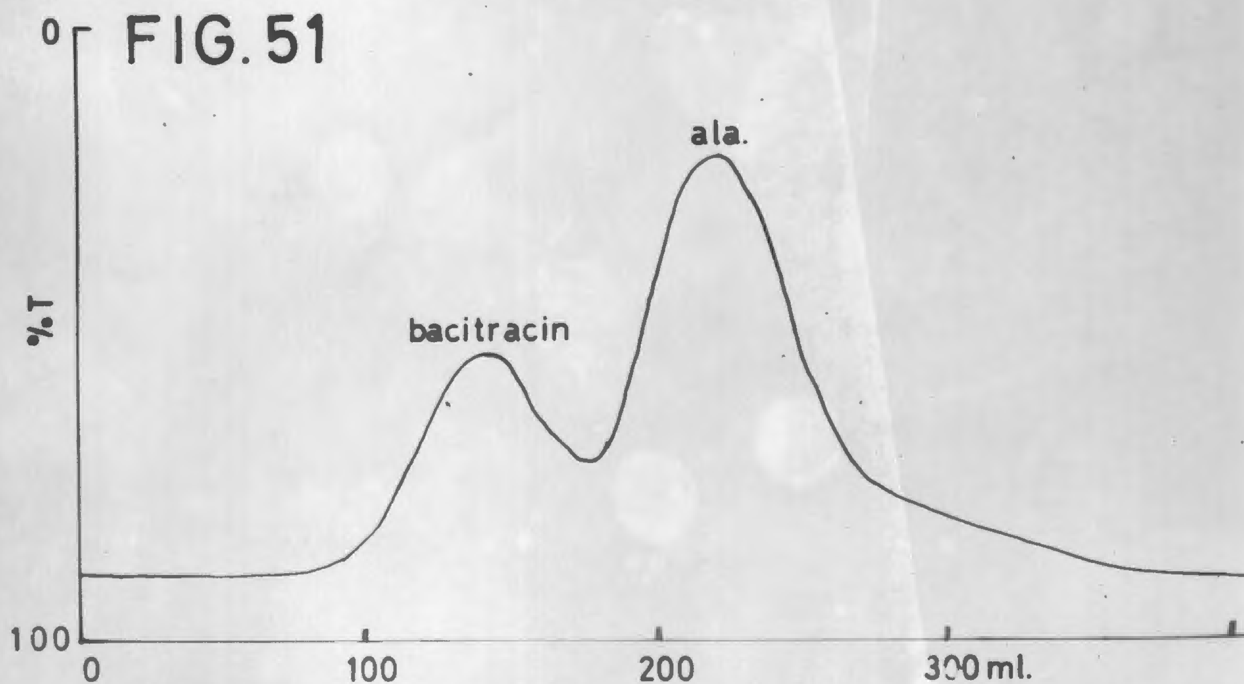


Fig. 51. Separation of Bacitracin and alanine on a column of Sephadex G-25 (3.5 x 4 cm). Eluted with 0.1M NaCl (Experiment 4).

FIG. 52

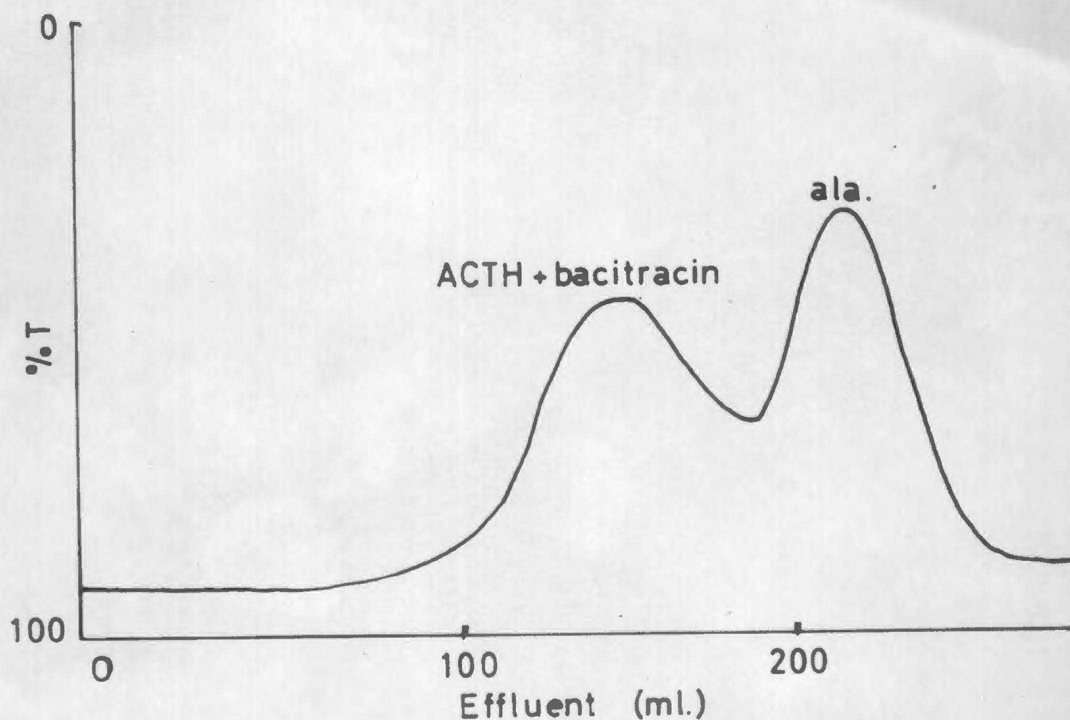


Fig. 52. Gel filtration of Bacitracin, ACTH and alanine on Sephadex G-25 (3.5 x 40 cm). Eluted with 0.1M NaCl. The first peak contains bacitracin and ACTH which have not separated.

The following set of experiments was designed to investigate the application of gel filtration to the separation of peptides occurring in urine from free forms of the amino acids.

Experiment 6: Gel filtration of Normal Urine.

An early morning specimen of fresh urine (450 ml.) from a normal male subject was reduced to 50 ml. in vacuo and made acid to litmus with N HCl. Urates were filtered off and the filter paper washed with a few ml. of water. This concentrate was applied to a column of Sephadex, G-25 (3.5 cms x 36 cms) and eluted with distilled water at a rate of 65 ml. per hour. The effluent was collected in 5 ml. fractions. Every fifth fraction was analysed for

1) Na^+ concentration in an EEL flame photometer,
2) α-NH_2 Nitrogen content, by the method of Moore & Stein (176)
3) Pigment content by measuring the extinction at 470 mu.

The results of these estimations are shown in the elution diagram fig. 53.

The major ninhydrin positive peak emerged slightly ahead of the salt peak and was assymetrical, suggesting at least two components. In addition a small shoulder which emerged before the main peak was suggestive of a higher molecular weight α-NH_2 compound. The pigment content of the fractions was more or less parallel to that of their α-NH_2 concentration, with two definite peaks in the man α-NH_2N peak; further evidence of heterogeneity.

Experiment 7: Gel filtration of Prefractionated Urine.

Fractions No. 63 - 67 which had been eluted from the anion exchange column during fractionation of bulked urine, according to the technique of Westall (155) - see page 118 - were pooled and subjected to gel filtration as in Experiment 6.

The α-NH_2 Nitrogen content of the eluted fractions was estimated, and the elution diagram drawn (fig. 54). This fraction was chosen, since paper chromatography had demonstrated that

FIG. 53 GEL FILTRATION OF NORMAL URINE

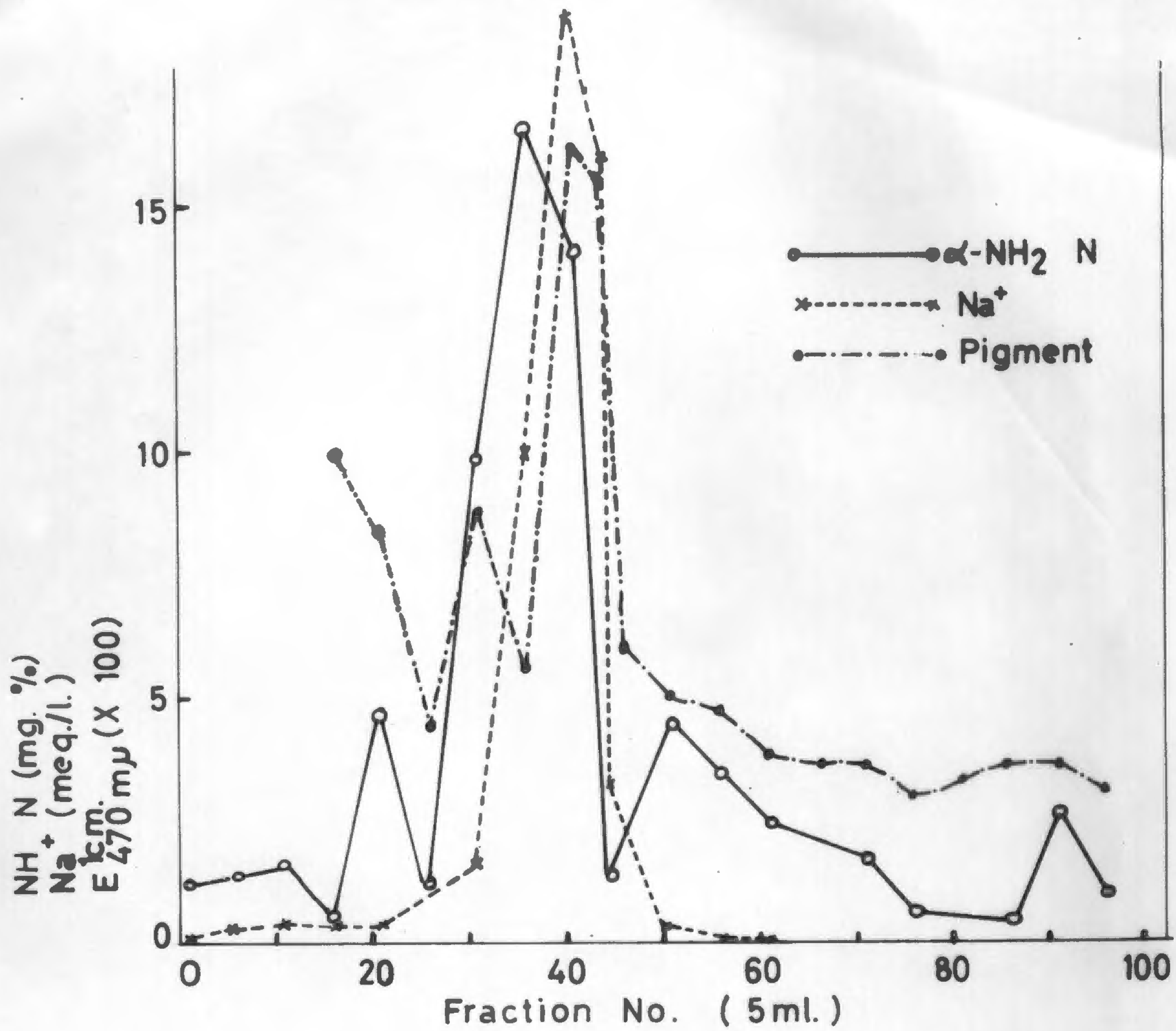


FIG. 54 GEL FILTRATION OF PREFRACTIONATED URINE

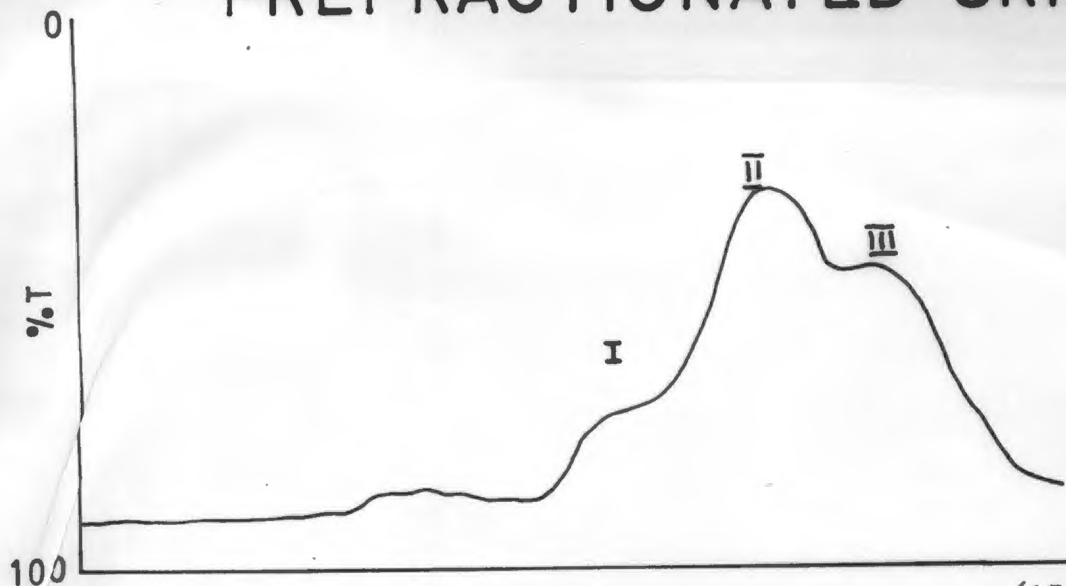


Fig. 54. Gel filtration of prefactionated urine (Westall)[155] on Sephadex G-25 (4 x 36 cm).

FIG. 55 GEL FILTRATION OF URINE ON LONG COLUMN (150cm.)

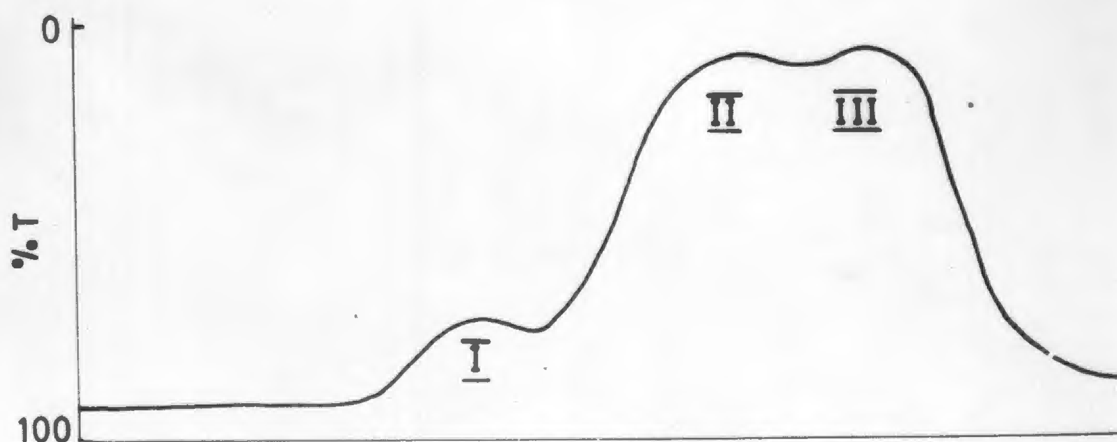


Fig. 55. Gel filtration of 8 ml. of urine on Sephadex G-25 (0.9 x 150 cm. column) (Experiment 8).
This volume of urine overloaded the column but at least three components were detected (I, II & III).

it contained several acid labile peptides. The complex elution diagram showed several minor components and a major peak, which probably consisted almost entirely of free glutamic and aspartic acids. Resolution was poor.

Although moderately large amounts of urine could be handled, the wide columns used above (4 x 36 cm) gave rather poor resolution. In order to improve this, column length was increased to 150 cm. and the width reduced to 0.9 cm. Owing to high resistance to flow, a pump was employed (Distillers Corporation Ltd. Type 1 Micropump) to apply the buffer at pressures of up to 20 lbs./sq. inch. The long column did improve resolution, but the handling capacity was reduced proportionately as the following experiments illustrated.

Experiment 8: Gel filtration of normal urine on a long (150 cm) column of Sephadex.

One hundred millilitres of normal adult urine was desalted by shaking in a mechanical shaker for 1 hour with 100 g. of Zeokarb 225 in the H^+ phase. The resin was filtered off, and washed with five changes of distilled water. The ampholytes were eluted from the resin with 100 ml. conc. NH_3 by shaking for 15 minutes. The ammonia liquor was concentrated in vacuo to dryness and the residue made to 25 ml.

An aliquot of 2 ml. of this concentrate (equivalent to 8 ml of urine) was applied to a column (0.9 x 150 cm) of G-25 Sephadex, and eluted with 0.2M Sodium Citrate buffer of pH 5.0, which was applied by means of a pump at a flow rate of 10 ml. per hour. The effluent was monitored for ninhydrin reacting material, and recorded on the Potentiometric recorder (as before).

The effluent (fig. 55) contained three components, which were not well separated, owing to effects of overloading.

Experiment 9(a):

Gel filtration of 0.4 ml. of urine concentrate (equivalent to 0.4 ml. of urine) was effected on a 150 x 0.9 cm column. Elution conditions and monitoring of the effluent were as in experiment 8.

The elution diagram showed a similar pattern to that obtained with larger volume of sample, but separation of components II and III was almost complete (fig. 56).

GEL FILTRATION OF BOUND α-NH_2N FRACTION IN URINE

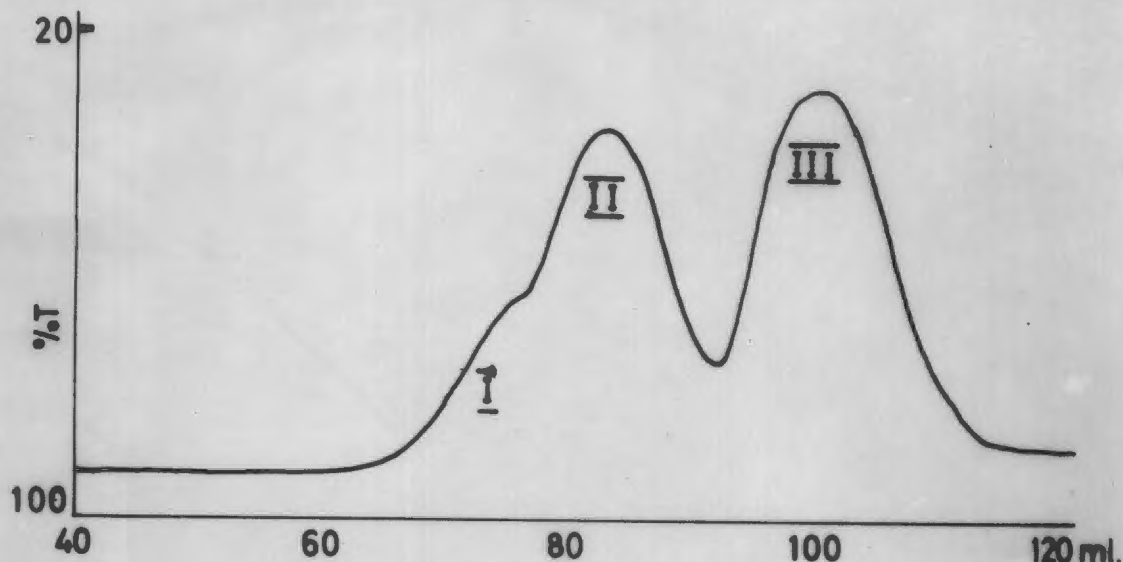


Fig. 56. Gel filtration of 0.4 ml. of urine on a 0.9 x 150 cm column of G-25 Sephadex. Three components are almost completely separated.

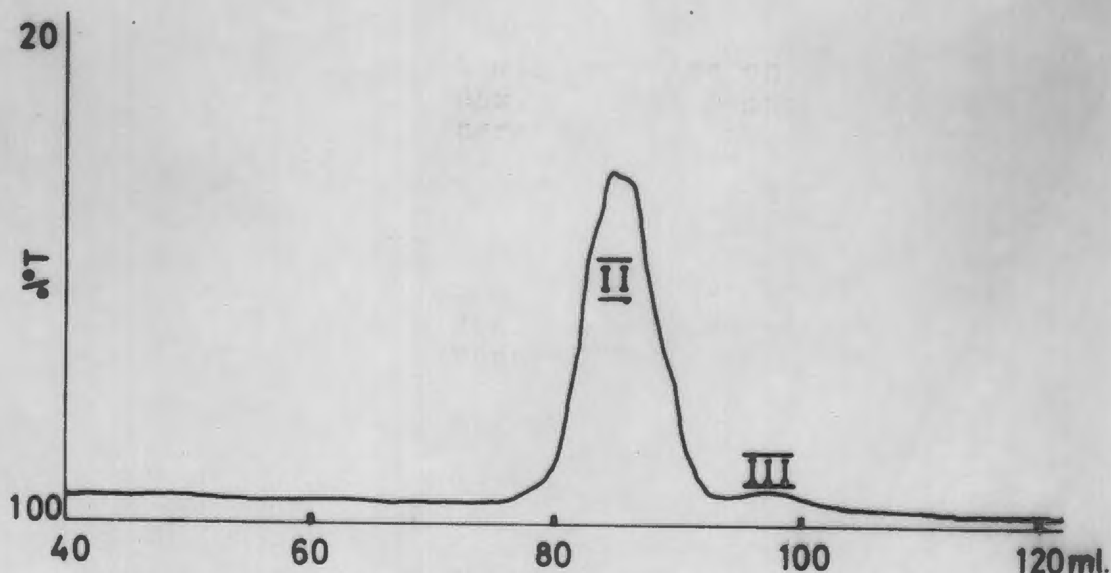


Fig. 57. Gel filtration of 0.2 ml. of hydrolysed urine. Components I and IIIhave disappeared - acid labile.

Experiment 9(b): Gel filtration of hydrolysed urine.

One ml. of urine was hydrolysed by 1 ml. of conc. HCl in a sealed ampoule at 105°C for 16 hours. After desiccation over KOH pellets, it was reconstituted, make alkaline and re-desiccated over conc. H_2SO_4 to remove ammonia. An aliquot of this solution, equivalent to 0.2 ml. of urine was again subjected to gel filtration, under conditions similar to those employed in 9(a). The effluent was monitored as before, and the elution curve obtained is shown in fig. 57.

The majority of the ninhydrin reacting material appeared as a single bifid peak, which contained more than one component (suggested by its bifid nature and by the shoulder on the upslope). The elution volume of this major peak corresponded with that of peak II. Peak III was represented by a small bump at its expected site of elution. Peak I had completely disappeared. Peak I and the bulk of peak III were therefore acid-labile, ninhydrin-positive materials, possibly of peptide nature.

Experiment 9(c):

Experiment 9(a) was repeated and was identical, except that the effluent was collected in 2.5 ml. fractions, instead of being continuously monitored.

Each odd numbered fraction was assayed for ninhydrin-positive material, using the modified reagent of Moore and Stein (194) on a 1.0 ml. aliquot. Another 1.0 ml. aliquot was assayed for total ninhydrin positive material after alkaline hydrolysis. This was done by heating to 90°C for 2.5 hours with 1.0 ml. of 2.5N NaOH, in a water bath, and then neutralised with 1.0 ml. of 2.5N (30% v/v) acetic acid. The hydrolysate was also measured for ninhydrin reacting material. The results of the free and bound material so obtained, expressed as equivalents of leucine per fraction, are shown in fig. 58.

The major fractions II and III could be identified, but resolution was less than that obtained with continuous monitoring. Fraction I was buried in the leading edge of II, but its presence was indicated by the change in slope of the curve at this point.

All tubes, except for fraction no. 16, showed release of ninhydrin-positive material after hydrolysis, but the majority of this was in fraction III (150% rise) with less in II (± 25% increase). This observation could not have been predicted, since bound forms of amino acids have higher molecular weight, and therefore should have been less retarded than free forms on the gel. Processes other than molecular sieving appear to have been operative.

Paper chromatography: Fractions 12 - 15, 16 to 18 and 19 to 24, representing peaks I, II and III respectively were pooled and desalted on Zeokarb 225 cation exchange columns. After elution with conc. NH_3 the solutions were taken to dryness in a vacuum desiccator and reconstituted in 0.1 ml. water. Aliquots (10 µl) were applied, along with a standard mixture of amino acids to the bottom of a sheet of No. 1 filter and developed in butanol - acetic acid - water by the ascending technique.

Ninhydrin positive spots occurred in all three peaks and the patterns were different (fig. 58). The greatest number of spots occurred in III, and three of these corresponded to those of free glycine, alanine and histidine. The major spot of II had a Rf. less than that of histidine/ornithine. This and the diffuse spot at the histidine locus in I migrated to positions compatible with peptide nature.

FIG. 58 CHARACTERISATION OF FRACTION I, II & III

Sephadex G-25 (0.9X150cm.)
8.0 ml. urine

fotal
free

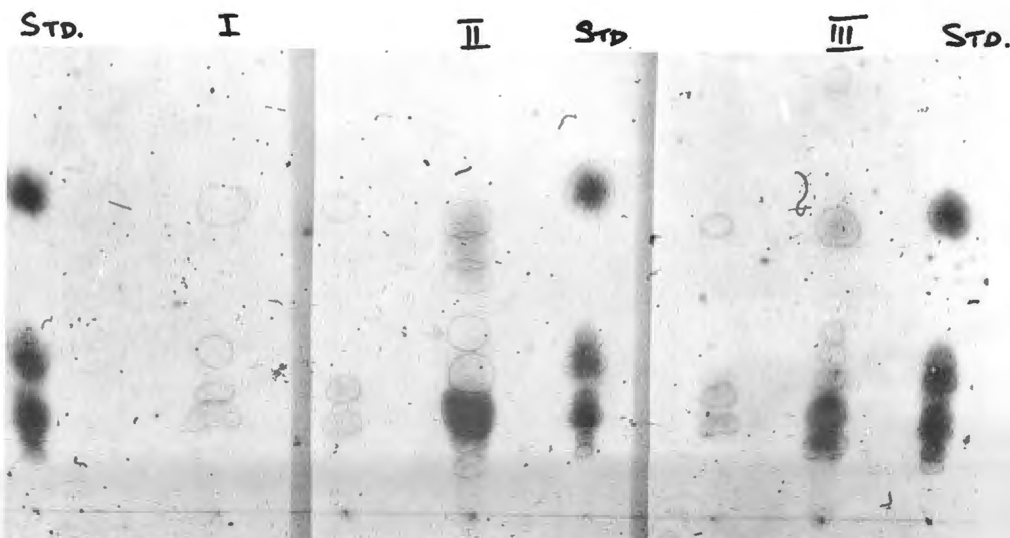
Leucine eqs. (μmoles per fraction)

10.0
7.5
5.0
2.5
0

I
II
III

3 6 9 12 15 18 21 24 27 30 33

fraction no. (2.5ml.)



COMMENTS ON THE TECHNIQUE OF GEL FILTRATION, AS APPLIED TO NORMAL URINE.

At least three ninhydrin reacting components, I, II and III have been separated from normal urine, by means of gel filtration on low cross linkage dextran gel (Sephadex G-25). Some bound forms of amino acids occurred in all three of these 'peaks' but the majority of this was in III.

The optimum load for filtration was of the order of 0.1 to 0.5 ml. of urine for a 150 x 0.9 cm. column of the gel. Increase of this load caused loss of resolution, whilst use of columns or larger diameter (e.g. 2.5 cm.) also resulted in poor resolution. Although a fraction could be prepared from urine, which consisted almost entirely of bound amino acids, the method was of limited use owing to its low capacity. The amounts of material which were thus made available, were insufficient for further fractionation and characterisation.

The technique of gel filtration of urine, although unsuitable for the needs of the present project, will be investigated at a later date.

Certain anomalies were obvious. Why is the fraction which contains the bound forms retarded to a greater extent than the free amino acids ? What is the nature of these bound forms ? Basic peptides have been shown to exhibit this anomalous behaviour on Sephadex columns[229]. These and other related problems may be a fruitful field for future investigation.

PREPARATION OF A NEUTRAL PEPTIDE FRACTION FROM URINE BY ABSORPTION ONTO AND ELUTION FROM THE DIETHYL-AMINO-ETHYL DERIVATIVE OF CROSS LINKED DEXTRAN (DEAE SEPHADEX).

Although the technique of Westall has the advantage of having a high capacity for urinary ampholytes through use of ion exchange, displacement chromatography, peptides are not separated from free amino acids. Conversely, gel filtration, which accomplished this separation, has low capacity. The logical conclusion was that a technique which utilised both gel filtration and ion-exchange mechanisms, might combine the advantages of these two physical processes.

The pK's (NH_3^+) of all the neutral amino acids are greater than 9.13, whilst those of most of the small peptides are less than 8.30 (222). Optimum separation of these by means of anion exchange should theoretically occur according to

$$pH_{opt.} = \frac{1}{2}\ pK'_{aa} + pK'_{pept.} \quad (230)$$

where pH_{opt} is the optimum pH of separation,

pK'_{aa} is the pK' of the amino acid,

and $pK'_{pept.}$ is the pK' of the peptides.

Carnegie[231] was able to separate neutral amino acids from neutral peptides of low molecular weight on the anion exchanger 'Dowex I' by elution with ammonium acetate at pH 8.65. Aromatic amino acids (phenylalanine and tyrosine) were strongly retained by non-ionic binding, and these, and traces of other free amino acids, contaminated the peptide fraction.

There are several theoretical advantages in using the diethyl-amino-ethyl (DEAE) derivative of dextran over the quaternary ammonium derivative of polystyrene (Dowex I). DEAE resins have high capacity near pH 8, and since the functionally charged centres are within the gel, pore size of the resin particle, which is determined by degree of cross-linkage, regulates admission of solute molecules, and there is fractionation of these on a size, or molecular weight basis. Aromatic adsorption, a surface phenomenon, is minimised, since the active sites are within the particle, rather than on the surface. Almost complete separation of neutral amino acids from neutral peptides has been achieved by Carnegie on DEAE Sephadex (A-25)[232], by using pH gradient elution with collidine acetate buffer. Traces of tyrosine and cystine contaminated this peptide fraction.

