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THE AMINO ACID SEQUENCE OF TRYPTIC PEPTIDES  
OF SHEEP HEART PHOSPHOFRUCTCKINASE.

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A thesis presented in partial fulfillment  
of the requirements for the  
degree of Doctor of Philosophy  
in Biochemistry at Massey University.

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ABBREVIATIONS

PFK	Phosphofructokinase
PTC	Phenylisothiocyanate
PTH	3-phenyl-2-thiohydantoin
PTC	Phenylthiocarbonyl
DMSO	Dimethyl sulphoxide
TFA	Trifluoroacetic acid
Fr	Fraction
MW	Molecular weight
DNS	Dansyl
Ep (6.5)	Electrophoretic mobility at pH 6.5
Ep (2.1)	Electrophoretic mobility at pH 2.1

ABSTRACT

Phosphofructokinase was purified from sheep heart. The sedimentation pattern of the purified enzyme was investigated over a protein concentration range 0.7 to 14.5 mg/ml. Two distinct 7 and 30 S boundaries were observed at all concentrations. A minor amount of 19 S material was also present. The 30 S boundary was asymmetric and its concentration dependence was characteristic of a polymerising system in rapid reversible equilibrium.

The dissolved crystalline enzyme usually sedimented as a single trailing 30 S boundary; the molecular weight of this component was estimated at  $1.5 \times 10^6$ . This value was consistent with x-ray data, which indicated unit cell dimensions of  $600 \times 250 \times 220 \text{ \AA}$ , implying a protein weight of greater than  $10^6$  daltons per asymmetric unit. In one experiment the 30 S component appeared to be undergoing a trimerisation to a 53 S form.

Sodium dodecylsulphate gel electrophoresis indicated a protomer molecular weight of 80,000 to 85,000, which was consistent with a corrected sedimentation coefficient of 3.8 S and a molecular weight of 90,000 for maleyl-phosphofructokinase, and with a corrected sedimentation coefficient of 3.9 S for the urea-dissociated enzyme.

When maleylation was carried out on carboxymethyl-phosphofructokinase in 7.5 M urea, the enzyme was further dissociated to a 40,000 molecular weight subunit. Peptide mapping of tryptic peptides; in which arginine-, histidine-, tryptophan- and tyrosine-containing peptides were located; was consistent with an 85,000 form composed of two identical subunits.

The enzyme was digested with trypsin. Forty-three different peptides were isolated using a combination of: gel filtration, ion exchange chromatography (on Dowex 50 and DE 32 cellulose), paper electrophoresis, and paper chromatography. The complete amino acid sequence was established for 38 of these peptides. The amino acid compositions (and

partial sequences) were established for the other five tryptic peptides. A summary of the amino acid sequence data obtained for the tryptic peptides is shown in Table V.

Seven carboxymethylcysteine-containing peptides were isolated in this investigation, while eight have been isolated from rabbit muscle (Coffee et al. 1973). Six of these peptides had very similar compositions between the two species. The rabbit enzyme contained two carboxymethylcysteine-containing peptides which were not found in sheep heart and the sheep enzyme contained a 20 residue peptide not found in rabbit muscle. This probably reflects genetic variation between the two species.

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## 1. INTRODUCTION

The foundation of protein chemistry was laid in 1902, when Fischer and Hofmeister independently proposed that the linkage between the amino acids of a protein was the peptide bond.

Although the phenomenon of biological catalysis was recognised as early as 1835, it was not until 1926 that these catalysts ( enzymes ) were shown to be, in fact, proteins. The work of Sumner, in crystallising urease, provided the impetus required to establish that enzymes were proteins.

A big advance in the field of protein structure came with Svedberg's development of the ultracentrifuge (Svedberg and Nichols, 1923; Svedberg and Peterson, 1940). From his studies with this instrument, Svedberg postulated that all proteins were composed of subunits. Although subsequent investigations have shown that this is not absolutely true, it is fair to say that the majority of proteins whose molecular weight exceeds 30,000, are composed of subunits. The term subunit is used to mean a dissociated form of the native enzyme. It is important to note that this dissociation does not result from the cleavage of a covalent bond, but results from the disruption of non specific interactions. For example, insulin, with a molecular weight of 11,466, is made up of two identical 5733 MW subunits; in turn each of these subunits contains two polypeptide chains linked through disulphide bridges.