A report on an investigation into the possibility of preparing a neutral peptide fraction from urine, utilising such a method, follows.

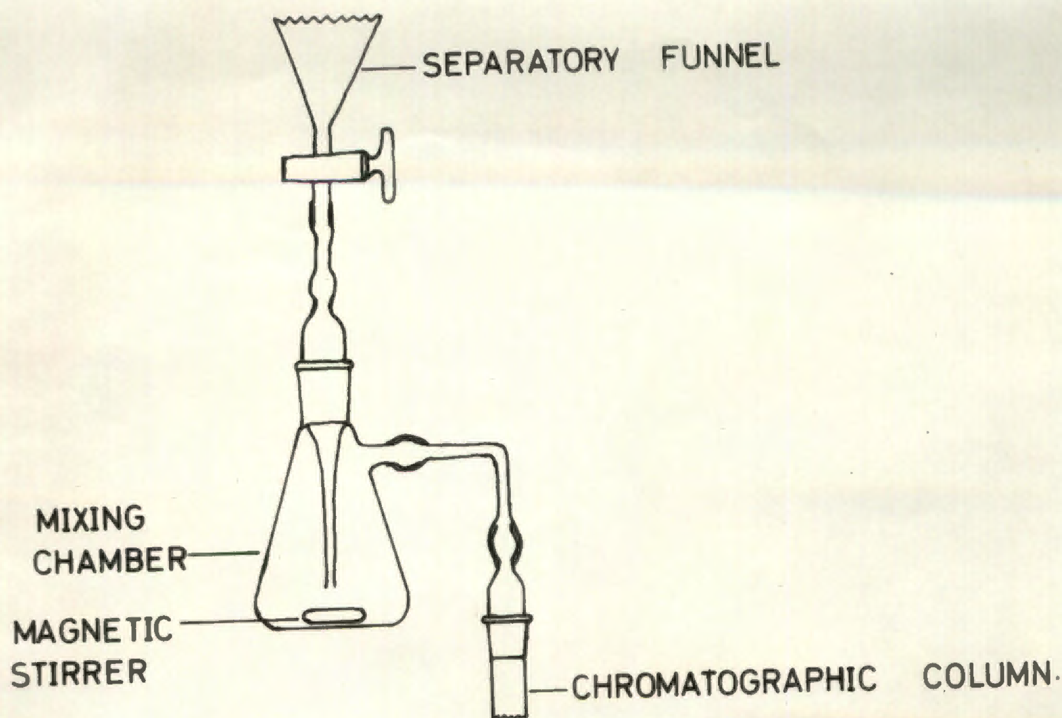
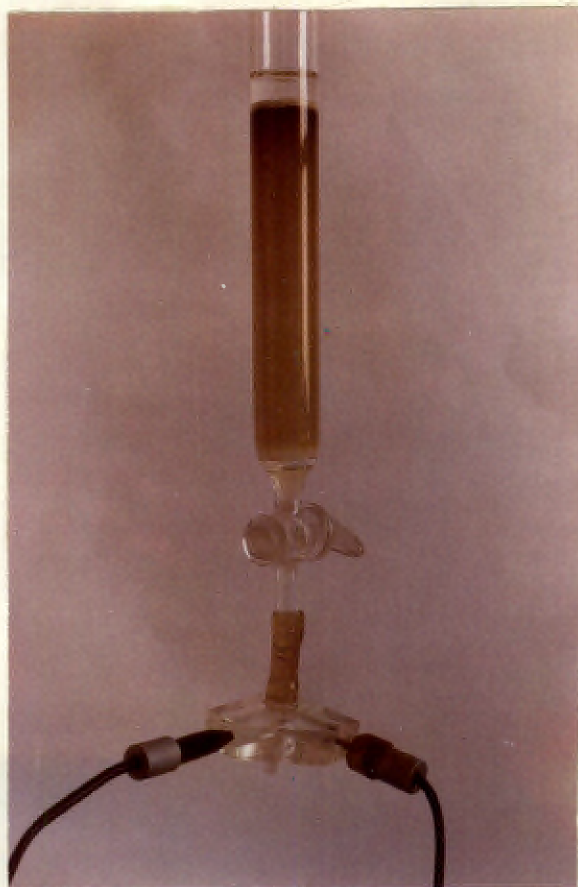


Fig. 59. Gradient elution device.

Fig. 60. Preparation column of DEAE-Sephadex (A-25) after application of sample. Urinary pigment is adsorbed onto the upper half. Note layer of 'phosphates'. Electrodes are for monitoring the effluent pH.



Experiment 10: Separation of a mixture of amino acids and a peptide on a column of DEAE SEPHADEX (A-25).

This experiment demonstrated the separation of a neutral peptide from free neutral and acidic amino acids.

A mixture of approximately 10 u moles, each of DL-leucine, DL-glycine, L-tyrosine, L-glutamic acid and glycylglycine in 5 ml. of pH 8.65 collidine acetate buffer was applied to an equilibrated column (2 x 16 cm.) of DEAE Sephadex (A-25).

Gradient elution was performed with the apparatus in fig. 59. The mixing vessel, containing a teflon-coated magnetic stirrer, was a 250 ml. flask. Gradient pH was performed with 0.1M acetic acid. The elution was allowed to proceed under gracity which achieved a flow rate of 85 m./hour.

The effluent pH was monitored on a Pye direct reading pH meter using a flow type elctrode (fig. 60). The effluent stream was subsequently split by pumping 30.0 ml. per hour into the ninhydrin analysing system of the automatic amino acid analyser. The remainder of the effluent stream (55 ml. per hour) was collected in 5.3 ml. fractions in a fraction collector. The output of the two photocells (570 mu and 440 mu) and that of the pH meter was recorded on a multichannel recorder. An aliquot (1.0 ml.) of each of the 52 fractions was analysed by the modified Moore and Stein reagent.

A tracing of the multichannel recording is shown in fig. 61. The calculated recovery of the dipeptide was 104%.

The neutral amino acids, leucine and glycine were well separated from the peptide, but tyrosine overlapped considerably. Neutral peptide was eluted soon after the pH started to fall (less than pH 8.2). Glutamic acid was eluted after the peptide. The elution diagram agrees with that of Carnegie (fig. 62) and demonstrates the separation of neutral peptides from both basic, neutral and acidic free amino acids. Cystine is eluted with neutral peptides and tyrosine overlapped. In spite of this the method appeared to be suitable for separation of neutral peptides present in urine, since both cystine and tyrosine occur in low concentration in that fluid.

Experiment 11: Separation of a neutral peptide fraction from normal urine.

<u>Sample</u>: Five ml. of early morning normal adult urine was made alkaline (pH 9) with NaOH and evacuated in a desiccator over conc. H_2SO_4 for 1 hour. The pH was adjusted to 8.55 with N. acetic acid and an aliquot equivalent to 2 ml. of original urine was chromatographed.

<u>Column</u>: DEAE Sephadex A-25 (100 - 200 mesh) equilibrated with 0.1 collidine acetate buffer at pH 8.65.

<u>Gradient pH elution</u>: The mixing flask contained 250 ml. of pH 8.65 buffer and was magnetically stirred. The gradient was formed with 0.1M acetic acid.

The effluent was collected in 1.4ml. fractions at a rate of 4 fractions per hour. The gradient was changed at fraction 39 by increasing the molarity of acetic acid to 1.0. Fifty-four fractions were collected.

<u>Analysis of fractions</u>;

The following estimations were performed on the effluent:

1. pH on the odd-numbered fractions.
2. Na^+ and K^+ on every 5th fraction.
3. Colorimeteric ninhydrin reaction on every fraction - expressed as leucine equivalents per fraction.

<u>Results</u>:

The results were expressed graphically in fig. 63.

Most of the Na^+ and K^+ appeared in the first five fractions as did the majority of the ninhydrin reacting material. With application of the sample, binding of ampholytes to the resin resulted in displacement of OH and there was an initial rise in pH up to 10 or 11. At tube 27 the gradient began (pH (8.0) and coincided with a small peak of ninhydrin-positive material which represented only a small fraction of the total. The two small peaks (tubes 45 and 52) corresponded to the expected elution behaviour of free aspartic and glutamic acids.

The small amount of material in tubes 27 - 32 behaved as neutral peptide (Cf. expt. 10). Since normal urine contained twice the weight of amino acids in bound as opposed to the free forms, the peptide peak was unexpected low. Explanations for this

FIG. 61 SEPARATION OF A DIPEPTIDE FROM FREE AMINO ACIDS ON A COLUMN OF DEAE SEPHADEX

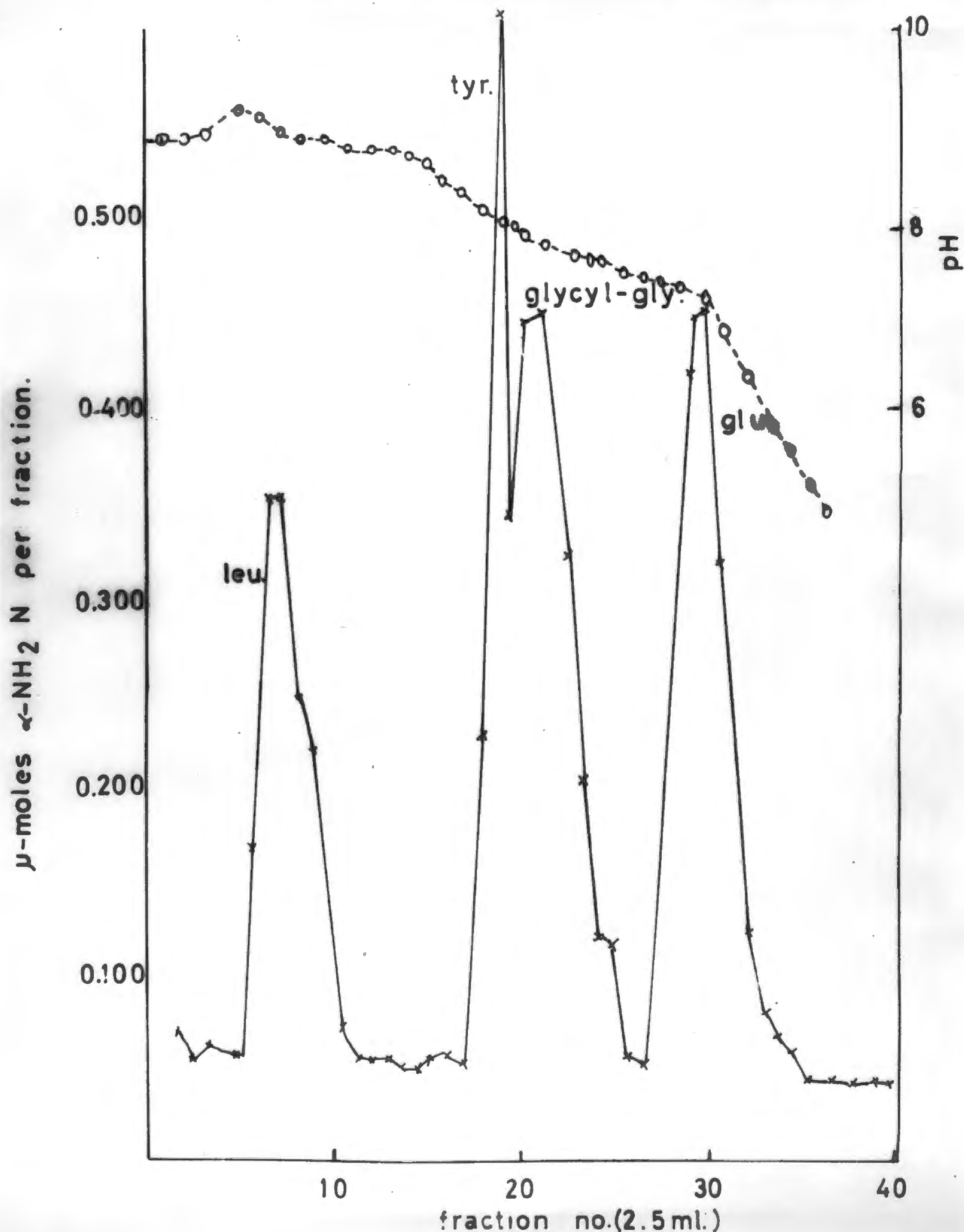
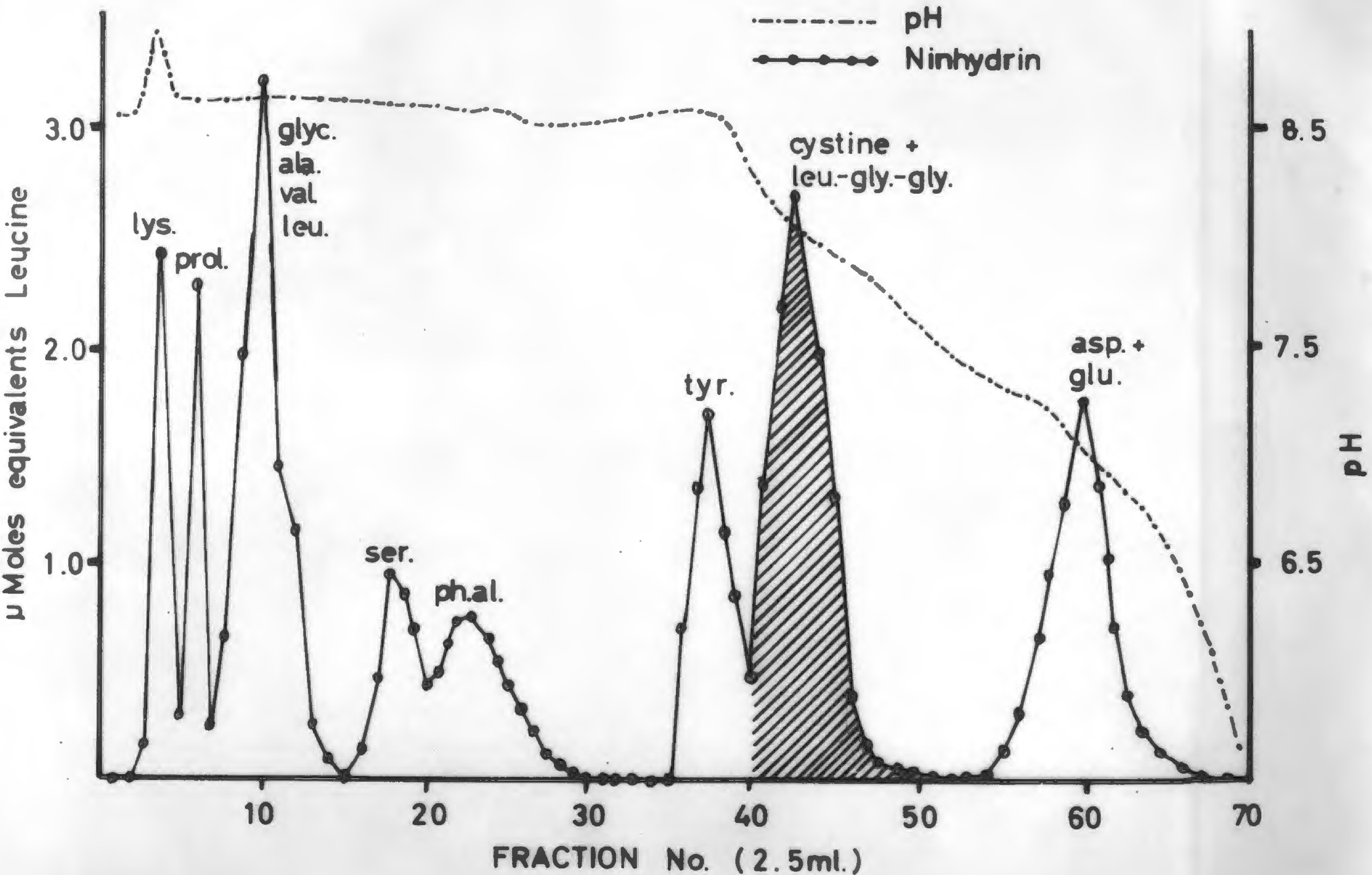


FIG.60 SERARATION OF A TRIPEPTIDE FROM FREE AMINO ACIDS ON A COLUMN OF DEAE SEPHADEX (A-25). CARNEGIE (232)



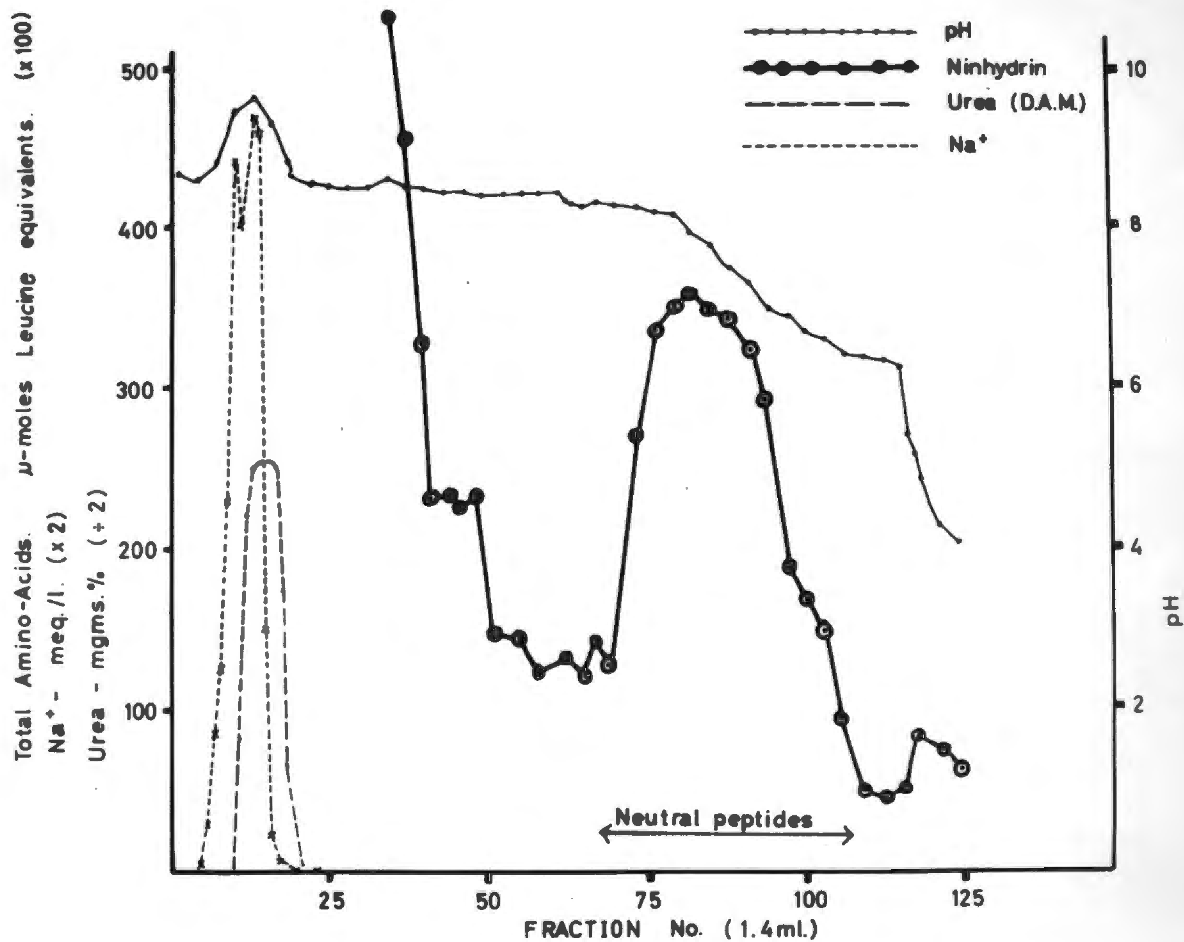


Fig: 63 Preparation of a neutral peptide fraction from normal urine (5 ml.) by gradient elution from DEAE Sephadex A -25

must include the following:-

either a) neutral peptides account for only a small proportion of total bound forms of amino acids,

or b) the colour yield for these peptides is low.

In order to prepare sufficient neutral peptide material for study, a larger volume of urine was used in the following experiment.

STUDIES ON THE NEUTRAL PEPTIDE FRACTION OF URINE.

In these and future descriptions, the term 'peptide fraction', or 'neutral peptide fraction', refers to material present in the effluent from the DEAE Sephadex column, which was eluted between pH 8.1 and pH 6.5

Bulk preparation of neutral peptides.

An early morning fasting specimen of urine, from a normal male subject, (410 ml.) was adjusted to pH 8.5 with 2N NaOH and placed in a vacuum desiccator over conc. H_2SO_4 to remove ammonia. This sample was applied in pH 8.65 buffer to a 3.8 x 30 cm. column of DEAE Sephadex and eluted by pH gradient as before, except that the mixing chamber was increased to 1 litre.

Alkalinisation of urine samples resulted in a precipitate of calcium phosphate. There was the possibility that the precipitate might adsorb peptides, especially those containing aromatic amino acids. To obviate selective losses, the precipitate was mixed with the sample and applied as a suspension to the column, forming a layer, 1.5 cm. thick on the top of the resin. Some urinary pigment was adsorbed onto the phosphate layer, but soon washed into the resin. Most of the urinary pigment stained the top 2 - 3cms. of the resin. During elution a small fraction of this pigment migrated down the column as a narrow sharp fronted band, but was not eluted prior to the peptide fraction (fig. 60).

The peptide fraction (460 ml.) was first concentrated in

vacuo on a cyclone evaporator to 120 ml. and finally taken to dryness in a rotary evaporator.

METHODS FOR SEPARATING INDIVIDUAL PEPTIDES.

Several different methods can be used to fractionate peptide mixtures. These include paper partition chromatography[233] high voltage electrophoresis on a paper support[234], gel filtration[235] and ion exchange chromatography[236]. The latter method has been chosen.

Peptides in whole urine have been fractionated on low cross-linked cation exchange resins (Dowex 50x2)[237]. This procedure gives an extremely complex elution diagram, and is useful only if specific reagents are used for detection. Peptides of proline and hydroxyproline have been thus isolated. In native urine, free amino acids and urea constitute ± 90% of the ninhydrin reacting material. These are absent from the prepared neutral peptide fraction, and the elution pattern from this fraction on Dowex 50x2, may be simpler to interprete, than from an equivalent volume of whole urine.

Chromatography of the peptide fraction on low cross linked cation exchange resin (Dowex 50x2).

The method of chromatography is as described by Moore and Stein for use in separating peptides from partial hydrolysates of oxidised ribonuclease (236).

Dowex 50 (x2) (200 - 400 mesh) was obtained from the Dow Chrmical Co., Michigan, in the Na^+ form. Five hundred grams of the resin was suspended in tap water and settled several times to remove 'fines'. The resin was then sitrred with 2N NaOH at room temperature for two hours, and converted to the H^+ form in 6N HCl, after washing in distilled water. This was finally

equilibrated on a Buchner funnel with sodium citrate buffer (0.2M) of pH 3.1. Six sections of 25 cm. each of resin were poured into a jacketted glass column (1.8 x 160 cm). The composition of eluting buffers was:

Table 29.

Buffer	Citric acid H_2O.	Acetic acid glacial	NaOH 97%	Sod. Acetate $3.H_2O$.	HCl conc.	Final vol.
	gm.	gm.	gm.	gm.	ml.	l.
pH 2.2 (0.2M)	105		42		80	5
pH 3.1 (0.2M)	714		282		393	34
pH 5.1 (2.0M)	3570	730	1600	4630		34

The dried peptide fraction was reconstituted with 120 ml. water and adjusted to pH 2.0 with ± 2 ml. of conc. HCl. The sample was pumped onto the resin with a Micropump, and washed in with 40 ml. of pH 2.2 buffer. A red band of pigment discoloured the upper 7 cm. of the resin. The effluent from the column which was collected during application of the sample, was hydrolysed with alkali and assayed for ninhydrin-positive material. None was detected. This assured that no peptide material was lost which was not bound to the resin, either by reason of large molecular size or acidic nature.

During chromatography, the jacket was maintained at 35°C by means of a thermostatted water bath and water pump. Elution was commenced with pH 3.1 buffer and the effluent was collected in 12.7 ml. fractions. After 70 fractions had been collected, a gradient device was attached to the inlet of the Micropump. The mixing chamber consisted of a 2 litre conical flask which contained pH 3.1 buffer and a magnetic stirrer. The gradient was formed with 1.0M, pH 5.1 buffer. At fraction 181 (2.3 litres effluent) a 2.0M, pH 5.1 buffer was substituted to continue the gradient. The buffer flow was stopped after 355 fractions had been collected (4.5 litres). During chromatography, the resin shrank due to increasing molarity of the buffers. This resulted in greater flow resistance. Initially the pumping rate was 40 ml/hour and the pressure 2 lbs. p.s.i. The flow was reduced eventually to 25 ml. per hour to prevent pressure rising above 5 lbs. p.s.i.

Analysis of fractions.

Samples were hydrolysed in alkali, neutralised and then assayed by the ninhydrin method. Alkaline hydrolysis has several advantages in this instance. The colour yields of

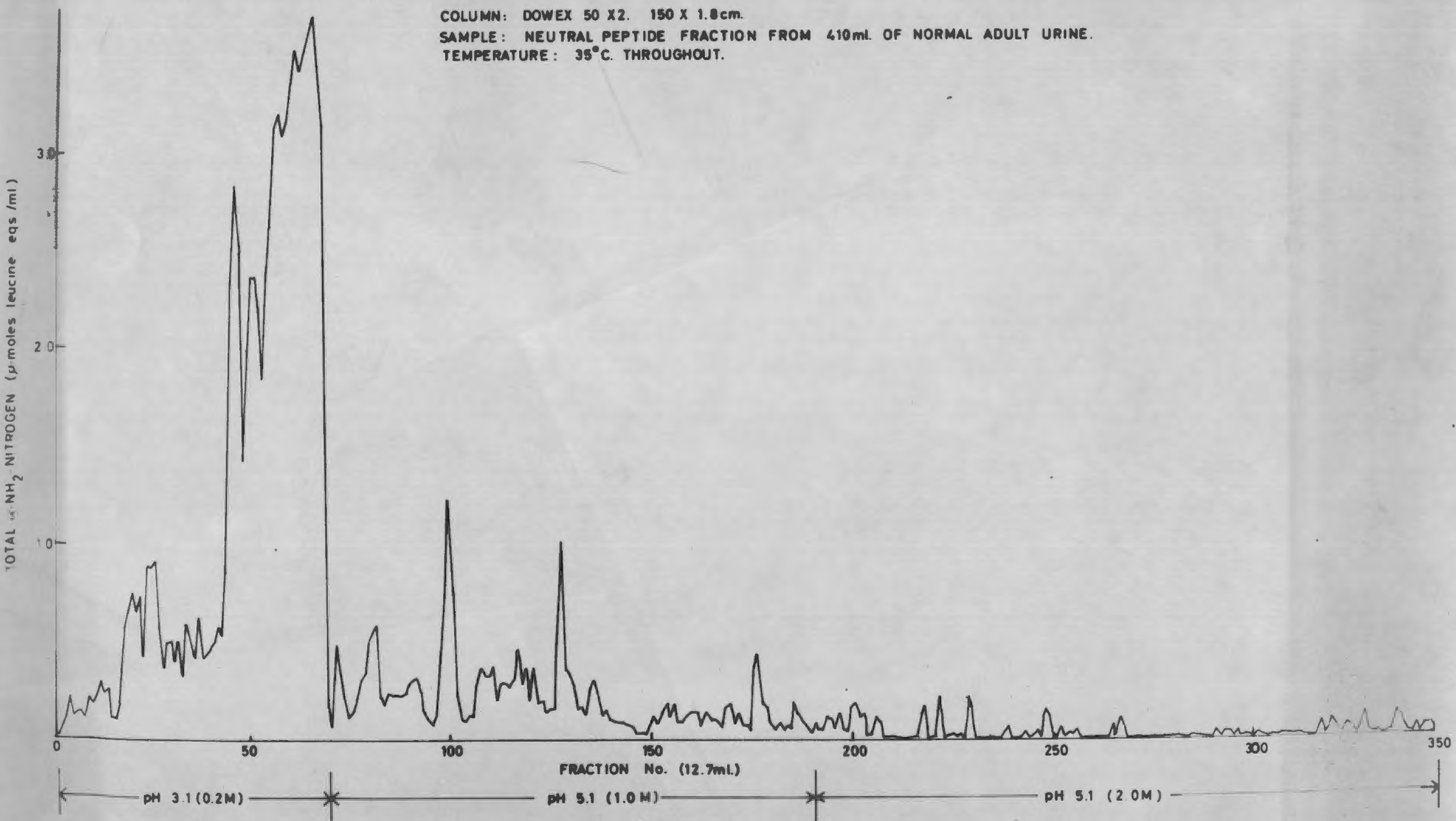
peptides are increased, depending on the number of residues per molecule. Some cyclic peptides are ninhydrin negative, since there is no free α-amino group. Ammonia absorbed by the acidic buffers is lost during alkaline hydrolysis.