The study of subunits has become increasingly important with the realisation that cellular control mechanisms, and regulation of enzymic activity, might operate at the molecular level through interactions between subunits of an oligomeric protein (Koshland and Neet, 1968). Frieden (1971) has listed no less than thirty-one enzymes in which reversible association — dissociation processes may be involved in controlling the rate of reaction in vivo.

Early methods of amino acid analysis of proteins were very laborious and required vast amounts of material. The ester distillation method of Fischer (1906) required 1 Kg of protein and took several months to complete. The introduction of automatic amino acid analysis on ion exchange columns (Spackman et al, 1958) was a milestone in protein chemistry. Since that time the equipment has been improved so that it is now

possible to analyse 5 n moles of each component of a hydrolysate in 3-4 hours, with completely automatic sample loading and column re-generation (Slump and Verbeek, 1968). Further improvements include automatic integration of amino acid peaks and the use of fluorescent products instead of ninhydrin to increase the sensitivity of the detection system.

Prior to the commencement of amino acid sequence determinations, it is essential to know three things:

1. The amino acid composition of the protein;
2. The smallest subunit of the protein. If there is a number of different subunits, these must be isolated and sequenced individually;
3. The number of polypeptide chains contained in a particular subunit. If a subunit contains two polypeptide chains linked through a disulphide bridge, it is necessary to either oxidise with performic acid, or <sup>reduce and</sup> alkylate the subunit in order to separate the polypeptide chains.

The first protein to have its primary structure determined was bovine insulin (Sanger, 1945; Sanger and Tuppy, 1951; Sanger and Thompson, 1953). The majority of amino acid sequence investigations since then have been conducted along the lines pioneered by Sanger in his classical study; that is, partial hydrolysis of the peptide chain by chemical and enzymatic methods and characterisation of the resulting peptides. Sanger had no method available for sequentially degrading the polypeptide chain; instead, his major technique was to label the N-terminus using 2,4-dinitrofluorobenzene and subject the peptide to partial acid hydrolysis. Schematically, this can be represented as;



During the 1950's, Edman developed a method for the sequential degradation of polypeptide chains (below). This represented a major advance in the techniques available for determining primary structure.

A wide range of enzymatic and chemical techniques are available for fragmentation of protein chains. The most widely used enzymes are trypsin (E.C.3.4.4.4.), which cleaves bonds adjacent to the C-terminus of lysine and arginine, and chymotrypsin (E.C.3.4.4.5.), which hydrolyses bonds adjacent to aromatic and leucine residues. Other less specific enzymes, including thermolysin, pronase, subtilisin and pepsin can also be used. A considerable interest has arisen in the chemical

modification of proteins to restrict enzymatic cleavage to a few well defined bonds. Examples of such modification include, the blocking of lysine by trifluoroacetylation (Goldberger and Anfinsen, 1962), maleylation (Butler et al, 1967) and succinylation (Li and Bertsch, 1960) and the modification of arginine using 1,2-cyclohexanedione (Toi et al, 1967) and phenylglyoxal (Takahashi, 1968).

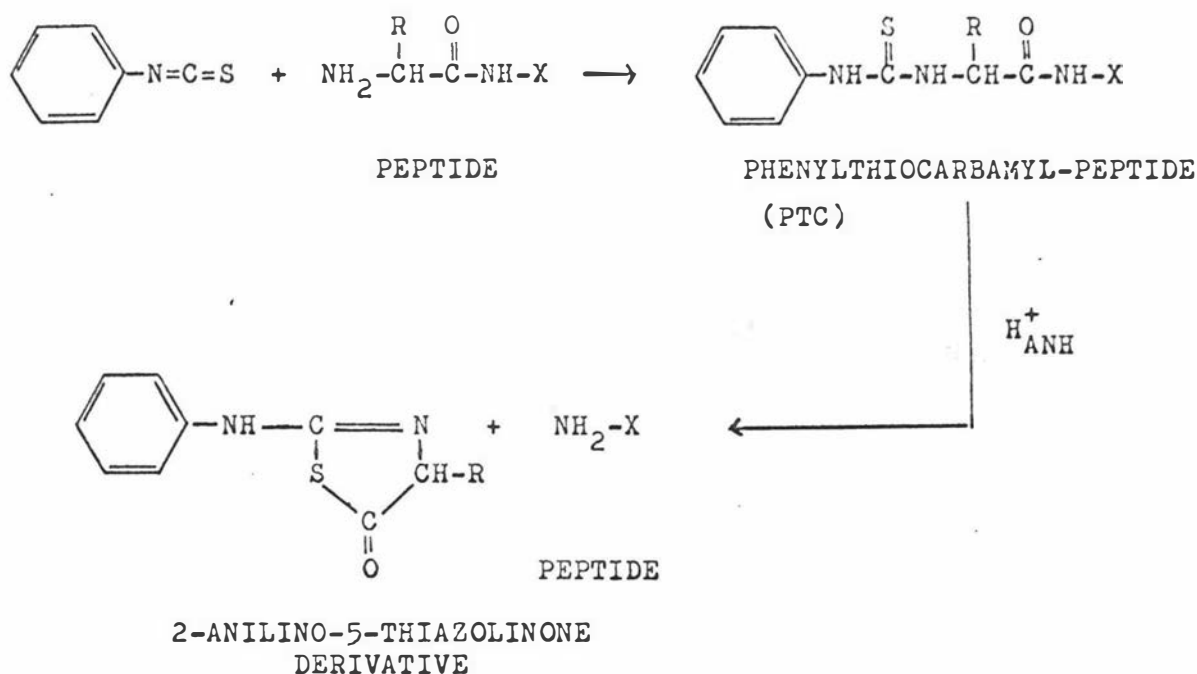
The only really satisfactory method of chemical cleavage involves the use of cyanogen bromide, which cleaves the peptide chain at methionine residues (Gross, 1967). Most other methods give poor yields or have undesirable side reactions associated with them.

The separation of the complex mixture of peptides obtained from fragmentation of a protein presents a challenge to the ingenuity of a protein chemist. No single method can give a complete separation, and a combination of several techniques is normally required. Gel filtration on columns of Sephadex (cross linked Dextran) or Biogel (cross linked polyacrylamide) permits a segregation of peptides into size groups (Hjerten, 1971). Ion exchange chromatography makes use of cellulose, dextran or divinylbenzene polymers with charged substituents such as diethylaminoethyl or quaternary nitrogen groups for anion exchangers and carboxymethyl, sulphonyl or phosphate groups for cation exchangers (Peterson, 1970, Schroeder, 1972).

Because both gel filtration and ion exchange chromatography can handle relatively large amounts of material, they frequently provide the first line of attack. Individual fractions from these separations are then frequently transferred to paper for further purification.

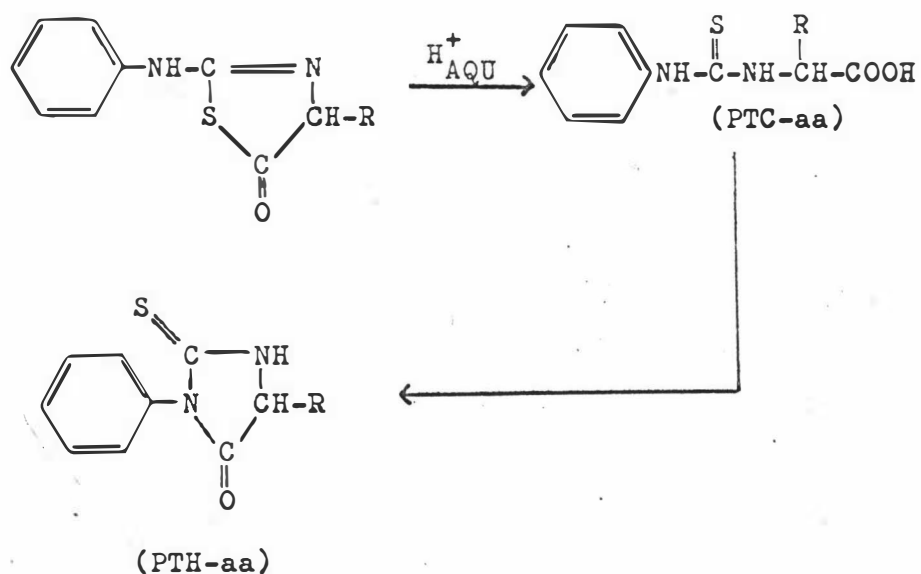
Paper electrophoresis will separate small molecules on the basis of size and charge (Offord, 1966), while separation by paper chromatography is based on the relative distribution of peptides between the aqueous phase on the paper and a mobile organic phase. Diagonal electrophoresis allows the separation and identification of peptides containing disulphide bridges (Brown and Hartley, 1966), lysine (Butler et al, 1967) and histidine (Milstein and Sanger, 1961).