An aliquot of the fraction (0.2 ml) was removed with a Seligman pipette, which was flushed with 1.0 ml. of 2.5N NaOH. The sample was hydrolysed at 90°C for 2.5 hours in a water bath. As soon as the tubes had cooled, 1.0 ml. of 2.5N acetic acid (30% v/v) was added to neutralise the hydrolysate. One ml. of modified Moore and Stein ninhydrin reagent (176) was added to each tube and heated in a boiling bath for exactly 15 minutes. Five ml. of 50% (v/v) ethanol were added and the tubes were allowed to cool, and then shaken in a mechanical shaker for 2-3 minutes in order to remove excess hydrindantoin. With each batch (100 tubes) a buffer blank and 1.0 ml. leucine standard, containing 0.1 u moles, was included. Results were expressed as leucine equivalents per fraction

The elution diagram (fig. 65) compares with that obtained for whole urine, and the pattern shows some similarities[220]. Removal of free amino acids has not, therefore, produced much simplification. Many peaks were noted corresponding to individual peptides, but the majority of these were eluted together (fraction 25 to 70) and asymmetry of other more discrete peaks, suggested that these contained more than one component. Re-chromatography of certain fractions, under different conditions of pH and temperature, should resolve more 'peaks' but the complexity was certainly greater than the mixture produced after partial hydrolysis of ribonuclease with HCl[237]. To resolve this peptide mixture Moore and Stein employed sequential chromatographic procedures.

It was obvious from these preliminary experiments that chromatography of peptides occurring in urine on Dowex (50 x2) had not the resolving power required to produce relatively pure samples of individual peptides for further analysis and characterisation.

FIG. 64 CHROMATOGRAPHY OF NEUTRAL URINARY PEPTIDES ON DOWEX 50-X2.



Analysis of the peptide fraction by the automatic column method of Spackman, Moore and Stein.

The technique of column chromatography developed by Moore and Stein[211] has proved suitable for separation of free amino acids and other material present in serum, urine and other biological fluids. When first described, the resin employed had a cross linkage of 8%[209]. Free amino acids and small peptides were eluted as sharp peaks, but octapeptides yielded broad zones, which made separation of higher polymers difficult. Later, a resin of lower cross linkage (4%) enabled larger molecules to penetrate the resin particles and be resolved from mixtures[210].

The automatic amino acid analysis procedure (Chap. 9) employs Amberlite CG 120 resin which has an effective cross linkage of approximately 8%. This system should therefore resolve peptides which contain up to 8 residues. During analysis of kwashiorkor urine by this technique, several acid labile peaks have been noted, some of which were broader than those of neighbouring free amino acids, suggesting that they were peptides of approximagely 8 - 10 residues.

Since amino acids and urea are the ampholytes in urine which occupy most binding sites on the cationic exchange centres of the resin, their removal during preparation of the peptide fraction should allow the column to be loaded with an aliquot of the peptide fraction derived from a large volume of urine, thus increasing the effective capacity of the system. When whole urine was analysed, no more than 5 ml. could be applied without overloading.

Fractionation of neutral peptides on Amberlite CG 120 cationic exchange resin.

Neutral and acidic components of the peptide fraction were resolved on the 150 cm. column with the 30 - 50°C. system, whilst basis peptides, if present, were run on the 50 cm. column at 30 - 50°C. using pH 4.26, 0.38M buffer.

The elution diagrams obtained from the peptide fraction of 100 ml. of urine are shown in fig. 65. No effects of overloading were apparent with this large sample. The elution diagram showed numerous discrete peaks, and shoulders on some of these which were incompletely resolved peptides. Most peptides were eluted with the 3.25 buffer whilst a few emerged with the front of the 4.25 buffer. Peaks were also noted at the sites where free amino acid contaminants of the peptide fraction were expected (aspartic and glutamic acids, cystine and tyrosine). Figure 66 is the elution pattern of an acid hydrolysate of one-tenth of the volume (10 ml.) of sample taken in figure 65. The difference between the sharpness of peaks and their different sites of elution were conclusive evidence that the peaks in the unhydrolysed peptide fraction pattern were ninhydrin-positive bound forms of amino acids. It remained therefore to isolate these bound forms and to prove that they indeed were peptides.

IDENTIFICATION AND CHARACTERISATION OF URINARY PEPTIDES.

A neutral peptide fraction was prepared from 30 ml. of urine from Case 4 which was collected during the early stages of protein repletion (day 5) and studied previously. After concentration and acidification to pH 2.2 the sample was applied to a 150 cm. Amberlite column and eluted with the 3.25/4.25, 30 - 50°C system.

The eluate was collected in 1.8 ml. fractions which were numbered from the time the sample had been applied.

Analysis of fractions.

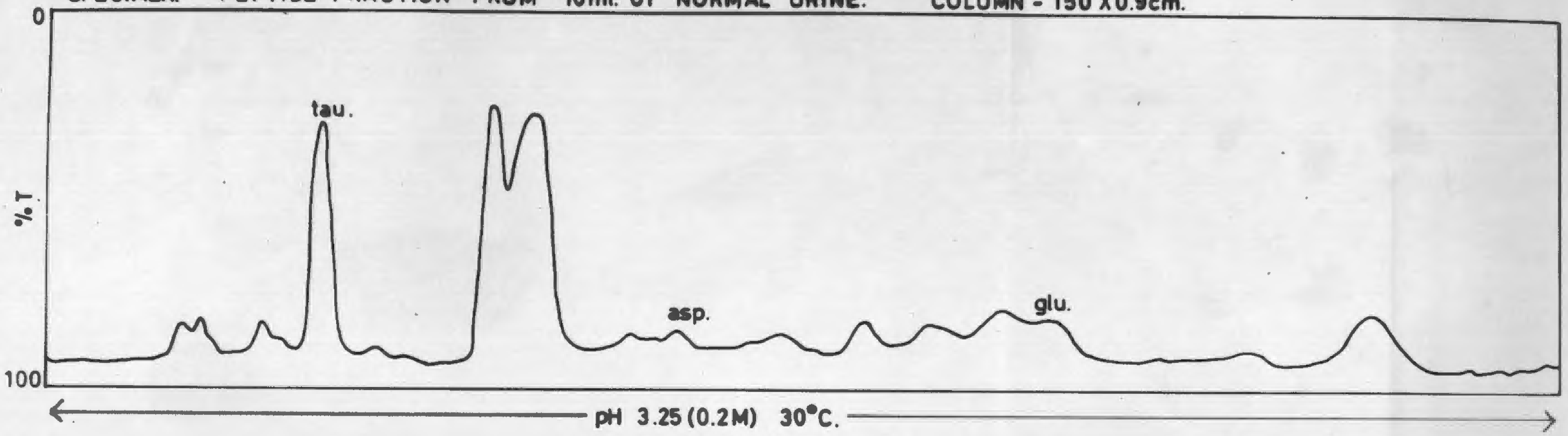
Each fraction was analysed for total α-amino Nitrogen after alkaline hydrolysis, as described previously on a 0.3 ml. aliquot.

Results.

The elution diagram (fig. 67) corresponded to that obtained with continuous flow analysis. Those fractions corresponding to the 5 peaks (labelled I - V) were desalted by adsorption onto a Dowex 1 anion exchange resin and eluted with diluted HCl. The

FIG. 65 CHROMATOGRAPHY OF URINARY PEPTIDES ON AMBERLITE CG-120.

SPECIMEN:- PEPTIDE FRACTION FROM 10ml. of NORMAL URINE. COLUMN - 150 X 0.9cm.



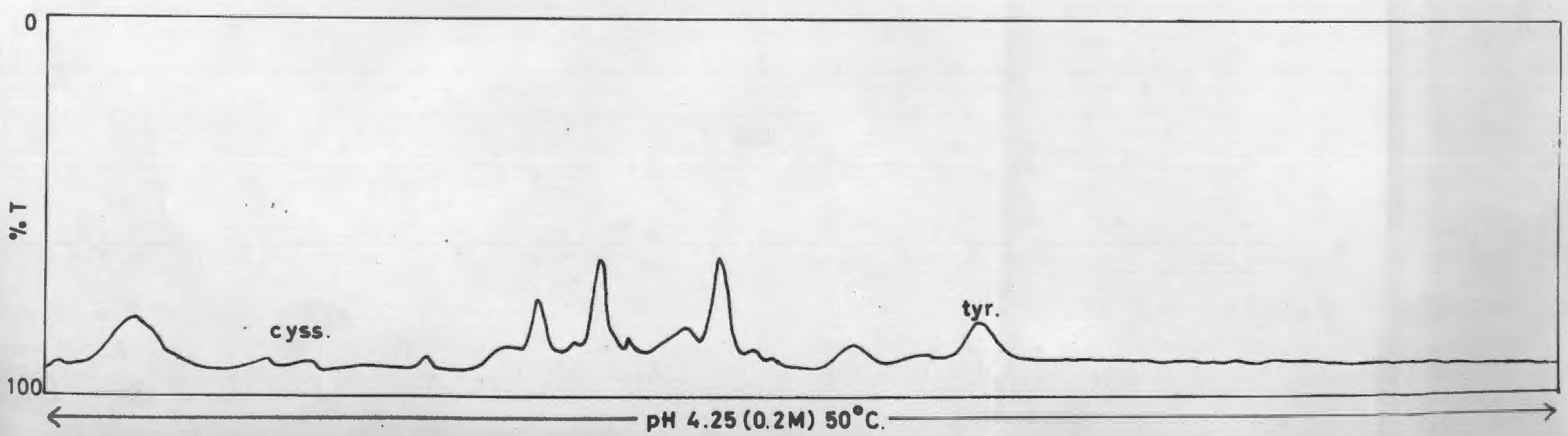
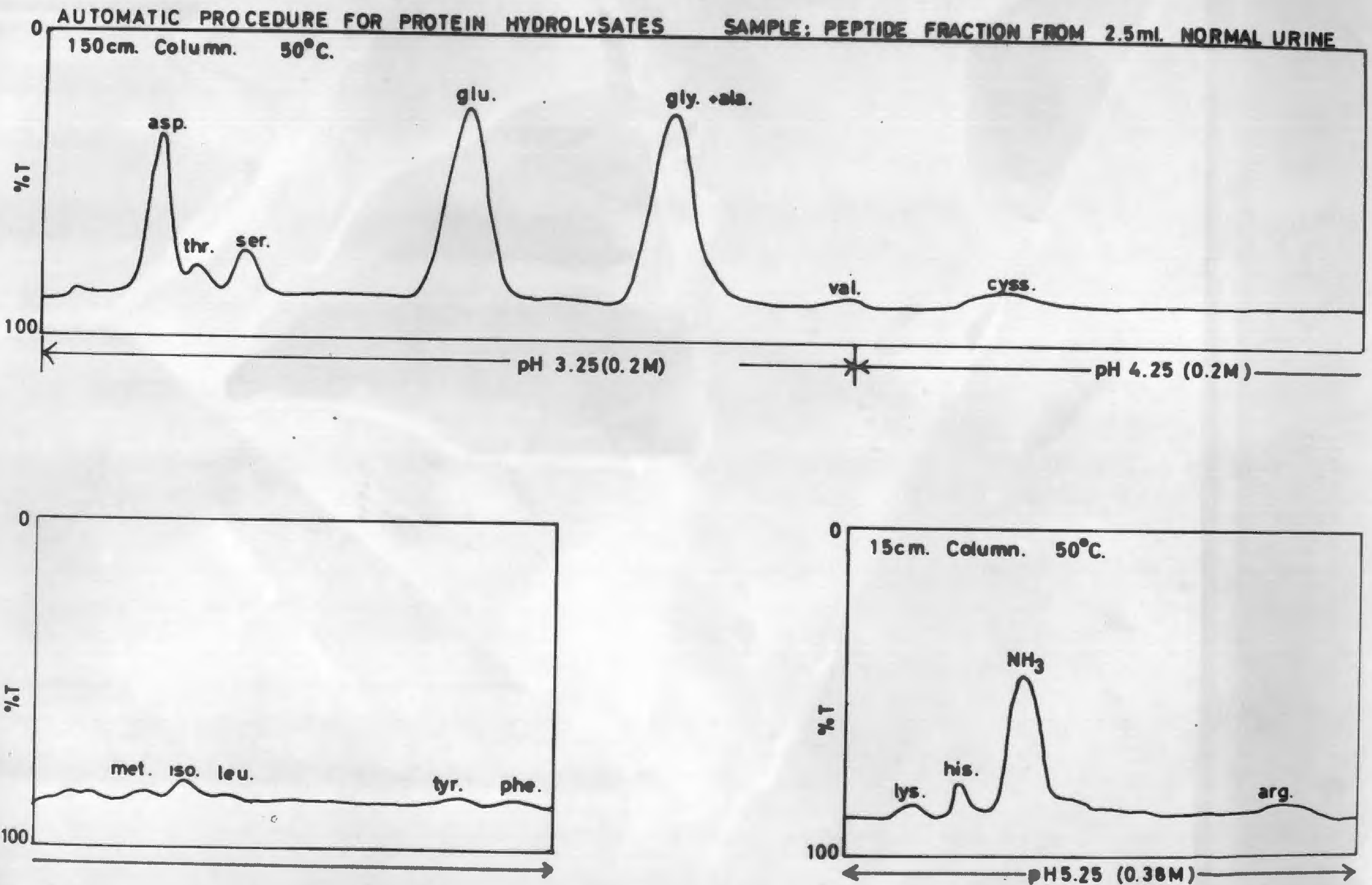


FIG. 66 AMINO ACID COMPOSITION OF NEUTRAL PEPTIDES IN URINE



desalted solutions were taken to dryness over KOH and then hydrolysed in 5.7N HCl in sealed tubes for 16 hours at 105°C. The acid was removed over KOH and the residue was dissolved in the minimum quantity of 10% isopropanol (±0.1 ml).

The whole sample was chromatographed by single way paper chromatography in Butanol : acetic acid : water (5:1:4).

The composition of the five peptide peaks is shown on the elution diagram (fig. 67).

The above pilot experiment demonstrated that column chromatography of the neutral peptide fraction, and analysis of individual 'peaks' by paper chromatography was a feasible method of characterising individual peptides. The procedure was used to characterise peptides from normal and kwashiorkor urine (Chap. 11).

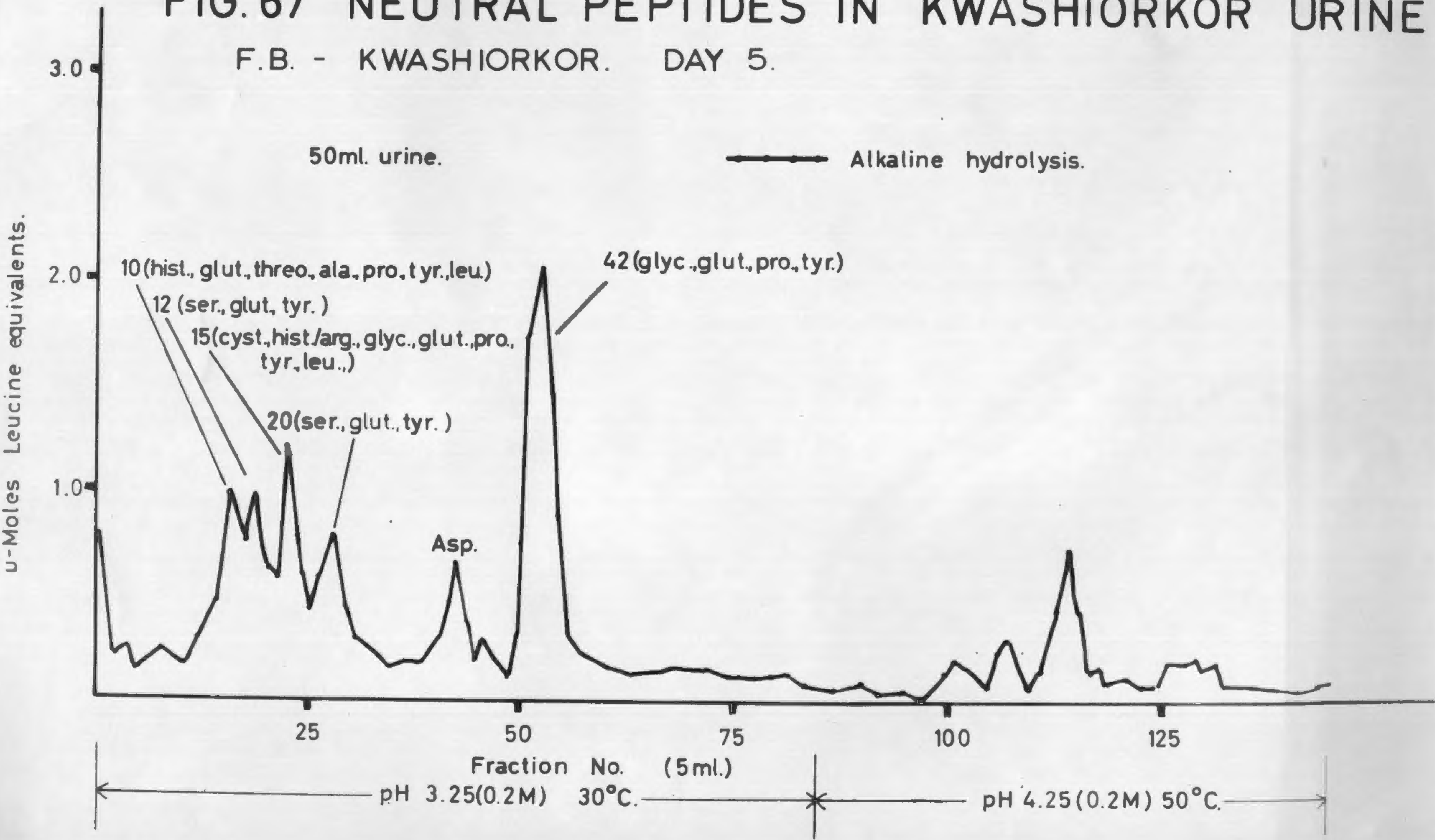


FIG. 67 NEUTRAL PEPTIDES IN KWASHIORKOR URINE

CHARACTERISATION OF NEUTRAL PEPTIDES ISOLATED FROM URINE.

This section describes methods used to characterise individual acid-labile peaks which constituted the peptide pattern described above. For descriptive purposes, peptides will be labelled according to their site of elution on the effluent curve of the Amino Acid Analyser, and their relationship to that of free amino acids.

Behaviour of some simple peptides on Amberlite IR 120 resin has been studied (238). Chromatographic behaviour of these was difficult to predict, since factors operative included a combination of ionic binding and partition chromatography. Nevertheless, large peptides (containing more than 8 residues) were poorly bound and were eluted early as broad peaks. Smaller acidic peptides were also poorly bound, but had sharper peaks. Neutral peptides, having less than 8 residues, behaved as free amino acids which had the same iso-electric points.

Site of elution on the chromatogram gave only general information regarding the identity of the peptide. Ideally the exact sequence of amino acid residues in the peptide should be established (primary structure). The number of specimens which had to be analysed, and the small quantities of each that were available (0.05 to 1.0 umole) rendered this impossible with the facilities which were available. It was therefore decided to characterise each peptide by means of its amino-acid composition, N-terminal amino acid and elution site.

Methodology.

Two possible methods of isolating the peptides from the effluent stream of the column were investigated. In the first the effluent was collected in fractions and each of these was sampled and assayed for total α-amino nitrogen. The fractions which corresponded to each peak were pooled, desalted and examined by paper chromatography.

Another method which was tried and found satisfactory, consisted of splitting the effluent stream. One-third (10 ml./hr) was pumped into the Amino Acid Analyser, whilst the remaining two-thirds (20 ml./hr.) was collected in a fraction collector. This had the advantage of having the elution diagram, from which fractions, which corresponded to individual peaks, could be selected and pooled. Some modifications to the existing Amino Acid Analyser were made for this purpose.

A T-piece was attached to the joint at the bottom of the column, from the side arm of which 10 ml. per hour of effluent buffer was pumped by a Micropump into the heating coil where it mixed with 10ml./hour of ninhydrin reagent.

Since the total flow-rate through the coil was only 20ml. per hour, the heating coil was changed for one which was calibrated to contain 6-7ml. so that colour developed for exactly 20 minutes. The remaining two-thirds of effluent (±20ml./hr.) was led to a fraction collector via fine (gauge 20) teflon tubing, and collected in 2 ml. fractions.

Calibration:

The split stream adaptor was calibrated against a standard amino acid mixture to establish integration constants at the new flow rates and altered buffer/ninhydrin reagent ratio. At the same time the degree to which resolution was impaired, by introducing another pump, was noted.

Measured flow rates:

Flow rate of buffer through column (30°C) - 29.9 ml./hr.
Flow rate of buffer pumped to heating coil - 10.8 ml./hr.
Flow rate of ninhydrin reagent - 9.6 ml./hr.

Fraction of total volume delivered to fraction collector =

$$\frac{29.9 - 10.8}{29.9.} = 64\%.$$

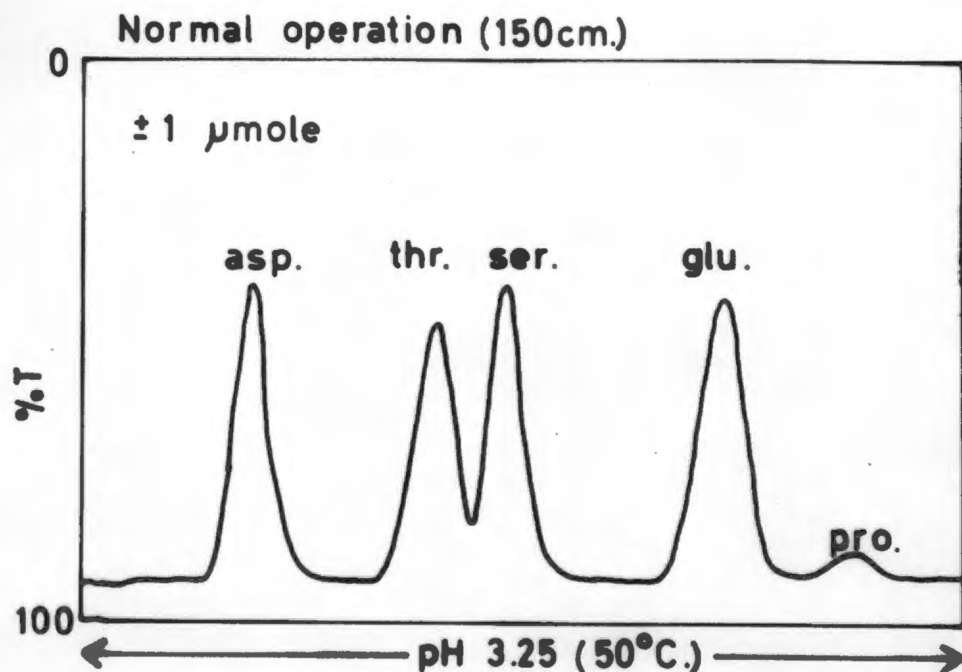
The machine was used to analyse a mixture of amino acids (2 μmole of each). The graph obtained, portion of which is reproduced in fig.68, showed definitely poorer resolution. Peaks were broader and their separation less. Overlap was minimal. The colour yields were higher with the split stream attachment. A comparison of integration constants obtained with and without the attachment is given in Table 30.

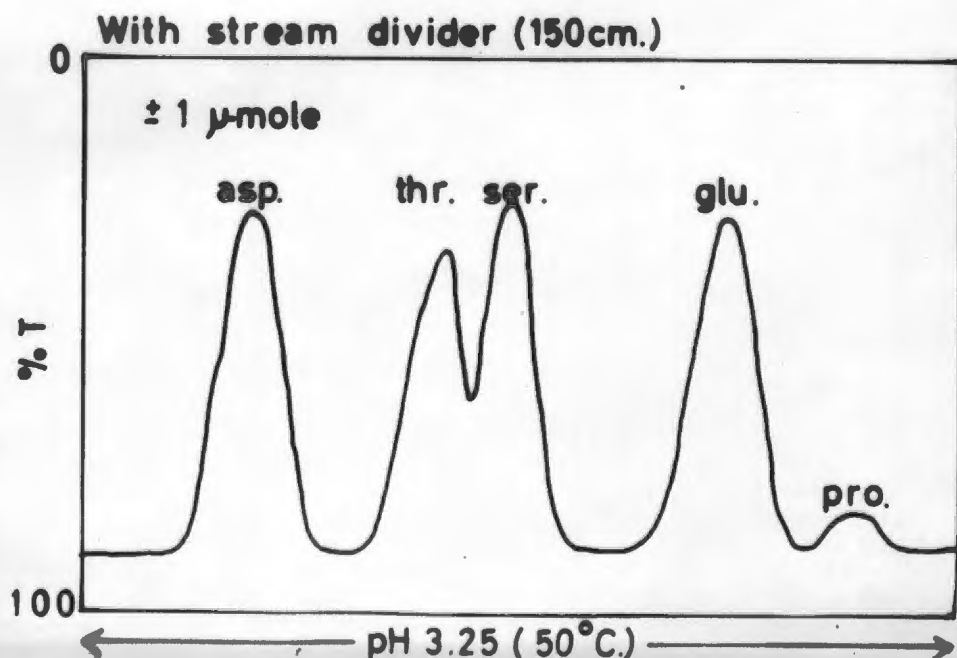
Table 30.

Integration constants with the split stream attachment.

Amino acid	Normal use	With split stream attachment.
Asp.	42.1	55.0
Thr.	41.6	50.4
Ser.	43.5	45.3
Pro.	9.7	6.6
Glu.	39.1	43.2
Gly.	37.6	49.7
Ala.	41.4	47.0
Cyss.	41.4	51.2
Val.	42.0	47.7
Met.	39.5	40.6
Iso.	41.6	48.4
Leu.	42.2	49.1
Tyr.	39.4	41.9

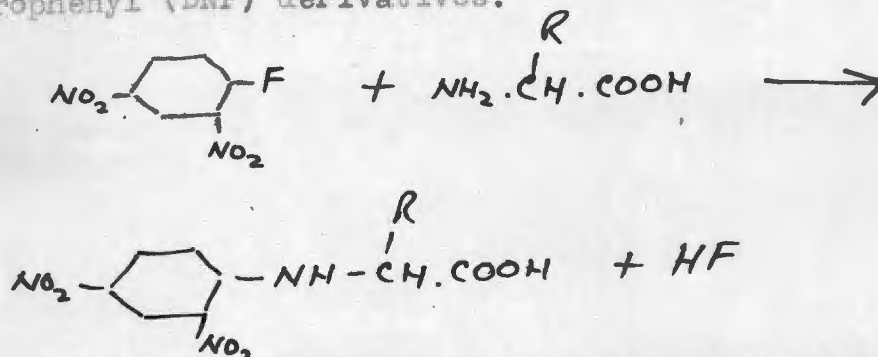
FIG. 69 STANDARD CURVE - SPLIT STREAM ATTACHMENT





DINITROPHENYL DERIVATIVES OF AMINO ACIDS AND PEPTIDES.

Fluorodinitrobenzene (FDNB) reacts in alkaline solution with α-amino acids, peptides and proteins to form dinitrophenyl (DNP) derivatives.



The DNP derivatives have an intense yellow colour and are easily extracted into organic solvents from acid solutions. They are also reasonably stable under conditions which disrupt peptide bonds.

The FDNB reaction was first used by Sanger to determine N-terminal amino acids of peptides and proteins (239), and was a key tool in the elucidation of the complete structure of insulin (2)(3). In Sanger's original procedure, the protein was shaken with excess FDNB in 66%(v/v) ethanol, containing sodium bicarbonate. Dinitrophenyl (DNP) groups can be introduced into protein in aqueous solution at pH 10 and 40°C without shaking by using 2, 4-dinitrobenzene 1-sulphonic acid as a dinitrophenylating agent. This procedure minimises denaturation (240). Excess FDNB in purely aqueous solution at 40°C and with shaking, maintains a constant saturated solution and the reaction is a first order one which may conveniently be followed by titrating the HF iberated to constant pH.

Besides free α-amino groups, FDNB reacts with side chains which project from the peptide, e.g.

ε- NH_2 of lysine

p - OH of tyrosine

imidazole N of histidine

and - SH of cystine.

Sodium bicarbonate may be replaced by trimethylamine in the reaction, obviating desalting prior to paper chromatography of the aqueous phase (241).

DNP peptides are conveniently hydrolysed in 5.7N HCl in sealed tubes at 105°C for 12 hours, since most DNP amino acids are stable under these conditions, with recoveries of 75 - 95%. Such hydrolytic procedures completely destroy DNP-proline, -glycine and -cystine.

In 1% $NaHCO_3$, DNP amino acids have light absorption maxima of 358 ±2mu, whilst DNP peptides absorb maximally at 350 mu. DNP-proline has maximal absorption at 385 mu; a fact which aids its identification. Chromatographic identification and quantitation of DNP amino acids has been accomplished on columns e.g. silica gel (242) or on paper for which numerous systems have been described (243). The method of choice was that of Biserte and Osteux (244), who employed toluene-pyridine-chloroethanol-aqueous ammonia as the first solvent, and 1.5M pH 6 phosphate buffer for the second dimension. Spots of individual amino acids may be eluted into 1% $NaHCO_3$ and determined spectrophotometrically.

DNP peptides can be extracted by ethyl acetate from aqueous solutions and this procedure has been used to isolate peptides present in salt solutions. Peptides in a partial acid hydrolysate of collagen have been separated by column chromatography on Dowex 50 (245). The DNP peptides could then be extracted from the effluent with ethyl-acetate. This obviated tedious desalting procedures.

The DNP method of amino end group analysis has the advantage of simplicity. Its main defect is, however, the liability of the DNP amino acids. DNP-glycine and DNP-proline are destroyed after a few hours in 5.7N H Cl at 100°C. The method is not suitable for sequence studies.

A fluorescent end-group reagent for proteins and peptides has recently been described by Gray and Hartley (246). The compound, 1-dimethyl aminonaphthalene-5-sulphonyl chloride (DNS-Cl) reacts with free amino and phenolic groups. Its derivatives are resistent to acid hydrolysis, can be separated by high voltage paper electrophoresis, and are located by their fluorescence in ultraviolet light. Quantities as small as 10^{-3} to 10^{-4} umoles of peptide can be handled i.e. 100 times more sensitive than the FDNB technique.

The FDNB method was selected for two main reasons. It is a fairly simple procedure and in a few extra steps the peptide can be dinitrophenylated and extracted from buffer.

The analytical problem was the separation of μ molar quantities of peptide from ±10ml. of 0.2M buffered salt solution, which also contained 0.1% phenol, 0.2% BRIJ 35 (polyoxyethylene lauryl alcohol) and in some cases 0.5% thiodiglycol.

This method was applied to pooled fractions.

Method.

The pooled fractions containing peptide, which varied in volume from 4.0 ml. to 20 ml. were washed three times with ether to remove excess phenol and thiodiglycol and then adjusted to pH 8-10 with 5% $NaHCO_3$. 0.1 ml. of FDNB was added and the mixture was shaken for 2 hours in a 40°C water bath. Washing with ether removed excess FDNB. The mixture was acidified with a slight excess of conc. HCl (the excess dinitrophenol is a convenient internal indicator) and then exhaustively extracted with ether (to remove dinitrophenol) and then with ethylacetate (to remove DNP peptide).

The organic extracts were evaporated to dryness on a water bath and the residual aqueous phase was discarded.

Observations.

The detergent caused considerable difficulty during extraction and emulsions of water in ether were produced, which broke only on long-standing.

Since the exact amount of peptide present was not known, excess FDNB was added to ensure complete substitution.ᶠ This naturally resulted in excess DNP-OH, which was extracted from the acid phase by ether. Since this indicator is colourless in acid solution, completeness of extraction was determined by mixing a drop of the ether extract with 1%(w/v) $NaHCO_3$.

ᶠ The presence of 0.2M salt solution provides buffering action, and the reaction cannot be followed by monitoring the pH.

DNP derivatives of free amino acids and of those peptides which are ether soluble will also be present in the ether extract, and subsequent chromatographic procedures were overloaded. DNP-OH may be sublimed off, but it was decided to first desalt the peptides on Dowex 2 prior to dinitrophenylation. This has the advantage of also removing BRIJ-35 which is non-ionic.

Desalting.

Two basic resins were used for desalting, Dowex 2 and Amberlie R140,

A column, 1 x 4 cm. was prepared and cycled according to the following scheme:-

Washed with water (±10ml.)

1 N HCl (10 ml.)

Wash till effluent neutral (±20ml.)

Regenerated with 15 ml. 2N (CO_2 free) NaOH.

Wash until effluent was less than pH8 (pH paper). The pooled fraction was then added and washed into the column with 20 ml. H_2O.

Peptides were eluted with 5 ml. of 5N HAc.

In preliminary investigations for completeness of desalting, washing, etc., the effluent was scanned at 260mμ. with an LKB 'uvicord' coupled to a Varian recorder.

Results:

Using tyrosine (1 μmole/ml.) as an indicator, both resins were loaded with 100 μmoles of amino acid without overload. Similar experiments with Bacitracin showed

that this peptide was only poorly bound to Dowex 2 but was to Amberlite IR 140. The eluted peptide in 3-5 ml. 1N HAC was taken to dryness over KOH in vacuo at room temperature.

Dinitrophenylation:

The dried peptide was dissolved in 0.5 ml.1%(v/v) triethylamine and then 50 µl of FDNB was added in 0.5 ml. of absolute alcohol. The mixture was allowed to stand, with occasional agitation, for 2 hours at room temperature. Excess FDNB was removed by three washings of 3 ml. of peroxide-free ether. After evaporation to dryness in vacuo, the dinitrophenylated peptide was dissolved in a few drops of glass-distilled 5.7N N HCl and transferred to a glass tube which was sealed. Hydrolysis was effected by heating at 105°C. overnight for sixteen hours in an oven.

Excess HCl was removed in vacuo over KOH, and the residue dissolved in 1 ml. of 1N HCl. The N-terminal DNP-amino acid was extracted with ether (3 x 3 ml.) and the combined extracts dried in an air current. The equeous phase was also taken to dryness.

Identification of ether soluble DNP-amino acids.

The combined ether extracts were evaporated in vacuo and transferred quantitatively to the paper chromatogram in a few drops of acetone. A compact spot was placed 4 inches from the short side and 1 inch from the long side of a piece of Whatman No.1 filter paper ($15\frac{1}{2}$" x 12"). The paper was curled into a cylinder, 12" high, and secured with a few staples.

Chromatography in the first dimension was performed by standing the paper cylinder in a 7" petri dish containing the upper phase of a mixture of 30 ml. of toluene, 9 ml. of pyridine and 18 ml. of 2-chloroethanol in a separatory funnel to which 8 ml. of 0.8 N ammonia (53 ml. conc. aqueous NH_3 per litre) was carefully added to form the lower layer. The solvent was allowed to equilibrate for 1 hour before use, and the lower aqueous layer discarded. The whole apparatus was placed in a sealed glass chromatography tank and allowed to run for 10 to 16 hours in the dark. The solvent rose to the top of the paper in the latter period. After drying at room temperature the spots were ringed and their probable identity established. Suitable controls (5µl of M/100 solution of known DNP-amino acids/ in acetone)

/- Standard DNP-amino acids were synthesised from pure amino acids, using standard techniques and recrystalised three times from the recommended solvents (243). A photomicrograph of crystals of DNP-valine is shown in fig.69.

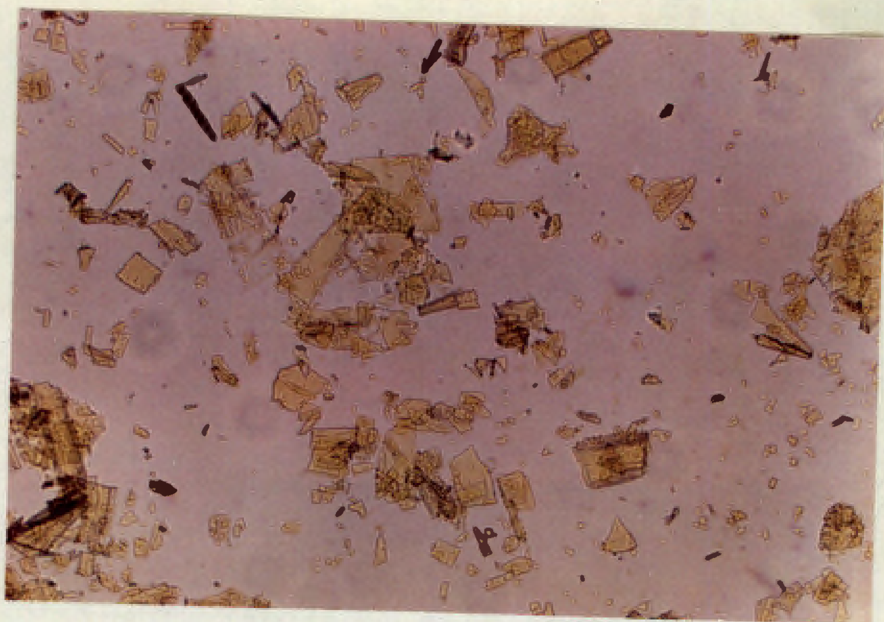


Fig. 69.

Micro-crystalline DNP-valine (x 240).
Recrystallised three times from ether-ligroin.

were spotted next to the unknown and the chromatogram was run overnight for ±12hr. in the second dimension by the descending technique using 1.5 M phosphate buffer (pH 6) which contained 138 g. of NaH_2PO_4 H_2O and 71 g. of Na_2HPO_4 in 1 litre of solvent.

A dish of water was placed in the bottom of the tank but no special precautions to ensure an airtight seal were necessary. The chromatogram was finally dried at room temperature for 2-3 hours.

When dense spots were encountered quantitation was attempted by cutting out the spot and an adjacent piece of paper (a blank). Both pieces were folded and dropped into 6" x 1" boiling tubes which contained 4 ml. of 1% Na HCO_3 and eluted by gently warming to 40°C in a water bath, for 20 minutes. The optical density of the solution was read at 360 mµ in a Beckman DU spectrophotometer. If DNP proline was suspected the maximum absorption wave length was determined. After subtracting the blank reading, the amount of DNP amino acid (in µmoles) was calculated by comparison with the optical density of pure DNP amino acid which was run through the whole procedure including hydrolysis and extraction. This corrected for unavoidable losses due to hydrolysis, partition, chromatography and photolysis.

The spot due to dinitrophenol was identified by bleaching with HCl fumes and was a useful marker. The chromatogram obtained from a standard mixture of known DNP amino acids is shown in fig.70, and the absorption spectra of some eluted spots in fig. 71.

<u>Examination of the aqueous phase</u> (247).

The aqueous phase, (in HCl.) after ether extraction, was evaporated to dryness in vacuo, and the residue applied to a 12" x 12" square of Whatman No.1 paper.

Ascending chromatograph in the first dimension was in the upper phase of an N-butanol/acetic acid/water mixture (4:1:5 v/v/v). After drying, the paper not occupied by amino acid spots was sprayed with pH 9.3 borate buffer (200 ml of 0.1 N boric acid and 114 ml of 0.1N NaOH). The second dimension was run by the ascending technique in a solvent mixture of 25 g of phenol, 25 g of m-cresol (both freshly distilled) and 7 ml of borate buffer. After drying for 4-6 hours in a fume cupboard, residual phenol (detectable by smell) was removed by rinsing in ether. Any yellow spots due to water soluble DNP derivates were marked.

FIG. 70 PAPER CHROMATOGRAPHY OF DNP-AMINO ACIDS

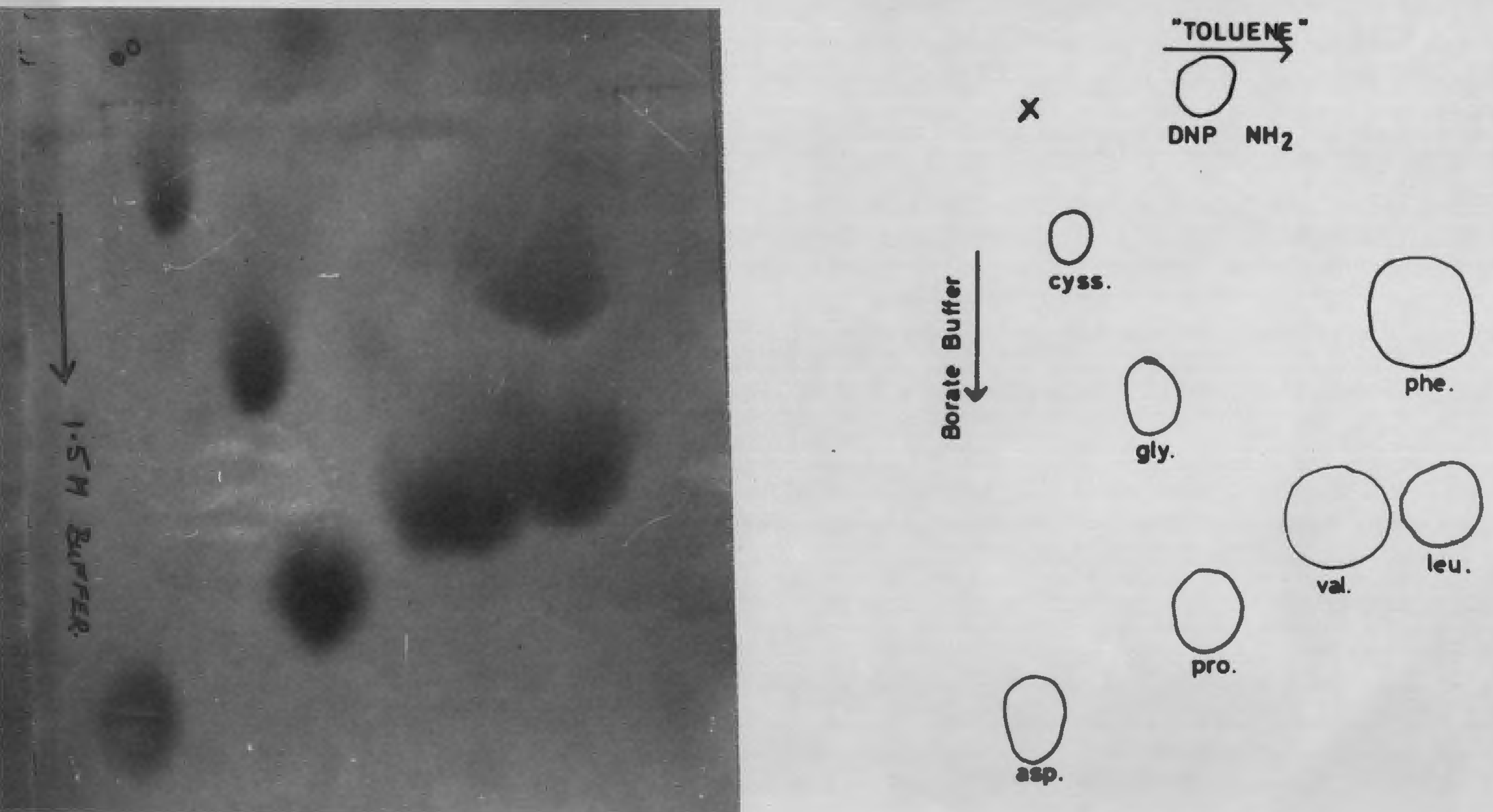
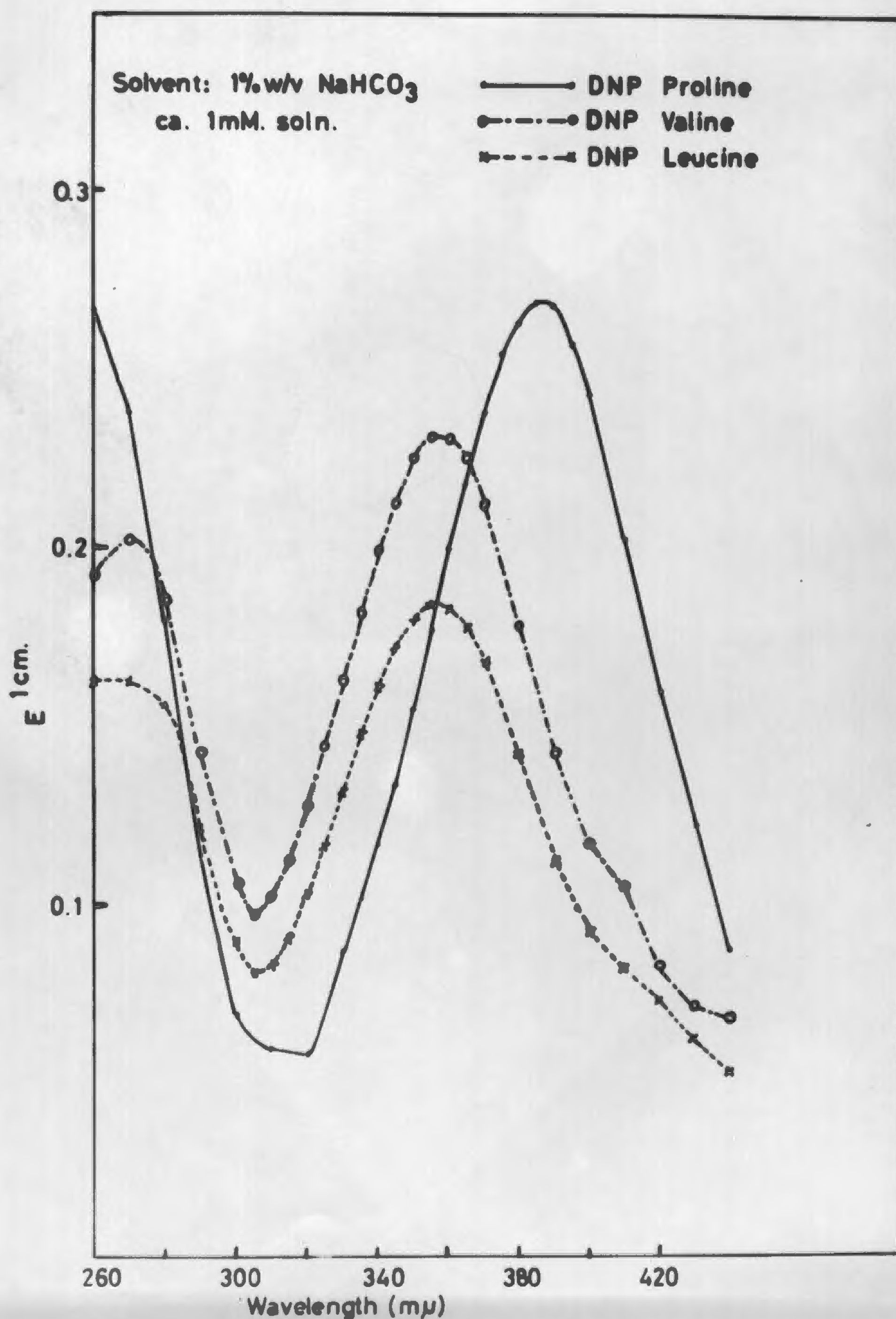


FIG. 71 ABSORPTION SPECTRA OF DNP-AMINO ACIDS



The chromatogram was sprayed with a mixture of 50 ml. of 0.1% ninhydrin in ethyl alcohol, 2 ml of collidine and 15 ml. of glacial acetic acid, and the colour developed over a hot plate or in a 105°C oven.

The DNP amino acids usually travelled in the brown 'fingers' of the phenolic front, and their identity was best established after the first dimension run.

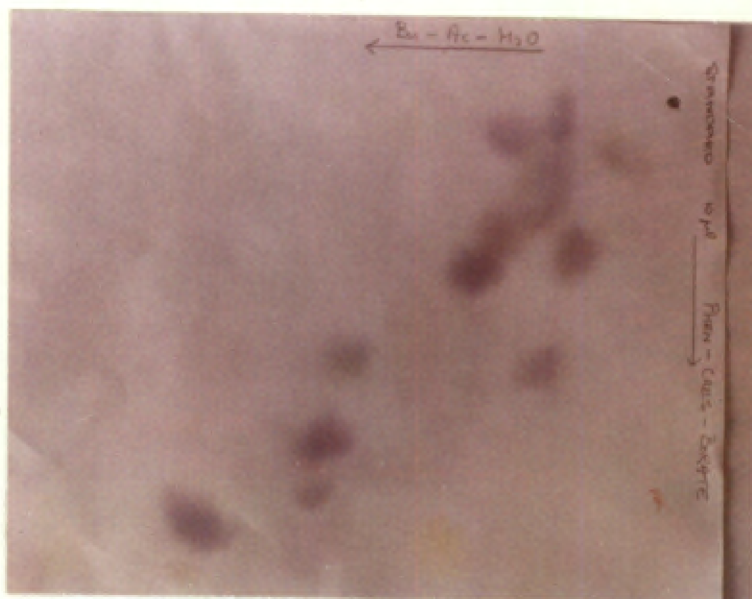
The pattern obtained from a standard amino acid mixture is shown in fig.72.

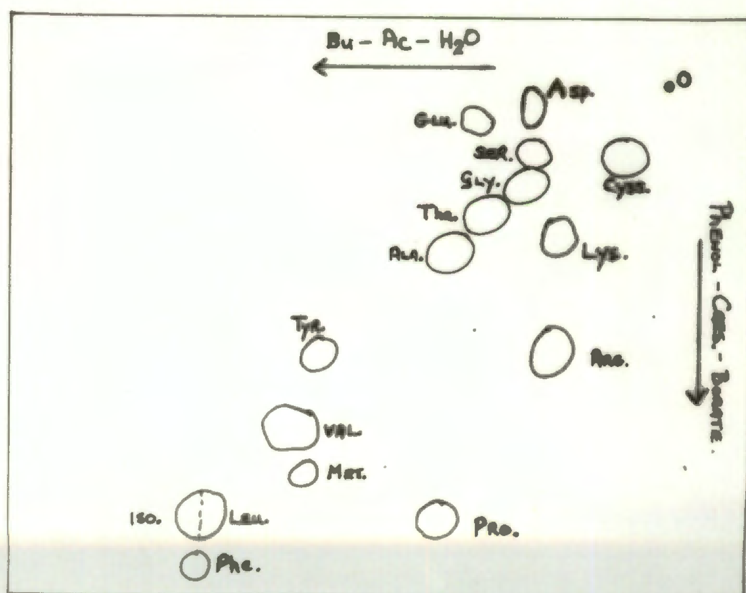
Owing to the small amount of each peptide available, a procedure was selected to give the most useful information concerning the peptide, but the following deficiencies are acknowledged.

Twelve hours of hydrolysis is the minimum time required to ensure almost complete hydrolysis of most peptides in 5N HCl at 105°C. Under these conditions most DNP amino acids are stable except for DNP-glycine -proline and cystine. DNP proline was completely lost whilst DNP glycine and cystine were mostly destroyed, but a small amount left was sufficient for identification. DNP arginine was incompletely resolved from DNP lysine and an aliquot of the aqueous phase should have been chromatographed in a tert-amyl alcohol-pH6 phthalate system which achieves this separation.

Losses of DNP glycine are unfortunate, since this amino acid has been identified in many normal urinary peptides, but for present purposes it was decided more important to look for essential amino acids in the N-terminal position of the peptide chain.

FIG. 72 PAPER CHROMATOGRAPHY OF AMINO ACIDS





Chapter 11.

Changes in Peptide Pattern during Protein Depletion, Repletion and Recovery.

The technique of separating neutral peptides from free amino acids, using gradient elution from DEAE 'Sephadex', has been applied to the urine specimens whose -amino nitrogen and bound amino acids had already been measured. The peptides were then fractionated and their pattern examined. This chapter describes the methods used, and the results obtained, on investigations of the urinary peptide patterns of children with kwashiorkor during the three stages of protein depletion, repletion and recovery. In a few cases individual peptides have been isolated and partly characterised.

Method.

The method utilised was that described in the previous chapter whereby a neutral peptide fraction was prepared from the urine, concentrated and analysed by the automatic column chromatographic system designed for analysing whole urine. The amino acid compositions of the peptides were determined by means of paper chromatography, and the N-terminal amino acid was identified by the FDNB technique.

An aliquot of urine (50 to 100 ml.) was adjusted to pH 8.6 with 1N NaOH and evacuated continuously by water pump in a desiccator for 1 hour, to remove excess ammonia.

A column of DEAE Sephadex (2.0 x 16 cms.) in the -OH form was prepared as described previously and equilibrated with 0.1M pH 8.65 collidine acetate buffer. The sample was applied, allowed to enter the resin under gravity and washed into the column with a few ml. of pH 8.65 buffer. After the sample had been applied, a 10 cm. layer of buffer was placed above the column and the gradient device, consisting of a 250 ml. flask filled with buffer, was connected. The gradient was effected with 0.1M acetic acid.

The pH of the effluent was monitored using Beckman electrodes connected to a 'Pye' pH meter and potentiometric recorder (as before). During application of the sample the pH rose sharply to 11, but fell again as soon as approximately twice the volume of the sample had been eluted. This volume and subsequent effluent, collected whilst the pH was above 8.2, was discarded. As soon as the effluent pH fell below this figure collection was begun,(*) and collection was continued until the pH was below 7.0. This fraction was referred to as the 'neutral peptide' fraction.

Concentration.

The average volume of the 'neutral peptide fraction' was 150 ml. This was concentrated to dryness in a rotary evaporator using a vacuum pump and solid CO_2/acetone mixture for cooling. The sample vessel was warmed slightly to 40°C. in a water bath. Drying took less than 1 hour to complete. When the sample had been nearly dried, the warming bath was removed and evaporation, and sublimation of collidine acetate completed. Dried peptide fraction was reconstituted in distilled water to a volume equivalent to one-tenth of that of the original sample of urine.

CHROMATOGRAPHY.

An aliquot of peptide fraction, equivalent to one-tenth of the 24 hr. urinary volume, was applied to the 150 cm. Moore and Stein column and eluted with the same conditions which were used to analyse neutral and acidic compounds i.e. pH 3.25/4.25 and 30°C/50°C., with buffer and temperature change at 415ml. (10.5 hrs.)

Characterisation of peptide peaks.

In a further few specimens, another aliquot of urine corresponding to 50 or 100 ml. was chromatographed on the Moore and Stein column (150cm.) under similar conditions to the above, but the effluent stream was split. One third was diverted for colour development, whilst the rest was collected in 2.5ml. fractions in a fraction collector. The fractions corresponding to 'peptide' peaks were pooled for subsequent analysis.

The pooled peaks were desalted on columns of IRA 140 anion exchange resin (Chap.10) and evaporated to dryness

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(*) A warning device, consisting of a microswitch and buzzer was arranged to trip as soon as the pen of the recorder reached this critical pH.

over KOH pellets. Peptide material was dinitrophenylated and hydrolysed, and the N-terminal and free amino acids identified by paper chromatography.

The DNP amino acids were identified from a standard map with reference (in second solvent only) to marked DNP amino acids.

All ether extracts contained dinitrophenol (DNP-OH) and dinitro-aniline ($DNP-NH_2$). The former provided a convenient reference point since it could be easily identified by bleaching with HCl fumes. $DNP-NH_2$ travelled with the 'toluene' solvent front.

Little difficulty was experienced in identifying the spots, except in the case of DNP-glumatic and aspartic acids, which overlapped and tended to run off the paper. With reference standards identification of these was more certain. When there was doubt the spot was labelled DNP-diCOOH.

Results:

Peptide patterns were obtained from the following specimens of:

1. A normal child (age 2½yrs.) on hospital diet.

2. Case 1 (H.L.) Day 3 (depletion).
   Day 5 (repletion).
   Day 15 (recovery).

3. Case 2 (A.O.) Day 2 (depletion).
   Day 4 (repletion).
   Day 15 (recovery).

4. Case 3 (J.C.) Day 3 (repletion).
   Day 15 (recovery).

5. Case 5 (T.I.) Day 6 (repletion).
   Day 24 (recovery).

The patterns are shown in figs. 73 to 82.

Peptide patterns.

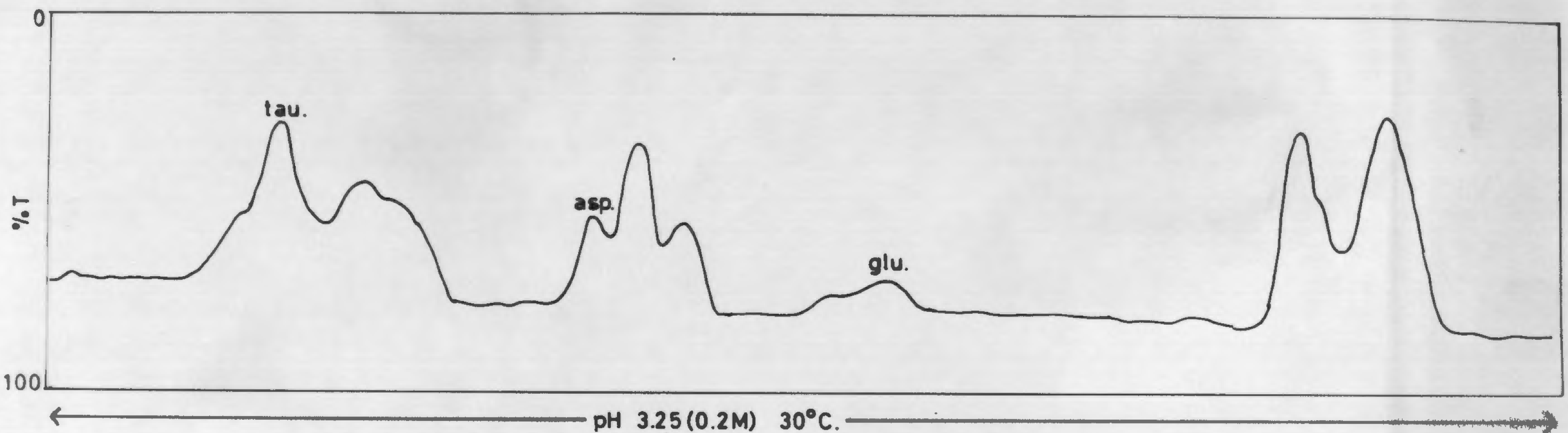
The neutral peptide patterns were so complex that it was impossible to analyse each component, especially since their composition was not known and thus no standards could be used as markers, The free amino acids, glutamic acid, cystine and tyrosine, which were expected to contaminate the neutral peptide fraction, could be easily located in most patterns and these served as internal reference points.

The patterns from a normal child and an adult superficially showed little similarity but if allowance was made for alterations in relative heights of the peaks, components of patterns could be matched. Four peaks from P-serine to urea were seen in both normal specimens. In the adult a high peak in this region was probably due to taurine.

A bifid complex in the region between urea and aspartic acid was seen in the normal as well as many of the abnormals. This corresponded to the peak seen here in unprocessed urine. (Phenylacetyl glutamine migrates to this position but is ninhydrin negative before hydrolysis.) Complexes corresponding to groups I, II and III were found in all normal and abnormal patterns, but in varying concentrations.