The chemical methods in current use for the sequential degradation of peptides rely on the phenylisothiocyanate (PITC) degradation of Edman (1949 and 1950). This procedure (see below) involves coupling of the N-terminal amino group of the peptide with PITC, followed by cleavage of the now labile N-terminal amino acid with anhydrous acid.



At this point, two approaches can be used to identify the amino acid.

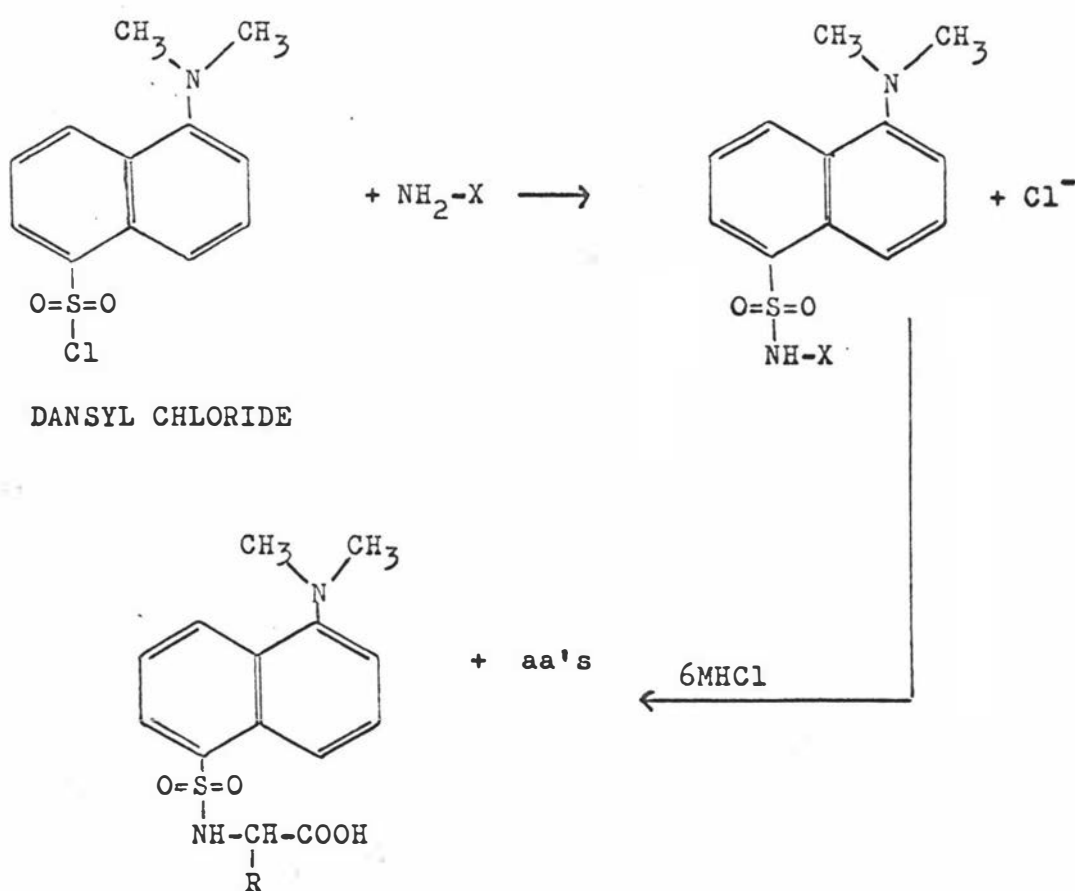
1. The unstable 2-anilino-5-thiazolinone derivative may be converted to the 3-phenyl-2-thiohydantoin derivative (PTH-aa), via the open chain form, using aqueous acid (Edman, 1956a; Edman, 1956b).



The PTH-amino acid can be identified by a variety of methods, including paper (Landmann et al. 1953) and thin layer chromatography (most commonly used)

(Cherbuliez et al. 1960; Edman and Begg, 1967), mass spectrometry (Richards and Lovins, 1972) and gas-liquid chromatography (Waterfield and Haber, 1970).