Protein repletion was accompanied by many changes in peptide pattern. Some peptides appeared, whilst others decreased in concentration after protein was given. In

FIG. 73 NORMAL CHILD. NEUTRAL PEPTIDE EXCRETORY PATTERN



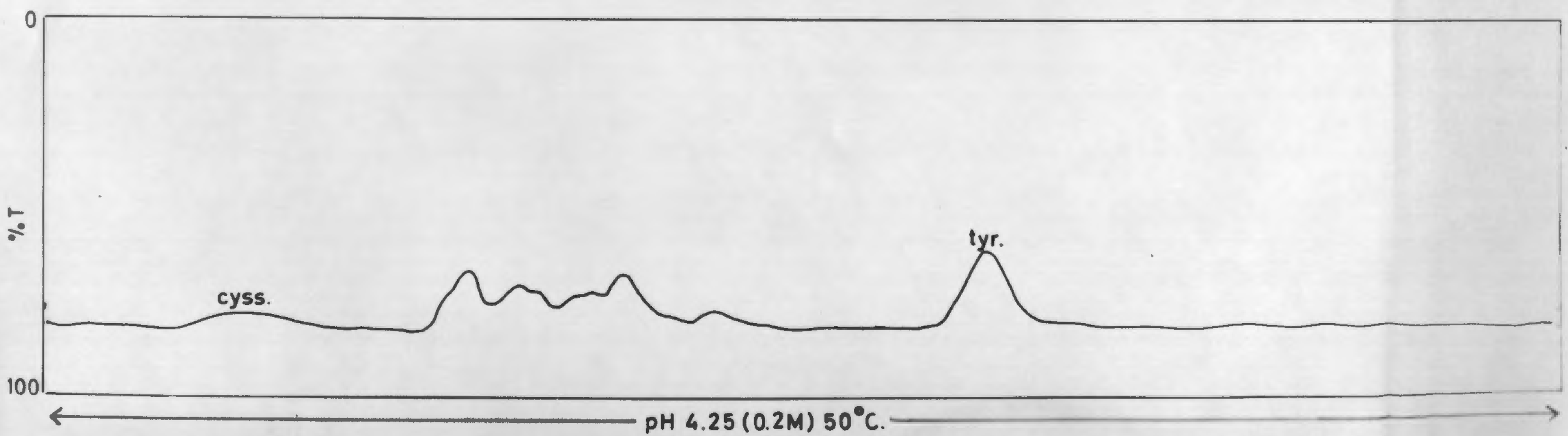


FIG. 75 CASE 1 .(H.L.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING REPLETION (DAY 5)

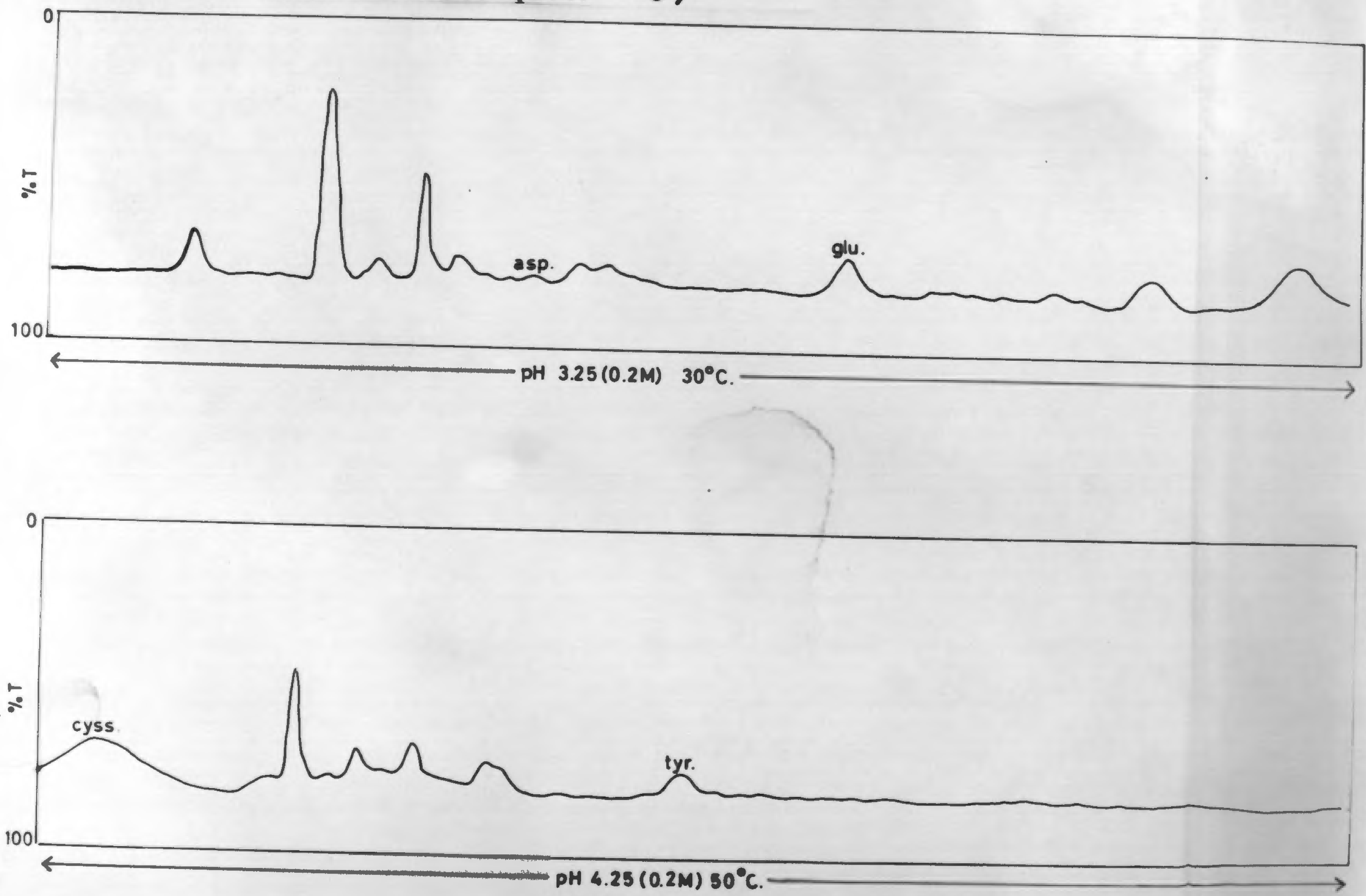
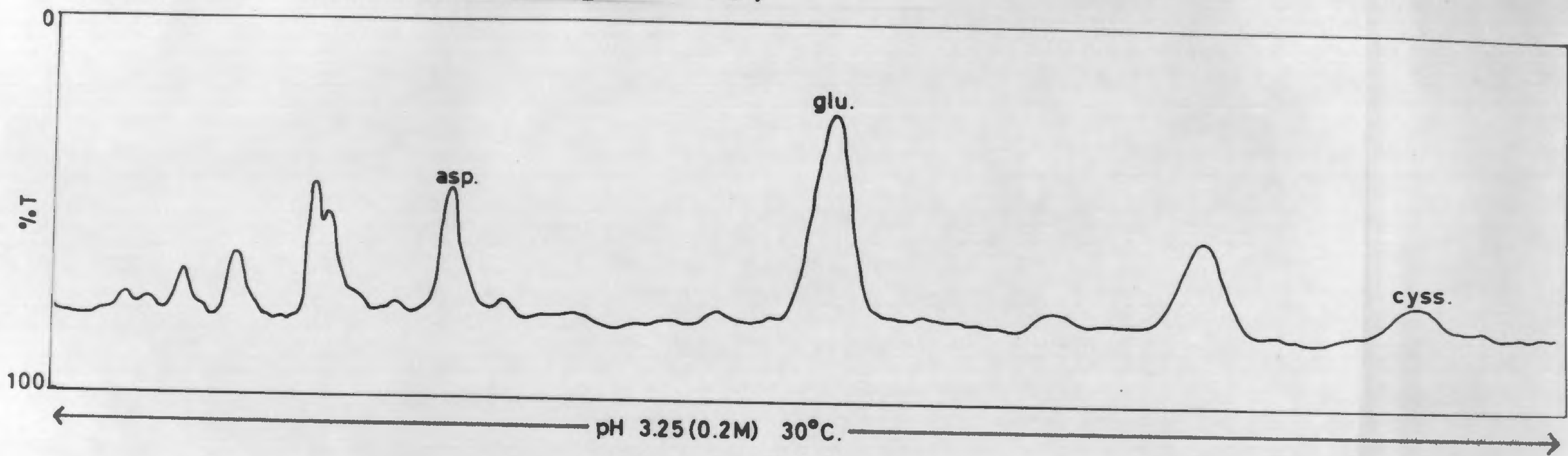


FIG. 76 CASE 1 .(H.L.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING RECOVERY (DAY 15)



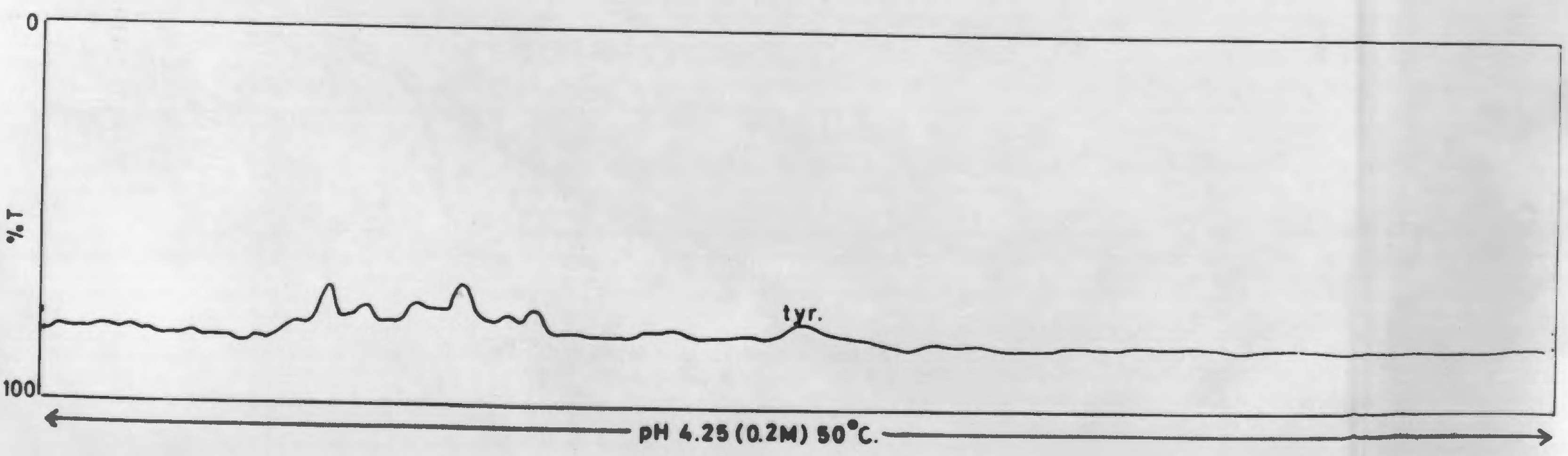
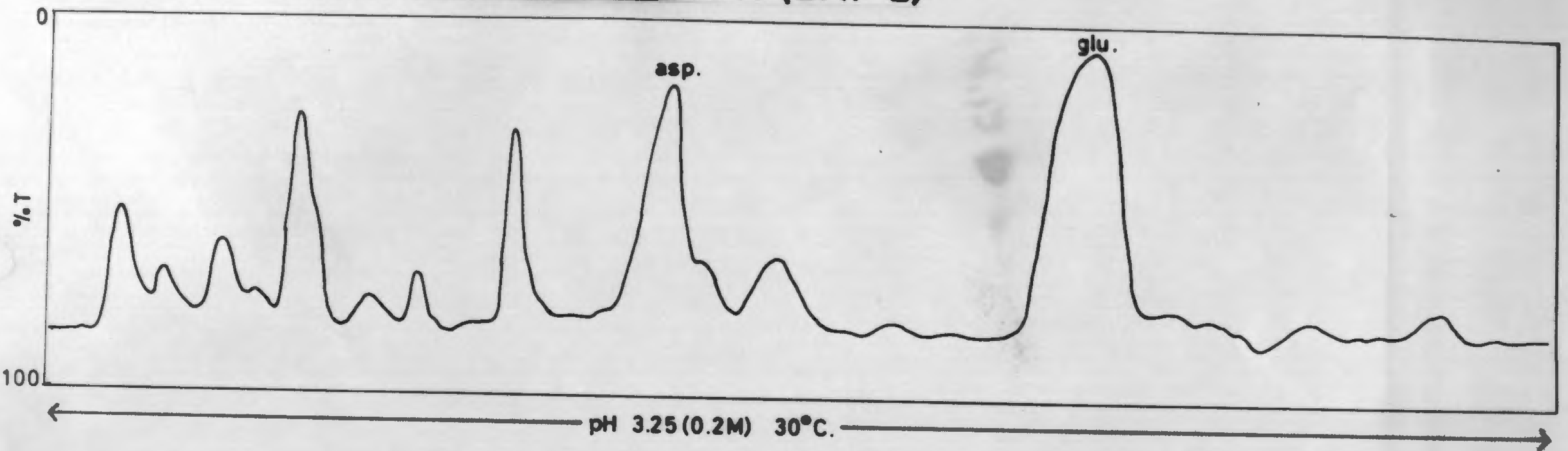


FIG. 77 CASE 2 .(A.O.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING PROTEIN DEPLETION (DAY 2)



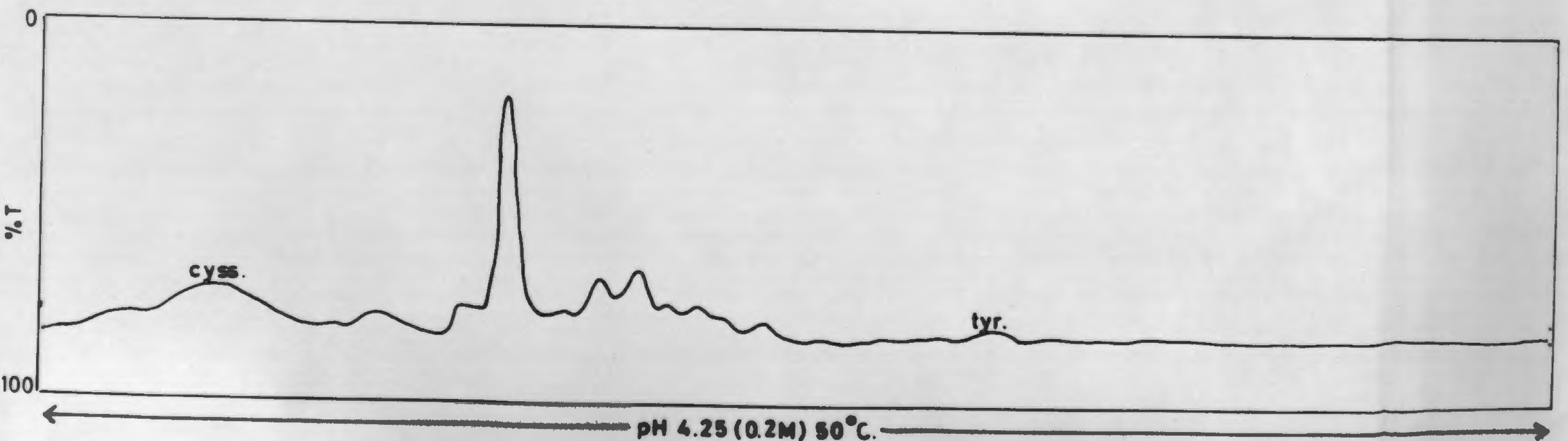


FIG. 78 CASE 2 .(A.O.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING PROTEIN REPLETION (DAY 4)

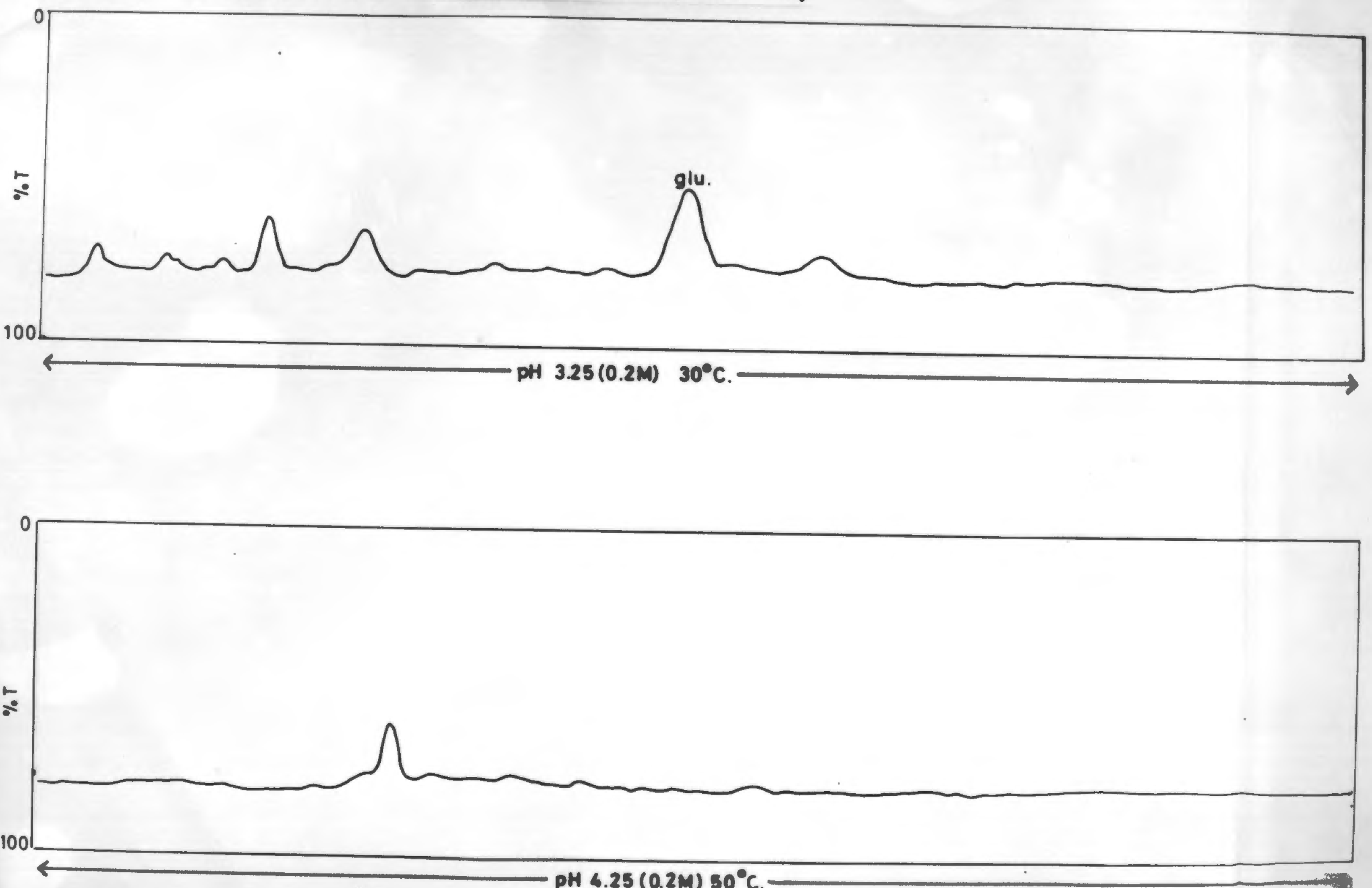


FIG. 79 CASE 2 .(A.O.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING RECOVERY (DAY 15)

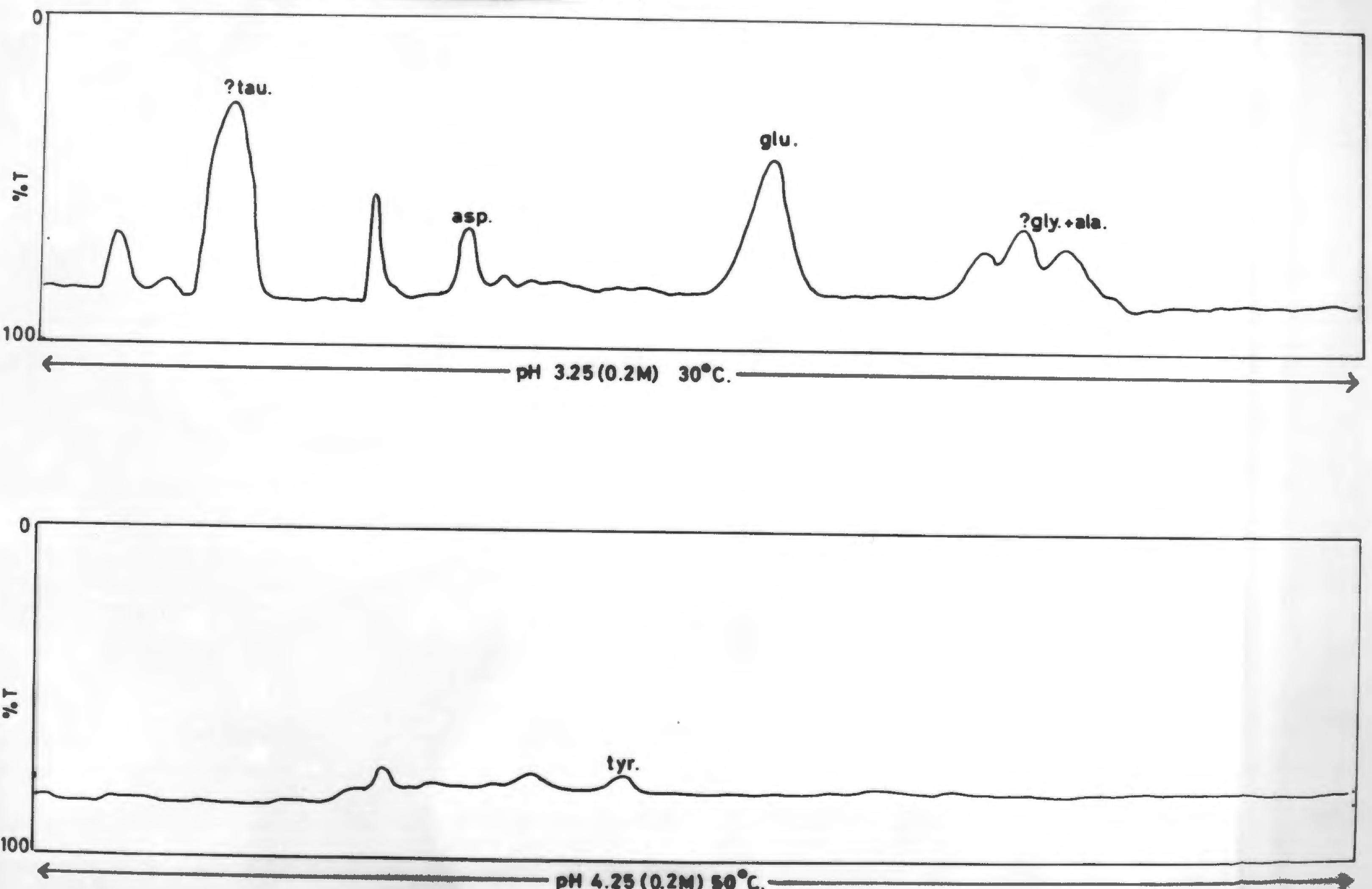


FIG. 80 CASE 3 .(J.C.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING PROTEIN DEPLETION

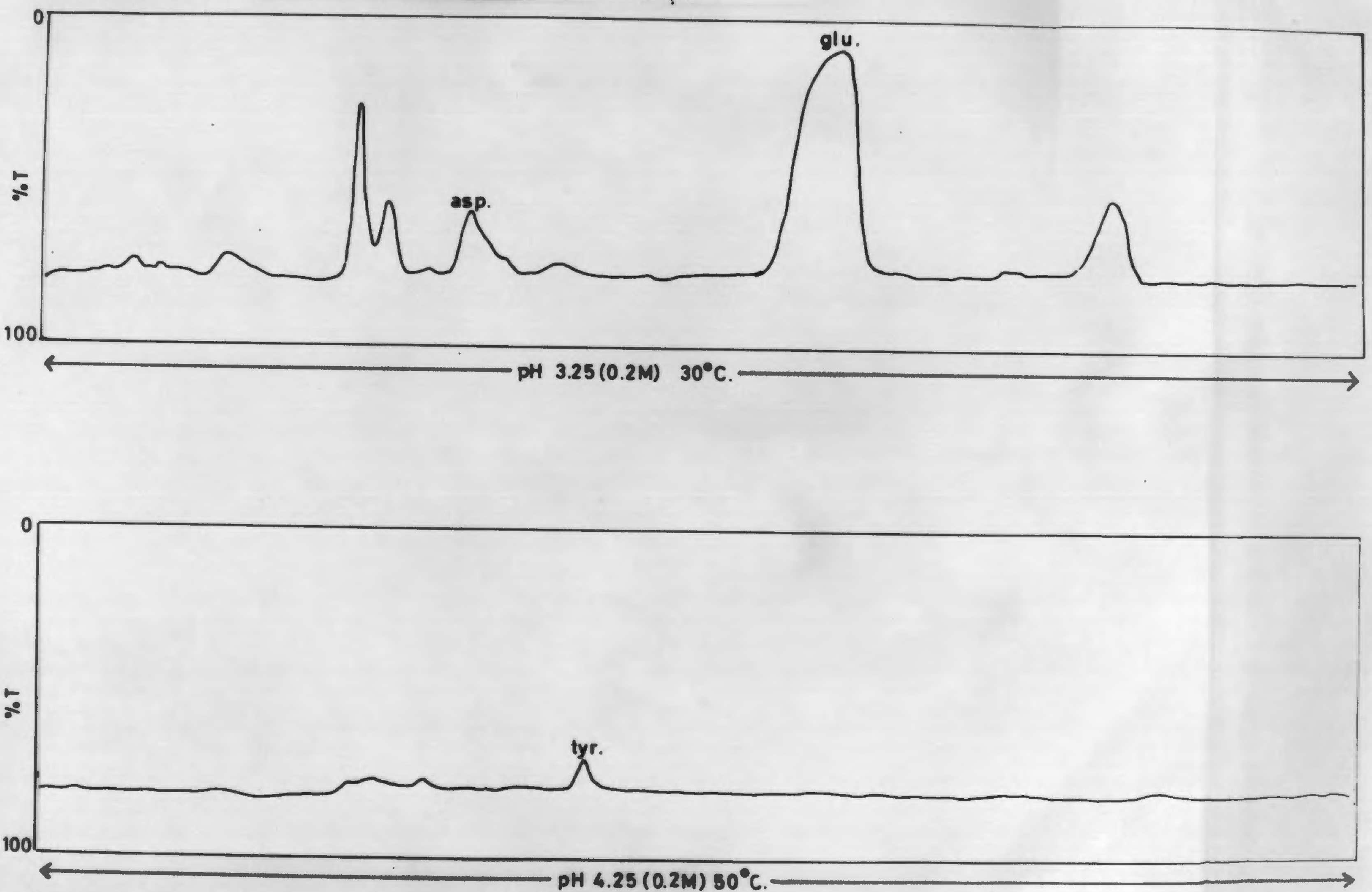


FIG. 81 CASE 3 .(J.C.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING RECOVERY (DAY 15)

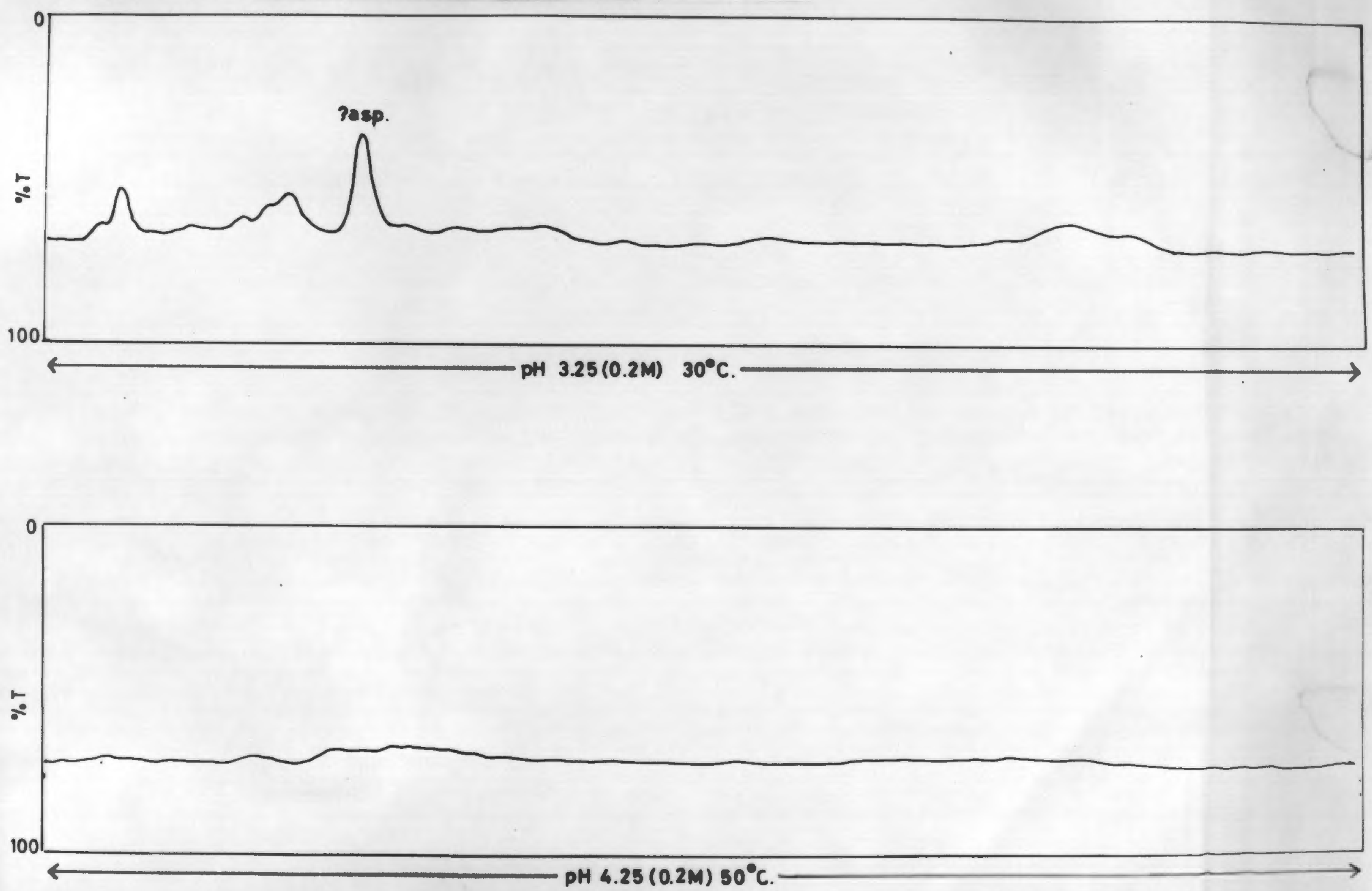
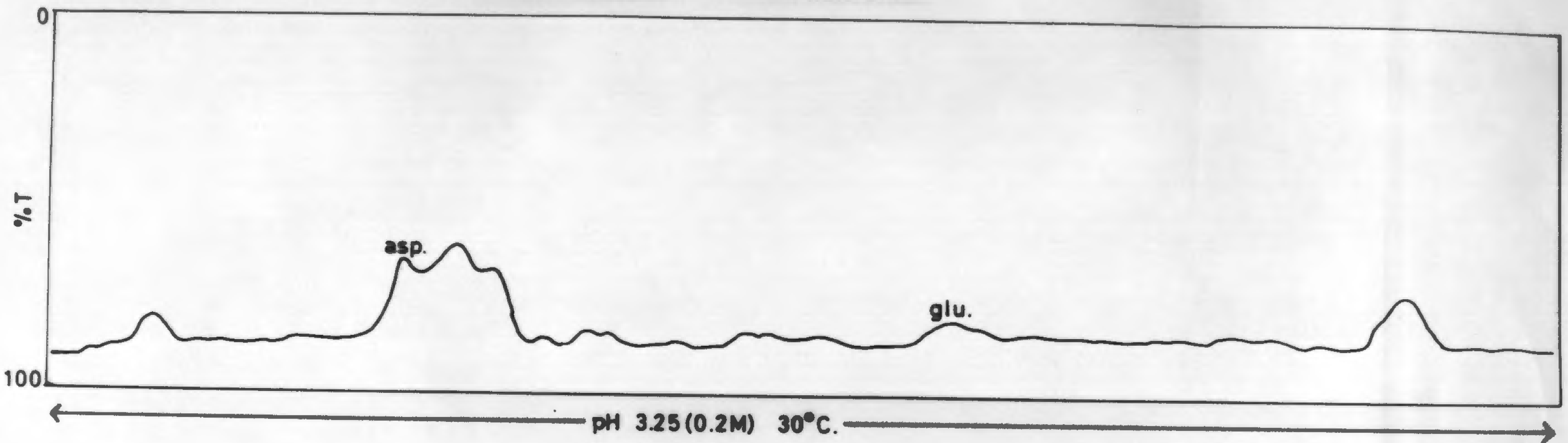


FIG. 82 CASE 5 .(T. I.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING PROTEIN REPLETION (DAY 6)



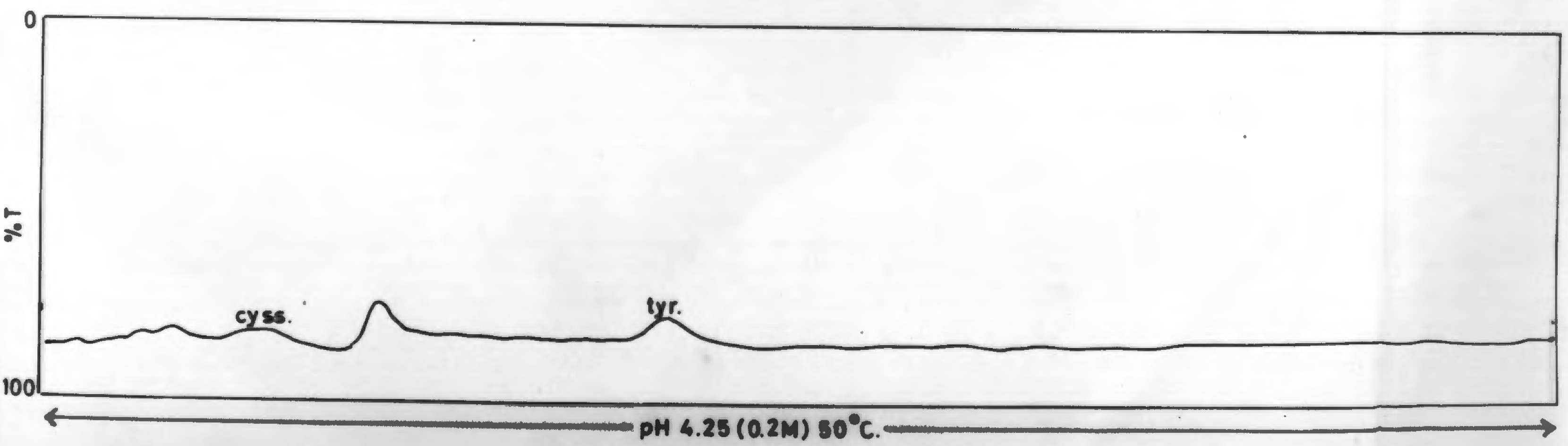


FIG. 83 CASE 5 .(T.I.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING RECOVERY (DAY 24)

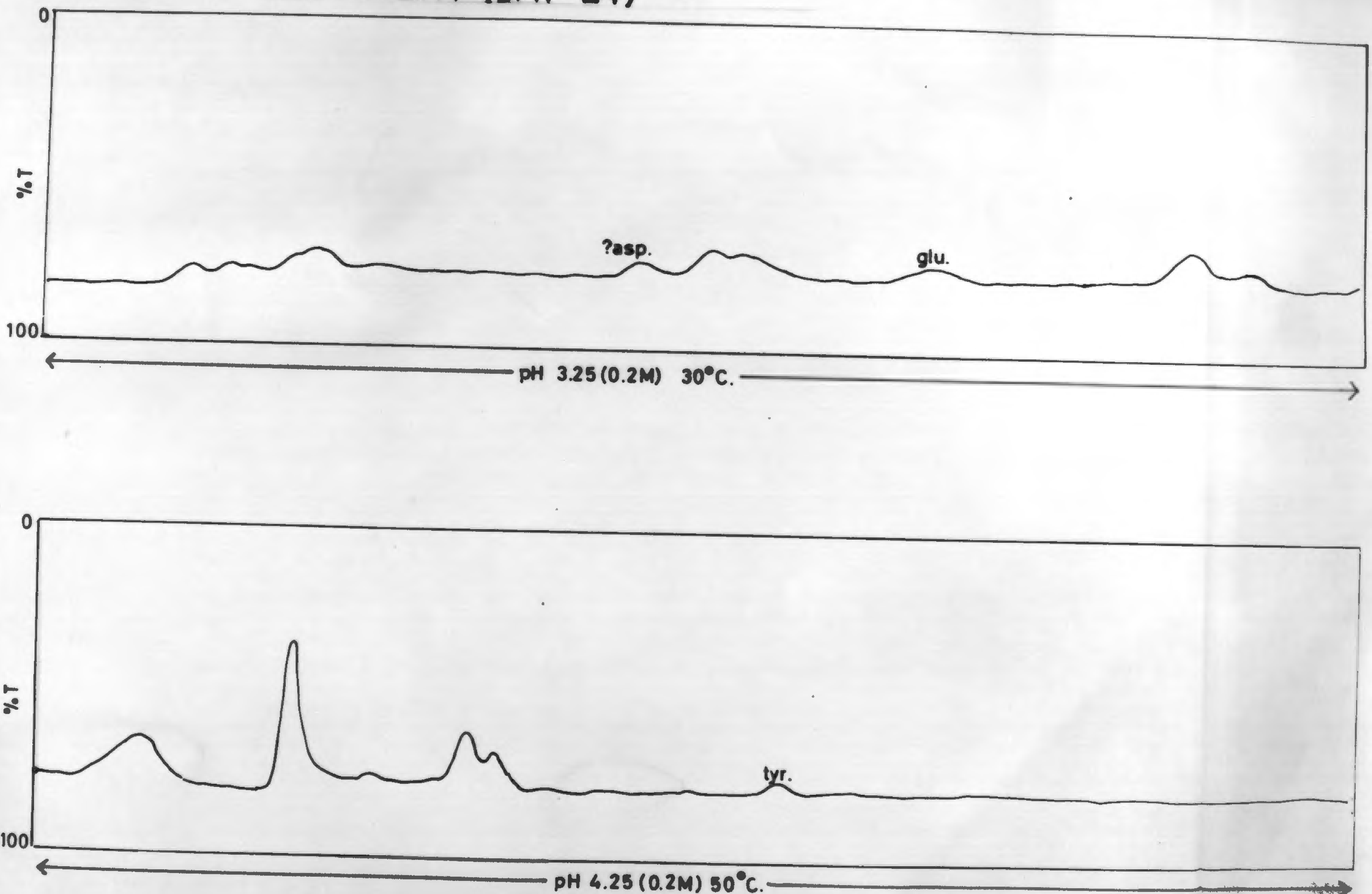
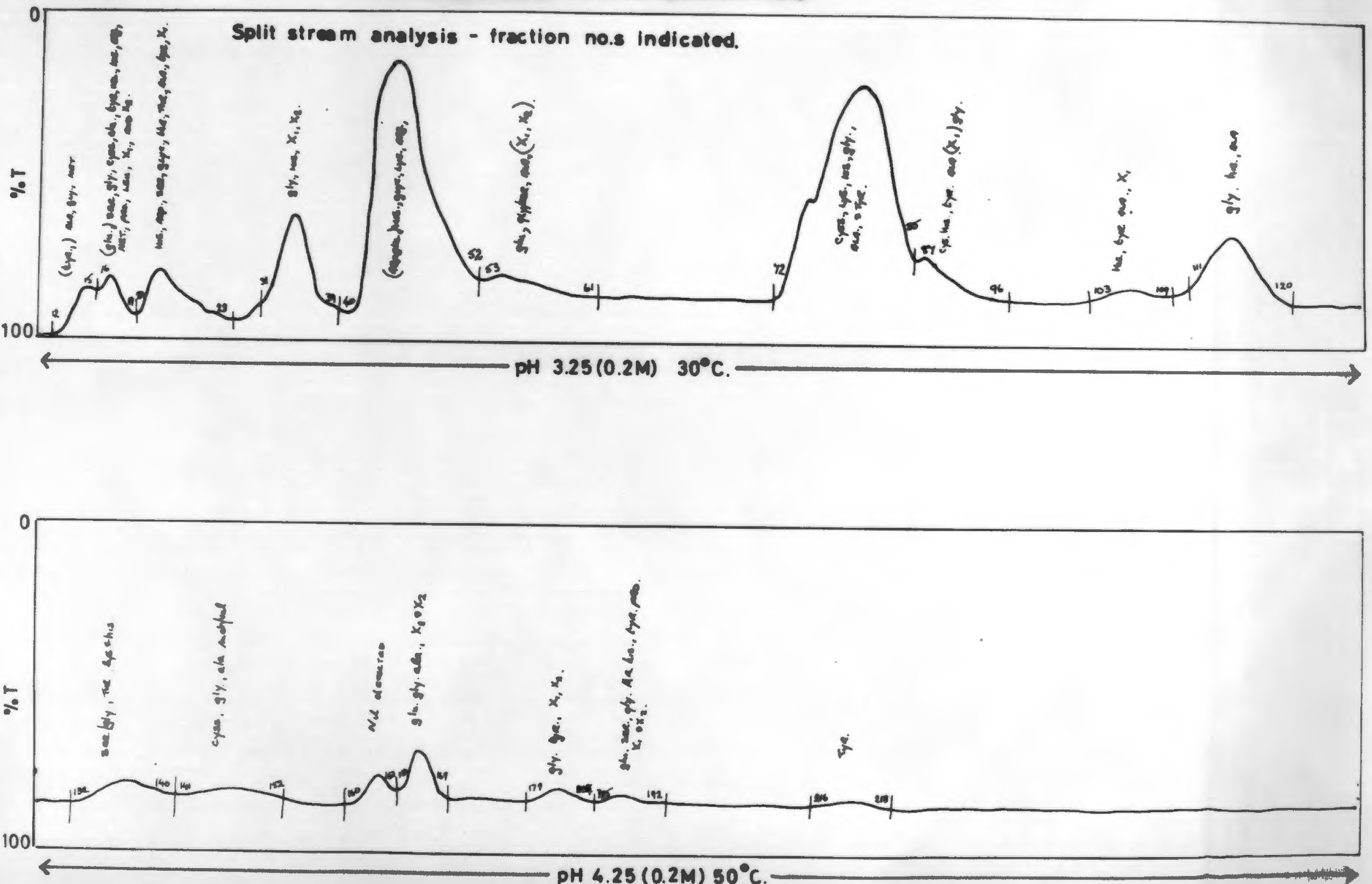


FIG. 84 CASE 2 .(A.O.) NEUTRAL PEPTIDE EXCRETORY PATTERN DURING PROTEIN REPLETION (DAY 15)



NOTES ON TABLES 31 - 36.

+, ++, +++, and ++++ = Intensity of amino acid spots on paper chromatogram.

(+) = Probably present.

* = Chromatogram partly obscured by poorly defined ninhydrin reacting material.

X_1 = Ninhydrin reacting material appearing at site of DNP - Cys. SO_3H.

X_2 = Spot between pro. and his. Identity unknown. Yields purple colour with ninhydrin.

X_3 = Brown pigment at site of Cys. SO_3H. No reaction with ninhydrin - usually associated with material at origin of the chromatogram which, behaved like a mucopolysaccharide.

Table 31.

Excretion of individual neutral Peptides - Normal child - (age 2½ years).

Fraction No.	Amount leu. eq/24 hrs. u-moles.	Composition	N-terminal Amino acid	Notes.
9 - 12	2.5	Asp/glu. +++ Ala/ser. +++ Thr. + Pro. ++	Nil	
13 - 17	8.5	Cys. SO_3H + His. + Glu/asp. ++ Gly. +++ Ala. + Tyr. ++ Pro + X_1 ++	? Glu.	
24 - 30	12.7	Gly. +++ His. ++ X_1 +++	gly.	
39 - 45	2.0	Overloaded Gly/Ser. +++ His/Lys. ++	Nil	Site of group II peptides.
52 - 56	0.9	Asp. + Gly/ser. ++ Tyr. +++ Leu/iso. +	? ala.	
63 - 66	1.8	Asp/Glu. + Ser. ++ Thr. + Gly. + Ala. + Leu/iso. +	? gly.	This and No. 67-77 not separated
67 - 77	3.2	Gly +++ Glu. ++ Ala +++ Tyr. ++ Met/Val. ++ Pro +	Nil.	

Table 31 (contd.)

86 - 89	1.1	Asp/glu +++ His/Lys. + Gly. ++ Ser. + ? Tyr. + X_1	? Gly.	May contain free gly.
95 - 101	1.3	?Tyr. X_1 X_2	nil	
104 - 111	1.0	Asp. + Glu. + Cyss. + His. ++ Ser. ++ Gly. + Ala. ++ Pro. +	nil	
120 - 124	0.8	Glu. + Cyss. + Gly/Ser. +++ Tyr. + Met. + ? Pro (+)	nil	
129 - 135	0.9	Glu. + Ser. + Gly. + Ala/Thr. ++ Tyr. +	?gly.	Cystathione peptide
141 - 146	0.6	Tyr. ++ Gly. +	nil	Site of leucine
150 - 154	0.4	Tyr. +++ ? Glu.	Tyr.	free tyr.

Table 32.

Case 1 (A.O.) Day 2. Excretion of individual neutral peptides.

Fraction No.	Amount leu.eq/24 hrs. u-moles.	Composition		N-terminal Amino acid.	Notes.
9 - 17	0.9	Asp. Glu. Ser. Gly. Tyr. Val/Met. Leu/Iso.	+ + + ++ + + +	nil	
18 - 21	1.4	Asp. Gly. Ala. Val/Met.	++ + + +	Ala. Leu (trace)	At least two peptides
22 - 29	0.9	? Tyr.	(+)	Nil	
30 - 33	0.2	Cys. SO_3H Glu. Gly. Ala.	(+) + + +	? gly.	? free Cys. SO_3H
34 - 37	0.1	nil detected		nil	artefact
38 - 47	5.5	Asp. Glu. Gly/Ser. Tyr. His. Leu/Iso. Phe. X_1	++ ++ ++ + ++ + (+)	glu	
48 - 56	3.4	Cyss. His. Thr. X_1	+ + + ++	phe.	
62 - 70	1.1	Asp. Glu. Gly. Val/Met. Ala. His.	+ + + + + +	nil	

Table 32 (contd.)

71 - 77	1.4	His. Cyss. Gly. X_1	++ * + ++ ++++	nil	ill-defined material on paper
98 - 108	6.5	Glu. Gly. OH Pro. Pro.	+ + ++ ++	? gly.	Hydroxy proline peptide
131 - 139	1.9	Asp. Cyss. Gly. Lys.	+ + + +	Asp/gly. Met.	Mixture of two peptides
160 - 163	1.7	Tyr.	++	tyr.	Free tyr.

Table 33.

Case 2. (A.O.) Day 4. Excretion of individual neutral peptides during protein repletion.

Fraction No.	Amount leu.eq/24 hrs. u-moles.	Composition.		N-terminal Amino acid.	Notes.
16 - 20	0.9	Cys.SO_3H	+++		Free Cys.SO_3H
21 - 25	5.3	Asp. Glu. Cyss. Ser. Ala, X_1	++ + + + + ++	glu/asp. + cyss.	contains some free Cys. SO_3
26 - 32	0.3	Asp. Cyss. Ala. Val. X_1	+ + + + +	nil	
33 - 38	0.2	Asp. Glu. Gly/Ser. Ala.	+ + ++	glu/asp.	
39 - 44	3.3	Leu.	+	nil	bound leu
45 - 50	4.0	X_1 Cyss.	++ (+)	Thr/Ser.	May be free Thr/Ser.
55 - 58	0.2	Glu. Gly. Ala. Phe. Arg. Lys. Tyr.	++ + + ++ + + +	? gly.	
59 - 65	1.0	Glu	++	Gly.	Glycyl-glu

Table 33 (contd.)

66 - 69	0.2	Ser.	++	Ser.	?free ser
70 - 74	1.1	Asp. Glu. Lys.	+ + 	Asp.	
95 - 103	8.9	Gly. His. Tyr. Lys.	++ +++ + (+)	nil	
104 - 110	9.2	Glu. Gly. Ala. His. Tyr.	+ + + ++ +	nil	Contains free glu.
117 - 125	1.2	Glu. Asp. Ser. Met. Pro.	+ + + + +	nil	
126 - 140	18.7	X_1 Tyr.	+ +		At site of free ala
150 - 165	1.2	Gly. Tyr. Val. Leu/Iso. Glu.	+++ + + + +	glu.	
174 - 180	4.2	Glu. Asp. Gly. Val. Leu/Iso.	++ + + + +	? gly.	At site of cysta thione.
186 - 190	0.6	Glu. Ala. Tyr.	 + +	nil	
191 - 199	0.8	Glu. Ser.		nil	At site of leu.

Table 34.

Case 2 (A.O.) Day 15. Excretion of individual neutral peptides during recovery.

Fraction No.	Amount leu.eq/24 hrs. u-moles.	Composition		N-terminal Amino acid.	Notes.
12 - 15	1.9	Tyr. Ala. Gly. Met.	++++ ++ + ++	nil	
16 - 19	2.3	Glu. Ser. Gly. Cyss. Ala. Tyr. Val. His. Arg. Met. Pro. Leu/Iso. X_1 X_2	++ ++ ++ (+) ++ ++ ++ ++ + ++ + + ++ +++	glu/asp.	almost complete complement of amino acids.
20 - 28	3.3	His. Asp. Ser. Gly. Thr. Ala. Tyr. X_1	++++ + ++ ++ + + ++ ++	? gly.	
31 - 39	5.4	Gly. His. X_2 X_1	+++ ++ + +	? gly.	
40 - 52	43.2	His. Gly. Ala. Lys. Arg.	+++ (+) (+) (+) (+)	asp/glu.	

Table 34 (contd).

53 - 61	6.3	Glut. Gly/Ser. Ala. Tyr. X_1 X_2	+ ++ + (+) ++ ++	nil	
72 - 85	54.1	Cyss. His/Lys.	(+) (+)	Glu.	Contains free glu.
87 - 96	4.4	Cyss. Lys. His. Gly. Ala. Tyr. DNP basics	+ + + (+) + ++ ++	nil	
103 - 109	0.6	His. Tyr. Ala. X_1	+* + + +	nil	
111 - 120	9.7	Gly/Ser/ Thr. Ala/Lys. His.	 +++ ++ +++++	nil	At site of free glycine
132 - 140	2.36	Ser/Gly. Thr. Cyss. His.	(+) + + +	glu/asp.	
141 - 152	6.7	Cyss. Gly. Lys. Met/Val.	+++ + ++ +	? gly.	
164 - 169	2.9	X_2 Glu. Gly. Ala. X_1	+++ + + + +++	nil	At site of cystathione

Table 34 (contd).

185 - 192	1.3	Glu. Ser. Gly. Thr. His. Tyr. X_1 X_2 Pro.	+ ++ (+) + ++ ++ ++ ++ (+)	nil	
210 - 218	0.7	Tyr.	+++	tyr.	Free tyr.

Table 35.

Case 5 (T.I.) Day 6. Excretion of individual neutral peptides during protein repletion.

Fraction No.	Amount leu.eq/24 hrs. u-moles.	Composition.		N-terminal Amino acid.	Notes.
17 - 20	3.9	Gly.	+	gly	
		Ala.	+		
		Val.	++		
		X_1			
35 - 40	6.4	Tyr.	++		At site of Group II peptides.
		X_1	++		
		X_2	++		
47 - 53	3.0	Glu.	+	gly.	
		Gly.	+		
		Ala.	+		
		Tyr.	+		
		X_1	+		
		X_2	++		
57 - 64	1.1	Gly.	+	? ala.	
		X_2	++		
		Tyr.	(+)		
75 - 82	0.9	Glu.		glu	Contains free glu
		Gly/Ser.	+		
		Ala.	+		
		His.	+		
107 - 113	2.1	Glu.	+	nil	
		Ser.	++		
		Ala.			
		His.	+++		
		X_1	+		
144 - 151	2.0	Gly.		nil	Site of cystathione.
		Tyr.	+		
		Glu.			
		X_1	+		
170 - 175	0.8	Results lost.		nil	

<u>Table 36.</u>

Case 5. (T.I.) Day 15. Excretion of individual neutral peptides during recovery.

Fraction No.	Amount leu.eq/24 hrs. u-moles.	Composition.		N-terminal Amino acid.	Notes.
[illegible] - 35	0.3	X_3	++*	nil	
89 - 95	6.7	Glu. Ser. Gly. Ala. Lys. Val/Met. Leu/Iso.	+ + + ++ + + +	nil	
116 - 122	3.8	Cyss. Asp. Glu. Iso. Phe. Lys. His. Gly.	+ + ++ + + + + (+)	nil	
123 - 129	1.1	X_3 Glu. Gly. X_1 His.	+++* + + + (+)	ala.	
150 - 155	11.5	Glu. Gly. X_2	+ + ++	? phe.	"Cystathione peptide"
161 - 165	4.3	Glu.	(+)	gly.	site of leu.
166 - 169	2.9	Tyr. Val.	(+) +	gly.	

this small series of cases, no pattern was detected which was characteristic of the depleted or repleted state and each case behaved differently on repletion as regards excretion of individual peptides. In spite of the diversity of peptide pattern and response to feeding, the following general observations could be made:-

1. Many neutral 'peptides' conformed in chromatographic behaviour to the acid-labile peaks observed when chromatographing small quantities of whole urine (2-5ml.). They corresponded to groups I, II and III.

2. The number and concentration of peptides in Group I increased with protein repletion. Case 2,(A.O.) showed the reverse of this. This case was also anomalous with regard to the excretion of bound $-NH_2$ nitrogen. A high bifid peak between urea and aspartic acid appeared to be characteristic of normal urine. Concentrations were low in the acute phase, but increase repidly to normal levels on repletion.

3. Increase of Group 2 complex which was especially noted with whole urine in Case 1 (H.L.) was also seen in the 'peptide' pattern of that case, and again in Case 4. This peptide has been shown to consist of glycine, proline, tyrosine and glutamic acid.

4. An increase in peaks at site of alamine accompanies the recovery phase. (Dramatically on Day 5 in Case 4).

5. An 'ethionine' peptide≠ disappears on treatment.

6. The number and amount of material in Group III (methionine to leucine) decreases with treatment.

The patterns on split stream analysis corresponded to those obtained in the conventional way but have been included and numbered to correspond with fraction numbers of peaks whose composition was determined (e.g. Fig.83 and tables 31 to 36).

The dicarboxylic acids, glutamic and aspartic acid, were present in virtually all the peptides examined. Glycine, serine and alanine were also present in the majority. Other amino acids were less common and appeared to be randomly distributed. Histidine was found in a few peptides, but lysine and arginine in only two instances. The N-terminal amino acid was most commonly glycine or alanine but glutamic or aspartic acid, and sometimes phenylalanine were detected. The free amino acids glycine, cystine and tyrosine were detected as N-terminal acids since these contaminated the neutral peptide fraction and appeared as peaks in the effluent chromatogram at the expected elution sites.

There was no obvious change in composition of the peptides accompanying protein repletion. Generally peptides from group I (eluted early) had more varied amino acid composition, including acidic, basic and neutral amino acids, whilst those eluted later had generally simpler structures.

≠ Indicates site of elution, not composition.

Although there was no information as to size of these peptides, those with many different amino acids in group I must have been relatively large (±8 residues), and these are the ones which increased during protein therapy.

The composition of the peptides was in agreement with the distribution of amino acids in the bound form (Chapter 9). Glutamic and aspartic acids, glycine, alanine and serine were the major amino acids constituting the bound fraction in all specimens of urine.

The compositions of peptides isolated during the present investigation on childrens' urine were not dissimilar from those isolated from the urine of normal adults 13. (158) , (160) in spite of differences in methodology. The peptides isolated by the latter worker were unusual in that they were rich in arginine and lysine. These authors also noted unknown ninhydrin positive substances in hydrolysates of urinary peptides. Carbohydrate and lipid bound peptide material has been noted in urine.

The behaviour of X1 suggested that it contained some carbohydrate. The nature of X2 was unknown.

Glycine and alanine were the only N-terminal amino acids found in the 8 peptides isolated by Sarnecka-Keller (160), and in two of these, none was found. Although these amino acids occupied the N-terminal position of some of the kwashiorkor peptides isolated, others were identified as well. These could not have been present as free acids,

since they were found in peaks which did not emerge at the expected site of that amino acid. The dicarbonxylic acids are known to occur in urine as weakly bound conjugates, and it is possible that these were split during dinitrophenylation, and were extracted into the ether phase, where they were detected after chromatography.

Chapter 12.

IMPLICATIONS OF EXPERIMENTAL RESULTS.

Although the number of cases studied was small it was fortunate that the urinary specimens of these had been examined by several procedures of increasing order of specificity. These were complementary and it was possible at this stage to correlate them in order to obtain a picture of peptide metabolism during various stages of protein depletion and its correction.

Correlation of Experimental findings.

The main hypothesis, that protein repletion would cause increased peptide excretion in urine has been substantiated on a small series of cases suffering from kwashiorkor. This increase was both with respect to excretion of peptides after recovery had taken place, and when compared with peptide excretion of a normal child, which was also examined.

Maximum excretion of peptides was on the 4th to 6th day following the beginning of protein repletion. Some individual peptides contributing to total increase in peptide material have been isolated and partly characterised. Many contained at least 8 - 10 amino acid residues (possibly more) and all the amino acids of protein hydrolysates were represented.

In addition to those peptides which were maximally excreted during the repletion phase, some peptides were

excreted in the depleted state and were diminished or not detectable during repletion and on recovery. Peptides exhibiting this behaviour were significantly lacking in essential amino acids.

A third group of peptides showed increased excretion when repletion was commenced, and continued to be excreted during recovery. They corresponded to some of the peptides found in normal urine.

Essential and non-essential amino acids were excreted in urine bound as peptides, but the latter were quantitatively more prominent. In spite of this it was found that when protein intake was low or nil, essential amino acids continued to be lost in urine in peptide form. This contrasted with excretion of free forms of essential amino acids.

En passant, the aminoaciduria of acute kwashiorkor was confirmed, including losses of β-amino isobutyric acid, which may be an index of tissue breakdown.

Significance of Experimental observations:

Before the metabolic significance of the findings could be discussed, it was necessary to consider to what extent the findings in urine reflected changes in endogenous metabolism.

In some of the cases studied it was obvious that alterations in urinary flow rates influenced the amount of peptide material excreted, and it might be argued that those

cases with diuresis were excreting peptides retained during oliguria. Such effects are unlikely for the following reasons:-

1. The concentration of bound amino acids was greater in some of diuretic specimens.
2. When there was no alteration in urinary volume, concentration of peptide material was highest after protein repletion.
3. There was no correlation between the ratio of bound to free forms of amino acids and urinary volume.

It has been postulated that the aminoaciduria of protein depleted states is a result of temporary dysfunction of the re-absorptive processes of proximal tubular cells of the kidneys (248). No similar differential reabsorption of peptides has been demonstrated in normal persons and the absence of peptides from serum indicates that these are rapidly cleared from the circulation. For these reasons it was considered unlikely that the findings in the urine were due to renal causes and it was concluded that the changes observed were of endogenous origin. If this were not the case, one would have to postulate differential absorption of peptides to account for increased excretion of some (peptides) whilst others decreased during protein repletion, or alternatively propose dual mechanisms.

Possible Enterogenous origins of Urinary Peptides.

Since peptides are rapidly cleared from blood into urine, absorption of these from the gut and changes in digestion or rate of absorption might influence the amount and the pattern of urinary peptide excretion.

Most experiments on the influence of diet on peptide excretion have been performed on dogs. Silber and Porter (250) found that 6% of orally administered peptides in a partial protein hydrolysate were absorbed from the gastro-intestinal tract and appeared in the urine. Completely hydrolysed protein did not increase bound amino acids in urine. Dent and Shilling (251) failed to find an increase in bound amino acids in portal blood in dogs who were fed partially hydrolysed casein. Christensens' (252) studies on partially gastrectomised dogs showed increases in conjugated amino acids in portal and jugular plasms but quantitatively only a minor part of protein was absorbed in peptide form.

The mechanism of amino acid and peptide absorption is not, however, a simple one. There is circumstantial evidence suggesting that whole protein may be absorbed from the intestine. This would explain passive immunisation of the new born mammal by absorption of γ-globulins from maternal milk. Absorption of protein antigens similarly may produce systemic allergic phenomenon. When dogs were

fed homologous protein (dog albumin) orally, there was no rise in either the free or bound amino acids of the portal blood, although all the protein left the gut within five hours, suggesting that whole protein was being absorbed (253).

Not all plasma (and urine) amino acid conjugates come from peptide fragments of partially digested protein. Oral administration of both free glycine and glutamic acid caused an increase in amino acid conjugates in plasma (254). These may be synthesised in the wall of the intestine.

The pancreas of kwashiorkor children is characteristically atrophic (255), and the exocrine digestive enzymes, amylase, trypsin and pancreatic lipase have very much lowered activity when measured in duodenal fluid (256). This would explain malabsorption of fat (steatorrhoea) and carbohydrate (lactorrhoea). The mucosa and wall of the small intestine is also thin and atrophic. It would not be surprising if, under these conditions, absorption of protein nitrogen was impaired. However, Cravioto and his co-workers have shown that in the early stages of kwashiorkor, even if complicated by severe diarrhoea, nitrogen absorption is only a few per cent below normal (99). Normal nitrogen absorption in the face of defective protein digestion implies that whole or fragments of proteins (peptides) are being absorbed and excreted in urine, which thus explains the peptiduria during protein repletion. Enzyme concentrations might be adequate to hydrolyse the small amount of protein intake

during the acute phase of the illness but inadequate for relatively large amounts given during repletion. Activity of digestive enzymes is rapidly restored by protein therapy and would explain why in the recovery phase, when equal or larger amounts of protein are being ingested, peptiduria returns to normal levels. This mechanism cannot of course, account for disappearance of some peptides during protein repletion.

If enterogenous absorption of peptides does account for increased peptiduria, one would expect the excreted peptides to have the same composition of the ingested protein. Casein and lactalbumin account for almost all of the total milk proteins and have similar amino acid composition (257).

Milk proteins are particularly rich in glutamic acid, proline, serine and the essential amino acids lysine, valine, leucine and isoleucine. The concentration of glycine is low. Glutamic acid did form a major portion of the bound urinary amino acid fraction and was present in most of individual peptides which were isolated at all levels of protein intake. Glycine and alanine were present in approximately equal quantities in the bound forms, which does not correlate with the low concentration of these acids in milk proteins. Although relatively greater amounts of essential acids were excreted in bound form than free, compared with glutamic acid they were not as abundant in urine as in milk protein (1 : 2). It was obvious therefore

that although an enterogenous cause may have accounted for some of the peptides which were excreted in increased amounts during protein repletion (especially those rich in essential amino acids) the majority of the urinary peptides were not derived from this source.

Peptides derived from tissue breakdown.

The concept of protein turnover implies that protein molecules are being continuously broken down. According to Monod (258) breakdown is entirely extracellular and on this basis it is easy to understand how protein degradation products might appear in the urine. It has since been shown that intracellular turnover is perhaps more important on a quantitative basis and is catalysed by cathepsin like activity. (See prev.) The products of intracellular protein breakdown are immediately available for reutilisation (recycling), and this mechanism would lengthen the apparent half life of that protein when turnover rates were measured. It is probable that partially digested protein can pass out from ageing cells, or those about to be replaced, in those organs where cell replacement is a normal procedure, e.g. epithelium, liver, kidney, etc.

Increased excretion of urinary peptides has been noted in wasting and following tissue damage. Increased peptiduria in burned patients has been known for a long time (136) and accounts for much of the negative nitrogen balance in this condition. Diffuse hepatic necrosis, which causes massive amino aciduria also results in pathological peptiduria (178).

Workers in Poland have been particularly interested in tissue breakdown fragments and have described pathological peptiduria in cirrhosis of the liver with jaundice (173), muscular dystrophy and following x-ray irradiation of tumours. Boulanger also isolated two peptides from patients with malignant tumours which were not present in normal urine. It may be inferred from these observations that at least some of the peptides which normally occur in urine result from tissue breakdown.

Unfortunately one cannot, by characterising individual peptides, decide whether these are degredation products or whether they are by-products of protein synthesis. In the case of at least one group of proteins, however, this question may be unambiguously answered. Hydroxyproline which occurs only in collagen and in elastin of connective tissue, is not incorporated into the peptide procollagen chain as such, but is only formed by hydroxylation of proline which has already been in the chain after synthesis is almost complete. Peptides containing hydroxyproline must therefore be derived from degradation of collagen. All hydroxyproline excreted in the urine is in bound form and measurement of urinary hydroxyproline is directly related to collagen breakdown.

Before protein was given, the children suffering from kwashiorkor excreted more bound hydroxyproline than normal or than during repletion-convincing evidence of increased degradation of connective tissue during acute kwashiorkor,

which could be reversed by protein repletion. Individual peptides which were excreted maximally before treatment was commenced, behaved as if they too were protein degradation products, although this is by inference only, since their amino acid composition is not characteristic. The excretion of free β-amino isobutyric acid also follows this pattern, i.e. much increased excretion during the early acute phase. This substance results from catabolism of the pyrimidine, thymine, and also indicates nucleoprotein degradation.

Children with untreated kwashiorkor also show clinical evidence of loss of collagen. Skin fold thickness is reduced and the skin is generally lax. This explains the tendency to dependant oedema, even whilst the rest of the body is dehydrated. The percentage body content of water (measured by H^3_2O dilution) is increased from 63 to 85% at the expense of body solids amongst which collagen is a major component (259). The percentage diminution in the quantity of collagen is thus greater than that of serum albumin, which is usually used as an index of the severity of protein depletion. There can be no doubt that at least some of the increase in bound α-amino nitrogen and some individual peptides, which have been isolated, were derived from increased tissue breakdown. From a teleological point of view, it is easy to understand why certain proteins, e.g. those of the brain, heart, serum and kidney, are conserved at the expense of others, which are less essential for life

(collagen), but the mechanisms whereby this conservation is achieved, are unknown and offer a challenging avenue for future investigation.

<u>Peptides derived from intermediates of protein synthesis</u>.

Originally it was hoped that some peptide or group of peptides derived from intermediates that were characteristic of the protein depleted state would be found in urine and that such peptides could influence the rate of protein synthesis and turnover.

Study of the peptide excretory patterns did not reveal any one which was characteristic of the protein-depleted state. Although peptide patterns varied from case to case, consecutive analyses did exhibit certain trends. Generally, the most dramatic change in excretion of bound α-amino nitrogen and of individual peptides occurred 3 to 5 days after protein repletion had been commenced. This is the time at which the serum albumin starts to return to normal but even more dramatic changes occur in certain globulin fractions which can be separated by starch gel electrophosis (260). If these proteins are taken as an index of protein synthesis in general, peak excretion of peptides coincided with the period of fastest protein synthesis and it seemed reasonable to consider whether these two processes were related. Unfortunately the only evidence which came to light during these investigations was indirect.

The composition of bound forms on the 4th to 6th days and of those peptides which were excreted in large quantities during this period were very similar to that of the activated nucleotide peptides which have been isolated from growing yeast cells (45). These peptides were also rich in the dicarboxylic acids in serine, glycine and alanine and contained random mixtures of all essential acids. Amongst activated nucleotides, van der Grinten found at least one which would stimulate and another which inhibited protein synthesis. Nucleotide bound amino acide or peptides have not, as yet, been detected in urine. In the procedures which have been used, they would have been measured as non-activated peptides. Methods of detection include isolation of the nucleotide by absorption onto activated charcoal, and preparation of peptide hydroxamates could be followed by electrophoresis to separate individual peptides, hydrolysis and amino acid analyses. This is an entire project on its own.

Variability of peptide pattern from case to case is not surprising. Urinary peptides could be derived from every conceivable protein in the body of which there are approximately 100,000 at a conservative estimate (221), but only peptides derived from proteins with highest turnover rates would be detected.

The hypothesis that lack of a single or limited number of essential amin o acids would be rate limiting in protein biosynthesis, has not been substantiated. This was also not

unexpected, since it appears that in this area (Western Cape), kwashiorkor is due to a deficiency of total nitrogen intake, rather than any single amino acid. Amino acid composition of the urinary peptides supports this, since every essential acid was represented.

For these reasons the syndrome of kwashiorkor does not appear to be an ideal one on which to test the 'peptides for proteins' theory. What is needed is a more precise model which could be rigidly controlled. A kwashiorkor-like syndrome has been reproduced in pigs which were on a low-protein high-carbohydrate diet (261). Such animals could be repleted with a diet deficient in lysine for example, and the urinary peptides analysed, as has been done in these studies on children. Urinary peptide patterns, such as in these experimental animals may be easier to interpret.

On general principles, a study of the reaction at ribosomal level should prove most rewarding. To this end, it is hoped that the next step in the investigation will be a measurement of incorporation of C^{14} labelled peptide into normal and protein-depleted ribosomes, to compare their uptake and rate limiting steps or substrates.

As previously discussed, those peptides which were excreted maximally before treatment was instituted, might have originated from protein degradation. These peptides were relatively deficient in essential amino acids, as was the whole bound amino acid fraction which was excreted during this phase. If essential amino acids are indeed

rate limiting, synthesis of peptide chains would be halted at that step in which an activated essential amino acid should have been linked to the C-terminal end of the chin. When these incomplete protein sub-units were stripped off the RNA template, as they must be (otherwise all protein synthesis utilising this template would be blocked), they might leak into the tissue fluids and eventually be cleared into the urine.

The peptides which were found in the urine of the kwashiorkor patients have a similar composition to those which have been postulated, and some may be abortive fragments of protein synthesis. Investigations on kwashiorkor patients have not answered this intriguing question and it is proposed, in future, to investigate more definitive systems, which can be rendered protein-depleted.

Physiological Action of Aberrant peptides.

Increased production of peptides in kwashiorkor, whether due to tissue breakdown, enterogenous causes or blocked protein synthesis, may be the explanation of several unexplained features of this disease. The control of many physiological processes is mediated by peptide hormones. du Vigneau and his colleagues (262) have especially studied species differences of the pituitary hormones ACTH, vasopressin and oxytoxin, and have found that parts of these molecules may be removed or replaced without significantly affecting their activity, and other peptides bearing only slight structural relationship to the parent peptide also have metabolic activity.

One of the clinical features of kwashiorkor which has yet to be explained, is the appearance of fluid retention and oedema. It does not seem unreasonable that some or many of the peptides released during protein depletion have fluid retaining properties. The amount of such peptide material is not likely to be detected by chemical means.

Circulatory collapse and even sudden death is a well-known complication of kwashiorkor and is especially likely to occur when protein feeding is first commenced. It is possible that during this period when protein synthesis is suddenly stimulated, an abortive protein fragment may 'come out' of the synthetic pathway, which possesses toxic activity for the myocardium or peripheral vessels. In order to detect these toxic peptides one would have to employ biological, as opposed to chemical methods.

Regulation of Protein turnover during Protein depletion.

In kwashiorkor, the only proteins whose turnovers have been studied are those of serum. The half-life of albumin is doubled (93) whilst the globulins show little change (263). Waterlow's experiment (264) on protein-depleted mice, using S^{35} methionine, showed that protein synthesis in the internal organs (liver and kidneys) took place at the expense of those in muscle and skin. Similar results were shown for rats (265) and, in these protein-depleted animals, radioactivity was lost more rapidly than normal from liver, kidney and plasma. In burned animals, redistribution of N^{15}-glycine followed an identical pattern

and Levenson and Watkin (266) concluded that the integrity of vital organs was maintained at the expense of less active areas, such as skeletal muscle. Other studies have shown that turnover rates of protein in tissues are variously affected by protein depletion. What are the mechanisms which bring this about?

Waterlow et al (267) have suggested that in protein depletion, those proteins with the highest turnover are decreased in amount, since the rate of synthesis, which is reduced by the supply of amino acids, lags behind the rate of breakdown. When a new equilibrium is reached at a reduced concentration of protein, the absolute catabolism of the protein will also be reduced. This would explain why proteins with fastest turnover are most affected and why, for example, the liver loses a large part of its protein in the first few days of protein depletion, but does not go on doing so indefinitely. Equilibrium is reached by reduction of the protein pool i,e. by purely passive mechanisms.

There is in vivo evidence that lowered amino intake per se, diminishes protein synthesis but in perfused organs Miller et al (268) have demonstrated that rate of synthesis is directly related to the concentration of amino acids in the perfusate.

The above 'passive' theory of Waterlow et al (267), explains how protein depletion selectively affects different tissue proteins but it is based upon the assumption that protein synthesis and catabolism are independent of one

another. Several considerations suggest that this a priori argument may not be correct.

By analogy with other biosynthetic pathways, whose end-product concentration or pool is accurately maintained, in spite of fluctuating physiological demands (e.g. blood glucose level), an active feedback loop must surely be required to increase albumin synthesis, when all serum albumin is removed in plasmaphoresis experiments. The degradative pathway from cholesterol to bile acids is inhibited in obstructive jaundice before biosynthesis is affected and accounts for most of the rise in serum cholesterol in this condition.

From teleogical and evolutionary points of view, it would be far more logical for an organism to conserve its protein by lowering the catabolic rate than by any other mechanism, since this involves least waste. In other words catabolism and anabolism are integrated. The findings of Purves and Hansen that the relative catabolic rate of serum albumin in children with protein depletion is much decreased has therefore important theoretical implications.(93)

This whole field is at the moment speculative. Several possible mechanisms whereby control of protein synthesis and breakdown are achieved in the face of limited amino acid supply must be investigated, since these may throw light on control of synthesis during growth and atrophy and loss of this control which is characteristic of neoplasia. One hypothesis which is being at present investigated is that the structure of the protein may be so altered as to be more

resistant to degradation. Potgieter, Hines and Kenoh have analysed serum albumin during the acute phase of kwashiorkor and found no significant differences in amino acid composition from that of normal children, but other changes such as secondary structure, sulphydryl and amide groups will also have to be investigated in this protein.

The fact that ATP is necessary for protein degradation, implies that this is an energy-dependent process, relying on the integrity of many enzymes for its production. The enzymes responsible for ATP formation may also be affected by protein depletion and thus indirectly diminsh protein catabolism.

Review of the findings of other workers has shown that peptides are undoubtedly intermediates in both protein synthesis and metabolism and the present experimental work has substantiated that changes in rate of protein turnover during protein depletion and repletion is accompanied by alterations in endogenous peptide production.

The experimental evidence which has been reported in this thesis has provided only circumstantial evidence concerning the role of peptides as intermediates of protein biosynthesis and as modulators of protein metabolism. In order to establish or negate the hypothesis, more definitive experiments will have to be performed.

Kwashiorkor in children, the model of protein depletion which was selected for study, is too variable and ill-defined from the biochemical point of view, and the original postulate that protein synthesis would be limited by lack of an essential amino acid has not been substantiated, neither has the concept of abortive fragments been resolved. Future work will have to be at a cellular level and studies on the incorporation of peptides into ribosomal protein are contemplated. Furthermore, it is planned to study the role of peptides in kwashiorkor-like syndromes in other animals and in a bacterial system, e.g. E.coli, in which a controlled limitation of protein synthesis can be achieved by omission of one essential amino acid. Armed with new information from such experiments, we can return to a re-investigation of protein depletion in man.

SUMMARY.

The following is a summary of the experimental findings and conclusions which may be derived from them:-

1. During protein depletion, children suffering from kwashiorkor continue to excrete nitrogenous products in their urine. Since nitrogen intake is very low, the bulk of excreted nitrogen must be of endogenous origin.

2. Protein repletion has a striking effect on excretion of nitrogen, and on its partition amongst its various forms of excretion. This effect was almost immediate and was maximal during the first week. This period coincided with return of plasma proteins to normal levels. Low excretion of total nitrogen and urea are a reflection of the slow turnover rate of protein during depletion.

3. In contrast to total nitrogen and urea, α-NH_2 nitrogen excretion was greater during protein depletion and returned to normal when protein was administered. Bound and free forms contributed approximately equally to the total but the ratio of bound to free α-NH_2 nitrogen was significantly higher in the acute phase than after recovery from protein depletion. In most of the cases studied maximum excretion of bound α-NM_2 nitrogen occurred immediately after protein was fed.

4. Individual amino acids which contributed to the total bound $-NH_2$ nitrogen were measured by an ion exchange column chromatographic procedure. The results reflected the findings on total amino acid excretion, and confirmed that bound forms of all amino acids measured, were excreted in increased amounts during the acute phase and were maximal 4 to 6 days after protein was given.

5. Three important exceptions to this general rule were noted for β-alanine, hydroxyproline and β-amino isobutyric acid which were maximally excreted in the protein depleted state. These levels were diminished to normal after 2 - 3 days of therapy. It was significant that these amino acids were derived from catabolism of protein and nucleoprotein, and this pattern of excretion suggested that in the protein depleted state, degradation of collagen nucleoprotein and muscle is accelerated. This inference is in agreement with morbid anatomical studies.

6. During protein depletion, essential amino acids continued to be lost in the urine - mainly as peptides.

7. Elution diagrams of column chromatography of ampholytes of urine showed numerous acid labile components, many of which were shown to be peptides. Acid labile material occurred at istes of elution corresponding to free amino acids, and in the case of

valine and methionine were the major component of these 'peaks' in kwashiorkor urine. Previous results on excretion of free amino acids in this and other diseases will have to be reassessed in this light.

8. A method was developed which separated neutral peptides in urine from free amino acids and was found to be suitable as a preliminary step in investigating peptides in this and possibly other biological fluids.

9. Neutral peptide excretory patterns from normal and kwashiorkor children have been compared. Three types of change were detected:

(a) Peptides maximally excreted in the protein depleted state were probably of catabolic origin, e.g. muscle breakdown.

(b) Peptides excreted immediately after protein was fed might be either of enterogenous origin or abortive fragments of protein synthesis.

(c) Peptides which were excreted only after the depleted state has been corrected, might be derived from normal protein catabolism and turnover which was reduced (to 50%) during protein depletion.

10. The composition of urinary peptides in kwashiorkor was not characteristic. The varied assortment of amino acids which were present in these, supports the contention that (in the cases studied) kwashiorkor is due to total nitrogen deficiency rather than lack of any one, or a group of essential amino acids.

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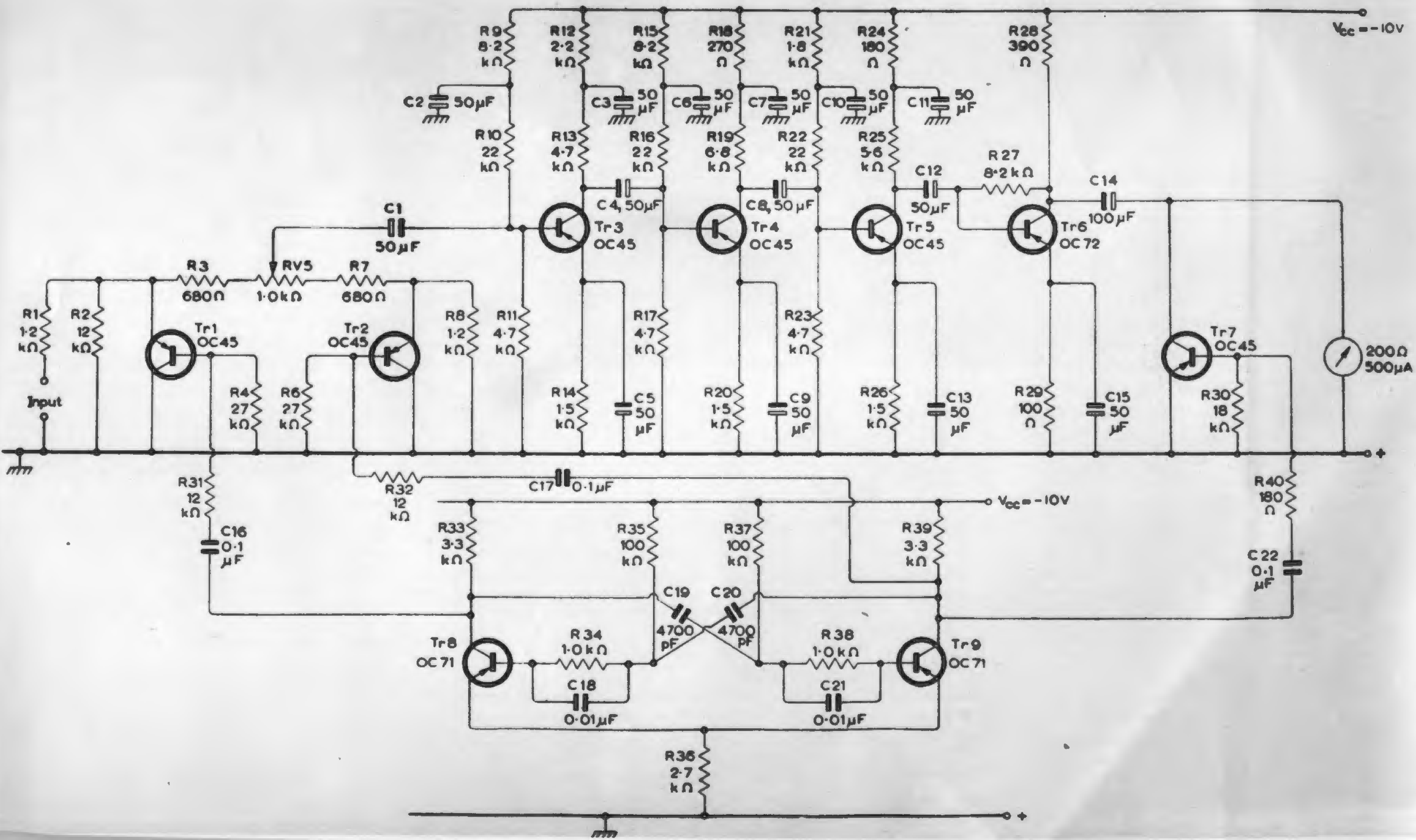
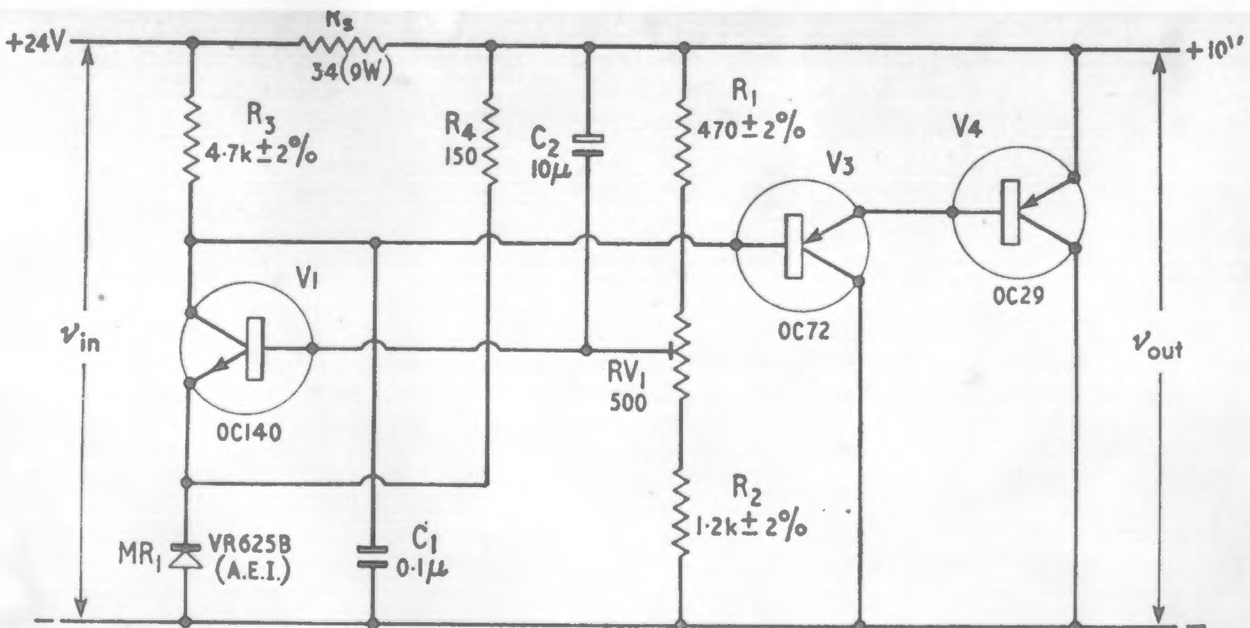
APPENDIX.

Appendix: Transistor D. C. Amplifier.(270)



Appendix: Transistor Regulated Power Supply.



APPENDIX.

Conversion Table. Optical Density to % Transmission.

(Derived Values)

%	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
10	1.000	995	989	984	979	974	969	965	961	957
11	.953	949	945	941	937	933	929	925	921	917
12	.914	910	906	902	898	896	890	887	884	881
13	.878	874	870	866	863	860	857	854	851	848
14	.845	842	839	836	833	830	827	824	822	820
15	.818	815	812	809	807	805	803	801	799	797
16	.795	792	789	786	783	780	777	775	773	771
17	.769	766	764	762	760	758	756	754	752	750
18	.748	746	744	742	740	738	736	734	732	730
19	.728	725	722	720	718	716	714	712	710	708
20	.706	703	700	698	696	694	692	690	688	686
21	.684	681	678	676	674	672	670	668	666	664
22	.662	660	658	656	654	652	650	648	647	646
23	.645	643	642	641	639	637	635	633	631	629
24	.627	625	623	621	619	617	615	613	612	611
25	.610	608	606	604	602	600	598	596	594	592
26	.591	587	585	583	582	581	580	579	578	577
27	.576	574	572	570	568	566	565	564	563	562
28	.561	559	557	555	553	551	550	549	548	547
29	.546	544	542	540	538	537	536	535	534	533
30	.532	530	529	528	527	526	525	524	523	522
31	.521	519	518	517	516	515	514	513	512	511
32	.510	508	506	504	502	501	500	499	498	497
33	.496	494	492	490	488	487	486	485	484	483
34	.482	480	479	478	477	476	475	474	473	472
35	.471	470	469	468	467	467	466	465	465	464
36	.464	463	462	461	460	460	459	458	458	457
37	.457	456	455	454	453	452	451	450	449	448
38	.448	447	446	445	444	443	442	441	440	439
39	.439	438	437	436	435	434	433	432	431	430
40	.430	429	428	427	426	425	425	424	423	422
41	.422	421	420	419	418	417	416	415	414	413
42	.413	412	411	410	409	409	408	407	406	405
43	.405	404	403	402	401	401	400	399	398	397
44	.397	396	395	394	393	392	391	390	389	388
45	.388	387	386	385	384	383	382	381	380	379
46	.378	377	376	375	374	374	373	372	371	370
47	.370	369	368	367	366	365	364	363	362	361
48	.361	360	359	358	357	357	356	355	354	353
49	.353	352	351	350	349	348	347	346	343	344
50	.344	343	342	341	340	340	339	338	337	336

APPENDIX.

Conversion Table. Optical Density to % Transmission (Derived Values).

%	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
51	.336	335	334	333	332	332	331	330	329	328
52	.328	327	326	326	325	324	323	323	322	321
53	.321	320	319	318	317	317	316	315	314	313
54	.313	312	311	310	309	309	308	307	306	305
55	.305	304	303	302	302	301	300	300	299	298
56	.298	297	296	295	294	294	293	292	291	290
57	.290	289	288	287	286	285	284	283	282	281
58	.281	280	279	278	277	276	275	274	273	272
59	.272	271	270	269	268	268	267	266	265	264
60	.264	263	262	261	260	260	259	258	257	256
61	.256	255	254	253	253	252	251	251	250	249
62	.249	248	247	246	245	244	243	242	241	240
63	.240	239	238	237	236	235	234	233	232	231
64	.231	230	229	228	227	227	226	225	224	223
65	.223	222	221	220	219	219	218	217	216	215
66	.215	214	213	212	211	210	209	208	207	206
67	.206	205	204	203	202	201	200	199	198	197
68	.197	196	195	194	193	192	191	190	189	188
69	.188	187	186	185	184	184	183	182	181	180
70	.180	179	178	177	176	175	174	173	172	171
71	.171	170	169	168	167	166	165	164	163	162
72	.162	161	160	159	158	157	156	155	154	153
73	.153	152	151	150	149	149	148	147	146	145
74	.145	144	143	143	142	141	141	140	139	138
75	.138	137	136	135	134	134	133	132	131	130
76	.130	129	128	127	127	126	125	125	124	123
77	.123	122	121	121	120	119	118	118	117	116
78	.116	115	114	113	112	112	111	110	109	108
79	.108	107	106	106	105	104	103	103	102	101
80	.101	100	099	098	098	097	097	096	096	095
81	.095	094	093	092	092	091	090	089	089	088
82	.088	087	086	086	085	084	084	083	083	082
83	.082	081	080	080	079	078	078	077	077	076
84	.076	075	074	074	073	072	072	071	071	070
85	.070	069	068	068	067	066	066	065	065	064
86	.064	063	063	062	062	061	061	060	060	059
87	.059	058	057	056	056	055	055	054	054	053
88	.053	052	052	051	051	050	050	049	049	048
89	.048	047	047	046	046	045	045	044	044	043
90	.043	042	042	041	041	040	040	039	039	038
91	.038	037	037	036	036	035	035	034	034	033
92	.033	033	032	032	031	031	030	030	029	029
93	.029	028	028	027	027	027	026	026	025	025
94	.025	024	024	023	023	023	022	022	021	021
95	.021	021	020	020	020	019	019	019	018	018
96	.018	017	017	016	016	016	015	015	014	014
97	.014	013	013	013	012	011	011	010	010	009
98	.009	008	007	007	006	006	005	005	005	004
99	.004	004	003	003	002	002	001	001	001	000
100	.000									

APPENDIX.

Calibration Table for Flowmeter. Seconds/ml.

ml.	.0	.1	.2	.3	.4	.5	.6	.7	.8	.9
25	89.8	89.4	89.0	88.7	88.4	88.1	87.6	87.3	87.0	86.7
26	86.3	86.0	85.7	75.4	85.1	84.7	84.3	84.0	83.7	83.4
27	83.1	82.8	82.4	82.2	81.9	81.6	81.3	81.0	80.7	80.4
28	80.1	78.8	79.5	79.3	79.0	78.7	78.5	78.2	77.9	77.6
29	77.3	77.1	76.8	76.6	76.3	76.1	75.8	75.6	75.3	75.1
30	74.8	74.6	74.3	74.1	73.8	73.6	73.3	73.1	72.8	72.6
31	72.4	72.1	71.9	71.7	71.5	71.3	71.0	70.3	70.6	70.4
32	70.2	70.0	69.7	69.5	69.3	69.1	68.8	68.7	68.4	68.2
33	68.0	67.8	67.6	67.4	67.2	67.0	66.8	66.6	66.4	66.2
34	66.0	65.8	65.6	65.5	65.3	65.1	64.9	64.8	64.6	64.4
35	64.2	64.1	63.9	63.7	63.5	63.3	63.1	62.9	62.7	62.5
36	62.3	62.1	61.9	61.8	61.6	61.4	61.3	61.2	61.0	60.9
37	60.7	60.6	60.4	60.3	60.2	60.0	59.9	59.7	59.5	59.3
38	59.1	58.9	58.8	58.7	58.6	58.4	58.3	58.1	57.9	57.8
39	57.6	57.5	57.3	57.2	57.1	56.9	56.7	56.6	56.5	56.3
40	56.1	55.9	55.8	55.7	55.6	55.5	55.3	55.2	55.0	54.9
41	54.8	54.6	54.5	54.4	54.3	54.1	53.9	53.8	53.6	53.5
42	53.4	53.2	53.1	53.0	52.9	52.8	52.6	52.5	52.4	52.3
43	52.2	52.0	51.9	51.8	51.7	51.6	51.4	51.3	51.2	51.1
44	51.0	50.8	50.7	50.6	50.5	50.4	50.2	50.1	50.0	49.9
45	49.8	49.7	49.6	49.5	49.4	49.3	49.2	49.1	49.0	48.9
46	48.8	48.7	48.6	48.5	48.4	48.3	48.2	48.1	48.0	47.9
47	47.8	47.7	47.6	47.5	47.4	47.3	47.2	47.1	47.0	46.9
48	46.8	46.7	46.6	46.5	46.4	46.3	46.2	46.1	46.0	45.9
49	45.8	45.7	45.6	45.5	45.4	45.3	45.2	45.1	45.0	44.9
50	44.8	44.7	44.6	44.5	44.4	44.3	44.2	44.1	44.0	43.9

SPECIMEN: VOL. APPLIED:

ELUTION PATTERN NO.: COLUMN: BUFFER SYSTEM:

PUMP NO. I: PRESSURE: lbs. FLOW RATE: mls/hour.

PUMP NO.II: PRESSURE: lbs. FLOW RATE: mls/hour.(Combined)

NEUTRAL AND ACIDIC COMPOUNDS.

COMPOUND	Baseline		Peak Ht.		Nett Ht.	Half Ht.		Width mm. X 10	H X W	C	μ moles = H X W/C.
	%	O.D.	%	O.D.	O.D.	O.D.	%				
PHOSPHOSERINE											
GLYCEROPHOSPHO-ETHANOLAMINE											
PHOSPHOETHANOLAMINE											
TAURINE											
METHIONINE SULPHOXIDES											
HYDROXYPROLINE											
ASPARTIC ACID											
THREONINE											
SERINE											
ASPARAGINE & GLUTAMINE											
SARCOSINE											
PROLINE											
GLUTAMIC ACID											
CITRULLINE											
GLYCINE											
ALANINE											
α-AMINO-ADIPIC & α-AMINO-n BUTYRIC											
VALINE											
CYSTINE											
CYSTATHIONE											
METHIONINE											
ISOLEUCINE											
LEUCINE											
GLUCOSAMINE											
TYROSINE											
PHENYLALANINE											
β-ALANINE											
β-AMINOISO-BUTYRIC ACID											

J. clin. Path. (1963), 16, 385

Separation of myoglobin and haemoglobin on a column of dextran gel

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Myoglobin and haemoglobin may frequently be found together in the urine of patients who have suffered from crush injuries. The separation of these two haemoproteins by differential solubility in salt solutions and their spectroscopic characterization are technically difficult. With this in mind, it appeared worth while to test whether the considerable difference in molecular size of the proteins (M.W. 18,000 and 67,000) could form the basis of a more satisfactory procedure. Gel filtration exploits the differing rates of diffusion of molecules within the interstices of a cross-linked gel to separate the molecules roughly according to their size. With the appropriate low cross-linked dextran gel, efficient separation was achieved, as the following experiment shows.

Human psoas muscle obtained after death was finely minced, and approximately 0·2 ml. of distilled water was added. The muscle was then crushed, frozen by adding solid carbon dioxide and then allowed to thaw. The juice was pressed out through cheese cloth and immediately saturated with carbon monoxide in the dark. The process was twice repeated to give, in all, a concentrated crude extract of muscle containing carbonyl myoglobin, carbonyl haemoglobin, and other soluble muscle constituents including proteins. Then 2 ml. of the extract was applied to a column (80 × 2 cm.) of low cross-linked dextran gel (Sephadex G-75, 100 to 200 mesh) which was developed with sodium chloride solution (0·05 M) saturated with carbon monoxide. Two red-coloured zones migrated down the column, well separated from each other at outflow, and, on passing from the column, were collected in 5 ml. fractions. Each of these was diluted with water, treated with sodium hydrosulphite ($Na_2S_2O_4$) and resaturated with CO, and the absorption maxima measured in a Beckman D.U. spectrophotometer. The wavelengths of maximum absorption for zone 1 were: α band 569 mμ, Soret region 420 mμ (human carbonyl haemoglobin gives 570, Lemberg and Legge, 1949), and 418 mμ (Hicks and Holden, 1929); for zone 2: α band 578 mμ, Soret region 424 mμ (carbonyl myoglobin (human) α band 577 mμ (Theorell and de Duve, 1947), Soret region (horse) 422 mμ (Colpa-Boonstra and Minnaert, 1959).

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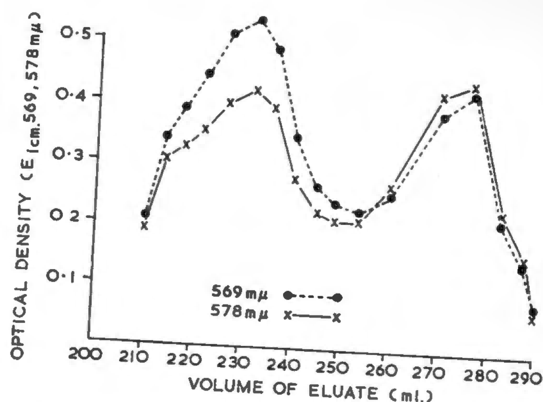

FIG. 1. *Separation of carbonyl myoglobin and carbonyl haemoglobin contained in 2 ml. crude human muscle extract. Zone 1 : HbCo. Zone 2 : MbCO.*

When the optical density readings at the absorption maxima, 569 and 578 mμ for HbCO and MbCO respectively, are plotted against the corresponding volumes of eluate (Fig. 1), the high degree of separation becomes apparent. Doubtless the residual slight overlapping could be eliminated by use of a longer column packed with a gel of finer mesh. No other coloured zones, as of cytochromes, were detected.

This simple procedure could be used as a rapid diagnostic manoeuvre in suspected myoglobinuria. If haemoglobin is added as a marker it will migrate ahead of myoglobin which can, thus, be positively identified.

SUMMARY

The carbonyl derivatives of myoglobin and haemoglobin contained in human psoas muscle were separated on a column of low cross-linked dextran gel. This simple procedure could facilitate the recognition of myoglobinuria.

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EXCRETION OF AMINO ACIDS IN THE BOUND FORM IN THE URINE OF PATIENTS SUFFERING FROM KWASHIORKOR

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Some years ago Westall[1] directed attention to the occurrence of small peptides in normal urine which he believed might be significant in intermediary protein metabolism. Prompted by Westall's observations and our own interest in disturbances in protein metabolism,[2,3] we thought that examination of the urinary peptides of protein-depleted kwashiorkor patients might provide important information about the condition. In particular, we wished to answer the following question:

Is there a pattern of urinary peptides characteristic of the protein-depleted state, changing to a recognizably normal one on clinical recovery?

Included in our enquiry is a search for evidence of partial blocks in synthesis or breakdown of body proteins caused by lack of dietary amino acids, especially essential ones, or of enzymes or co-enzymes.

A recent publication by Edozien and Phillips[4] described a comparative study of the partition of nitrogen in spot urinary specimens of subjects on normal, low-protein and kwashiorkor diets. Their paper did not attempt to explore the issues with which we are concerned, but the relevant findings will be discussed later.

MATERIALS AND METHODS

The patients studied were diagnosed clinically as having severe kwashiorkor and requiring hospitalization. Only males have been studied, owing to the difficulties of 24-hour urine collections in females. Six non-European males have been investigated up to the present time.

The clinical material consisted of:

Case 1, H.L. African male aged 1 year 3 months. Weight 16 lb. 8 oz. Admitted with florid kwashiorkor. Hb. 14 G./100 ml., albumin 2·09 G./100 ml., globulin 2·62 G./100 ml.

Case 2, A.O. Coloured male aged 1 year 9 months. Weight 14 lb. 8 oz. Moderately severe kwashiorkor. Hb. 12 G./100 ml.

Case 3, J.C. Coloured male aged 2 years 4 months. Weight 20 lb. 4 oz. Mild kwashiorkor.

Case 4, F.B. Coloured male aged 4 years. Weight 23 lb. 14 oz. Severe kwashiorkor. Hb. 10 G./100 ml., albumin 1·74 G./100 ml., globulin 1·88 G./100 ml.

Case 5, T.I. Coloured male aged 1 year 9 months. Weight 20 lb. Moderately severe kwashiorkor. Hb. 9·5 G./100 ml., albumin 1·38 G./100 ml., globulin 2·56 G./100 ml.

Case 6, P.J. Aged 1 year. Weight 13 lb. 8 oz. Admitted with kwashiorkor complicated by severe dehydration from gastroenteritis. Hb. 7 G./100 ml., serum sodium 165 mEq./l., serum chloride 146 mEq./l., blood urea 49 mg./100 ml., serum calcium 6·6 mg./100 ml. Only the initial 24-hour urinary collection was made since the patient became extremely ill and died 1 week after admission.

Cases 1 – 5 were given clear feeds on the day of admission (day 1). On days 2 and 3 skim milk was offered which was changed to half-cream and full-cream milk on days 4 and 6 respectively.

During the collection of the 24-hour urine specimens, the patients were on metabolic beds. No preservatives were added to the specimens, which were kept at –10°C. as soon as collection was completed.

Total nitrogen was determined by the micro-Kjeldahl procedure. Urinary ammonia was measured by alkaline distillation, and urinary urea by the carbamido-diacetyl reaction using the technicon auto-analyser.[5] All amino-acid assays were made on urine which had been deproteinized with tungstic acid by addition of 1·0 ml. of 0·67N H_2SO_4 and 1·0 ml. of 10% sodium tungstate to 10·0 ml. of urine. After standing overnight at 4°C., the mixture was filtered and the resulting filtrate was stored at –10°C. Urinary free and total α-amino nitrogen was measured by the colorimetric ninhydrin method.[6]

Acid hydrolysis was performed in sealed tubes containing equal volumes of urine and concentrated HCl in a thermostatic oven at 120°C. for 18 hours. The hydrolysate was filtered to remove humin.

Analysis of individual amino acids was performed by ion-exchange chromatography on Amberlite CG 120 and the effluent analysed for ninhydrin-positive material by the automatic technique of Spackman *et al.*[7] Single-channel recording of the effluent was used, with a 2·9 mm. flow cell at a wavelength of 570 mμ. At this wavelength, hydroxyproline and proline gave colour constants of 1·93 and 4·20 respectively, compared with 37·1 for leucine. 98% confidence limits for these 2 substances in the 1 μ mole range is estimated at $\pm$ 20%. The 30° - 50° C. system was used. For the 150 cm. neutral and acidic column, temperature and buffer change was made at 11·5 hours (345 ml. of effluent) and the buffer break-through occurred after cystine. The temperature change for the 50 cm. column was made at 14 hours (420 ml. of effluent). A 2·00 ml. aliquot of deproteinized urine (equivalent to 1·67 ml. of original urine) was first dried over KOH in a vacuum desiccator, and then dissolved in 2 ml. of citrate buffer (0·2 M: pH 2·2) before being applied to the column. For the basic amino-acid analysis on the 50 cm. column, the aliquot was brought to pH 10 - 11 with 2N KOH to remove ammonia before desiccation.

The bound amino acids were analysed in a 2·00 ml. aliquot of hydrolysate (equivalent to 0·835 ml. of original urine). Here also ammonia was removed at pH 10 - 11 before the basic amino-acid analysis. The least quantity of proline and of hydroxyproline which could be detected in the applied sample was 0·100 μ moles, whereas the corresponding value for leucine and other amino acids of high colour yield was 0·01 μ moles.

Results were calculated as μ moles of amino acid excreted per 24 hours.

TABLE I. 24-HR. EXCRETION OF URINARY NITROGENOUS COMPOUNDS

Case No.	*Day*	*24-hr. volume (ml.)*	*Total α-NH_2 N (mg./ 24 hrs.)*	*Free α-NH_2 N (mg./ 24 hrs.)*	*Bound α-NH_3 N (mg./ 24 hrs.)*	*Ratio of bound/free α-NH_2 N*	*NH_3 N (mg./ 24 hrs.)*	*Urea N (mg./ 24 hrs.)*	*Total N (mg./ 24 hrs.)*
1 (H.L.)	1	23·5	13·3	5·95	7·4	1·24	96	57	241
	3	239	71·7	47·6	24·1	0·52	378	336	1,410
	5	887	265	113	152	1·34	434	1,812	4,200
	15	557	108	55·7	42·3	0·76	986	2,060	3,403
	17	370	47·3	24·0	23·3	0·97	1,095	1,280	2,586
	19	438	90·7	23·1	67·6	2·92	2,203	628	3,066
2 (A.O.)	2	190	53·7	14·4	29·3	2·04	181	222	551
	4	846	158	64·0	94·0	1·47	255	614	1,286
	14	460	39·5	13·6	25·9	1·90	160	496	1,359
	15	281	45·5	29·0	16·5	0·57	123	894	1,206
	16	236	21·5	10·0	11·5	1·15	1,412	520	2,556
3 (J.C.)	1	292	71·5	35·0	36·5	1·04	169	678	972
	3	153	37·9	20·6	17·3	0·84	174	1,028	1,637
	5	35	10·0	5·0	5·0	1·00	45	284	364
	13	470	40·8	21·2	19·6	0·92	158	1,120	1,936
	15	448	64·2	32·6	20·6	0·63	390	3,440	4,880
	17	408	39·2	17·7	21·5	1·22	178	1,520	2,340
4 (F.B.)	1	785	91·0	18·4	72·6	4·00	390	1,580	2,410
	3	340	64·0	26·7	37·3	1·40	290	604	1,357
	5	998	117·8	73·1	44·7	0·61	303	1,893	2,409
5 (T.I.)	2	217	84·8	34·8	50·0	1·44	—	1,420	—
	4	261	49·1	22·4	26·7	1·19	—	1,520	—
	6	245	77·8	16·7	61·1	3·66	—	1,160	—
	22	467	58·8	30·0	28·8	0·96	—	2,690	—
	24	463	37·1	18·0	19·1	1·06	—	1,480	—
	26	424	45·8	19·5	26·3	1·40	—	1,630	—
6 (P.J.)	1	105	27·1	20·0	7·1	0·35	239	361	925

RESULTS

Twenty-four-hour excretions of free and bound α-amino nitrogen were estimated in all 6 cases. In 4 of these, collections were available during the acute phase and on recovery just before discharge from hospital. In 1 case urines were not available during the phase of recovery, while Case 6 died 7 days after admission.

The observations on α-amino nitrogen excretion, together with 24-hour urinary volumes and total nitrogen content are shown in Table I. Differences between total and free α-amino nitrogen are reported as bound α-amino nitrogen. The ratio of bound to free forms has been calculated.

Case No. 1 was further analysed for urinary excretion of individual free and bound amino acids by ion-exchange column chromatography during the acute phase of the illness on the 1st, 3rd and 5th days after admission. In addition the excretion on the 15th day was assayed as an index of the recovery phase. The results are shown in Table II.

Two groups of acid-labile materials which reacted with ninhydrin were noted. The first occurs in the area of the effluent curve immediately preceding taurine and urea at an effluent volume of 60 - 100 ml. at the site of expected emergence of phosphoserine, phosphoglyceraldehyde and phosphoglyceroethanolamine. Maximum concentrations of these compounds were noted on day 1, the urine of which was the most concentrated. At least 3 acid-labile components have been noted (Figs. 1 A and B). Smaller amounts of these components were noted on days 3, 5 and 15 and the concentrations are roughly inversely proportional to the 24-hour urinary volume.

A second complex of acid-labile compounds was noted in the site of elution usually occupied by aspartic acid, threonine and serine. At least 2 broad peaks were identified which differed markedly from the sharp peaks and 'clean' symmetrical pattern of the free amino acids after acid hydrolysis (Figs. 1 C and D). The maximum concentration of this complex was found on the 3rd day.

DISCUSSION

When we examine our data on the urinary excretion of ammonia and urea in this small series of kwashiorkor patients, it is clear that there is a small rate of breakdown of protein at the time of admission of the kwashiorkor subject to hospital. This is in accordance with recent determinations which show a very slow turnover rate of such patients' serum albumin[8] and a quickening of the catabolic rate during protein repletion.

The average daily excretion of free amino acids covering the first week of observation was higher than corresponding normals[9] and higher than those of the subsequent convalescent stages.

Similar relationships held also for bound forms; the maximum daily excretion of both free and bound amino

TABLE II. URINARY EXCRETION OF AMINO ACIDS IN μ MOLES PER 24 HOURS

	Day							
	1 (23·5)*		*3* (239)		*5* (887)		*15* (557)	
	Free	*Bound*	*Free*	*Bound*	*Free*	*Bound*	*Free*	*Bound*
Amino nitrogen (mg./24 hrs.)	6	7·4	47·6	24·1	113	152	55·7	42·3
Taurine	58	[<0·3]‡‡	167	24	163	38	1	29
Hydroxyproline	[<1·4]	79	[14–42]	141	[<53]	635	[<33]	225
Aspartic acid	6	35	32	170	[<5·3]	462	8	326
Threonine	—‡	19	—‡	119††	—‡	391††	13	123
Serine	—‡	30	—‡	253††	—‡	682††	3	187
Asparagine + glutamine**	4	—	481	—	869	—	45	—
Sarcosine	1	[<0·3]	[<1·4]	[3–8·5]	[<5·3]	71	31	20
Proline	[<1·4]	51	[<14]	292	[<53]	238	[<33]	82
Glutamic acid	2	104	35	504	89	2,321	43	776
Citrulline	[0·1–0·4]	0·5	[<1·4]	[3–8·5]	[5·3–16]	—	[3·3–10]	[<6·5]
Glycine } ***	54 (Glycine + Alanine)	110 (Glycine + Alanine)	656	223	1,438	772	236	1,152
Alanine }			126	54	686	443	57	333
α—Amino-adipic acid / α—Amino-n-butyric acid } ***	0·6	[<0·3]	3·2	16	80	[<10·6]	4	11
Valine	[0·1–0·4]	7·5	[1·4–4·2]	39	[5·3–16]	100	[<3·3]	60
Cystine	[<0·1]	23	[1·4–4·2]	51	46	92	[<3·3]	152
Cystathioneine	0·6	1·0	21	[<3]	[5·3–16]	14	[<3·3]	[6·5–20]
Methionine†	1·7	3·3	29	[3–8·5]	[5·3–16]	11	7	35
Isoleucine	1·9	3·0	24	[3–8·5]	[5·3–16]	85	[3·3–10]	44
Leucine	0·5	12·5	11	58	[5·3–16]	226	[3·3–10]	111
Tyrosine	[<0·1]	5·6	3·7	47	[5·3–16]	146	[3·3–10]	131
Phenylalanine	[<0·1]	6·0	4·2	43	[5·3–16]	139	[3·3–10]	145
β-Alanine	[<0·1]	3·4	[1·4–4·2]	[<3]	[<5·3]	38	[3·3–10]	100
β-Aminoisobutyric acid	11	12	12	28	[5·3–16]	87	[3·3–10]	[<6·5]
Hydroxy lysine	[0·1–0·4]	3	5	10	—	—	8	[<6·5]
δ-Amino-n-butyric acid	2	[<0·3]	17	[<3]	83	80	21	[<6·5]
Lysine	—	—	2·3	[<3]	—	—	[<3·3]	26
3-Methyl histidine	2	[<0·3]	—	—	—	—	304	45
Histidine	15	[<0·3]	602	169	1,030	[<10·6]	217	10

* Figures in parenthesis thus ( ) represent the 24-hour urinary volume for that day in ml.
‡‡ Values enclosed within brackets thus [ ] could not be determined more precisely, for technical reasons.
** Asparagine and glutamine are eluted together as a single peak. The results are expressed as glutamine.
*** In high concentrations, glycine and alanine are not completely resolved. α—Amino-adipic and α—amino-n-butyric acid are eluted as a single peak.
† Includes methionine sulphoxides.
‡ Threonine and serine are obscured in these unhydrolysed specimens by an unidentified component (Fig. 1).
†† These are total excretions, i.e. free plus bound forms.

acids was attained around the 5th day of treatment, which coincides in fact with the greatest rate of change of serum-protein pattern.[2] Although in several cases great fluctuations of urinary volume appear to dictate the excretion of free and bound amino acids, the lack of correlation between the ratios of bound to free forms and urinary volume (Fig. 2) implies an even greater influence of other factors. Since peptides are rapidly cleared from the circulation, this

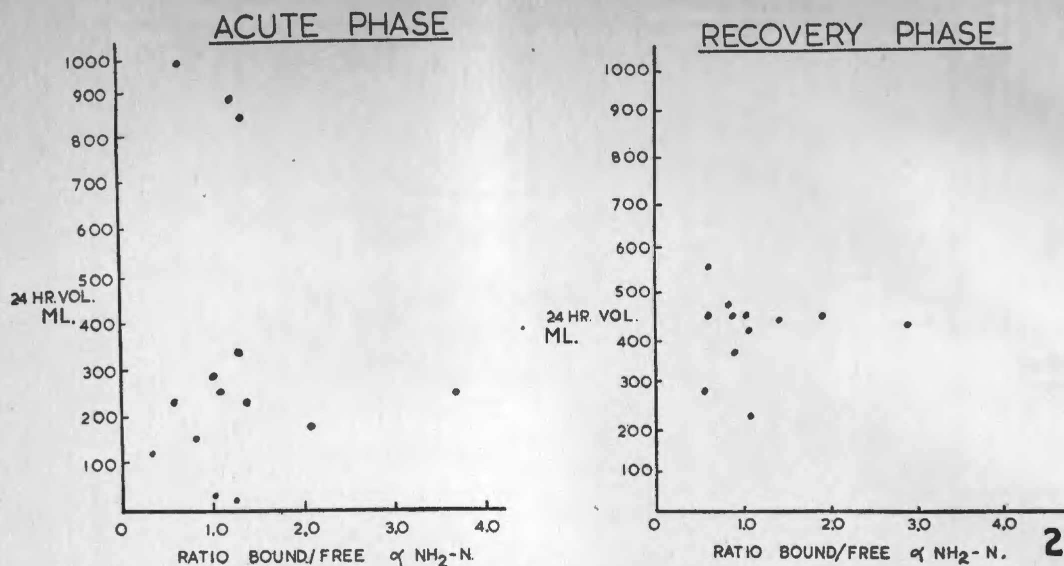

Fig. 2. Lack of correlation between the ratio of bound to free α-NH_2 nitrogen and the 24-hour urinary volume is shown in the acute and recovery phases. Both parameters show a wider scatter during the acute phase.

Fig. 1. Effluent pattern from 150 cm. column (30° - 50°C. system). The figures along the ordinates indicate percentage transmission through the flow cells, while those on the abscissae represent millilitres of effluent. Labelling of the peaks is derived from the site of the peaks of corresponding pure standard compounds on the effluent curve. Owing to the increased amounts of ninhydrin-positive material released after acid hydrolysis, exactly half the unhydrolysed sample volume has been used for analysis of the hydrolysed specimen to avoid overloading the system. The patterns are those from Case 1 (H.L.).

A. Day 1. 24-hour volume 23·5 ml. Unhydrolysed specimen. Sample 1·67 ml.
B. Day 1. Hydrolysed specimen. Sample 0·835 ml.
C. Day 3. 24-hour volume 239 ml. Unhydrolysed specimen. Sample 1·67 ml.
D. Day 3. Hydrolysed specimen. Sample 0·835 ml.

Acid-labile material is noted in 2 distinct areas of the effluent pattern. The first, before taurine and the second between aspartic acid and asparagine/glutamine. The peak marked '?' is broad and unlike the sharp peak of threonine seen after hydrolysis. Peptides from dipeptides to octapeptides can be expected to yield broad peaks. The tripeptide, reduced glutathione, in tissue extracts, is found in this position, but has never been demonstrated in urine.

ratio may furnish an indirect index of impaired protein metabolism at the peptide level.

As regards the composition of the bound forms, certain observations appear worthy of special mention. Essential amino acids are being lost in the bound form from the body even in the grossly protein-depleted state. In normal adults essential amino acids are excreted mainly in the bound form.[10] The only comparable values available for children[9] show a similar pattern. The ratios of bound to free essential amino acids which we have found are higher than their quoted controls. The one exception found is histidine, which is excreted mainly in the free form. Proline and hydroxyproline are excreted in grossly increased amounts in the first week. The fact that the maximum excretion of proline occurred on the 3rd day while hydroxyproline is excreted maximally on the 5th day, is presumptive evidence of at least 2 compounds containing these amino acids in bound form in the urine. It is significant that 2 peptides containing both hydroxyproline and proline have been isolated from the urine of patients suffering from rheumatoid arthritis.[11] Our findings provide incontrovertible evidence of continued collagen degradation in the protein-depleted state.

With the enrichment of the diet with both dispensible and non-dispensible amino acids, it is important to note that the excreted peptides become relatively richer in essential amino acids such as valine, phenylalanine, and leucine as compared with glycine and alanine. This finding is also consistent with the appearance of such bound forms as abortive peptide fragments arising as intermediates in protein turnover. The content of the basic amino acids, lysine, arginine, and histidine in bound form was uniformly low although anserine was readily detectable.

It is proposed to make a more detailed examination of the peptides which contribute to the bound-amino nitrogen of the urine, as a contribution to the knowledge of protein metabolism in kwashiorkor.

SUMMARY

The urinary excretion of free and bound forms of amino acids has been investigated in 6 patients with kwashiorkor.

During the acute phase of the disease there is a continued loss of free and bound amino acids and there is an absolute increase in the losses of bound forms in the acute, as compared with the recovery phase. The ratio of free to bound forms remains relatively constant.

In the single patient investigated in more detail, essential amino acids were found in the urine in the acute stage and were present mainly as bound forms.

There is evidence of increased breakdown of collagen in the protein-depleted state.

These findings are discussed with regard to a possible block at the peptide level of protein metabolism in protein-depleted states caused by lack of essential amino acids.

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