2. The second approach involves investigation of the peptide produced by the cleavage reaction. Here a small sample of the peptide is taken and the missing residue identified by the Subtractive-Edman procedure (Konigsberg, 1972), or the Dansyl-Edman procedure (Gray, 1972) may be employed to determine the N-terminal of the new peptide. The Subtractive-Edman procedure is not suited to routine sequence determinations, as it relies on amino acid analysis to identify the missing residue. This results in the need for a large (20 nm) sample, also amino acid analysis on each cycle is very time consuming. With the Dansyl-Edman procedure, a 0.1-1nm sample of peptide ( $\text{NH}_2\text{-X}$ ) is taken and reacted with 1-dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl chloride), in order to label the N-terminal residue. The peptide is then completely hydrolysed with hydrochloric acid.



The dansyl amino acid may then be identified by chromatography on polyamide sheets (Woods and Wang, 1967).

Mass Spectrometry offers an alternative approach to peptide sequence determinations (Shemyakin, 1968; Lederer, 1968; Shemyakin et al. 1970). Peptide chains of up to ten residues can be sequenced directly following N-acetylation and permethylation. However, this is far from being a routine procedure. Under ideal conditions, mass spectrometry has the advantages:

1. Due to their different volatilities, mixtures of peptides can be sequenced without prior purification;
2. Amide and acid side chains can be differentiated directly;
3. Peptides with blocked N-terminals (which would not couple with PITC) can be sequenced;
4. Unusual amino acids can be identified.

With all these advantages, it may appear surprising that this technique has not been used more often in sequence studies. It does, however, suffer from serious drawbacks:

1. The method of peptide blocking is not suitable for all side chains. Peptides containing arginine are not presently amenable to mass spectrometry; problems are also associated with quaternisation of histidine. Difficulties are encountered with peptides containing cysteine;
2. Leucine cannot be distinguished from isoleucine;
3. Unexpected fragmentations commonly occur, making interpretation of the spectra difficult;
4. Large amounts of peptide (about 0.5 mg) are required for analysis.

The amount of sequence data that can be determined for a peptide is generally limited to about 10 residues. In the case of mass spectrometry, this limitation is caused by the

decreased volatility of large peptides and the lack of stability of their molecular ions. Systems involving the Edman degradation are limited by oxidative desulphuration of the PTC-peptide. When this occurs, the cleavage reaction is inhibited (Ilse and Edman, 1963). Also, the presence of glutamic and aspartic acid can effectively reduce the amount of peptide available for coupling and cleavage respectively.

Because there is a limit to the amount of direct sequence data obtainable from a large peptide or protein, it is necessary to fragment proteins prior to sequence analysis. The isolation and purification of these peptide fragments is often the rate-limiting step in sequence determinations.

An automated sequenator has been developed by Edman and Begg (1967). This instrument performs the Edman coupling and cleavage reaction with an efficiency of about 98% per cycle, as opposed to about 90% for the manual degradation. This allows longer sequences to be determined directly; e.g. the first 60 residues of apomyoglobin D (Edman and Begg, 1967). This type of instrument would be unsuitable for sequence studies on PFK, which appears to contain a blocked N-terminal residue (Paetkau et al. 1968).

The assignment of amide groups poses no problem when identifying PTH-amino acids or when mass spectrometry is used in sequence studies. However, the hydrolytic conditions (6 M HCl, 110°C) used in conjunction with the Dansyl-Edman technique, result in the conversion of glutamine and asparagine to glutamic and aspartic acid respectively. The loss of this information can be avoided if aminopeptidase is used to cleave the N-terminal amino acid. The N-terminal residue can be determined directly on the amino acid analyser. Alternatively, The electrophoretic mobility of a peptide, before and after removal of a suspected amide, can be used as a basis for assigning amide groups.

Phosphofructokinase (PFK)(or, ATP: fructose 6-phosphate 1-phosphotransferase. EC 2.7.1.11) catalyses the phosphorylation of fructose-6-phosphate to give fructose-1,6-diphosphate. This reaction is a rate-limiting step in the glycolytic pathway, and as such, is ideally suitable as a major metabolic control point. As is the case with other phosphokinases, PFK has a requirement for ATP and magnesium ions. In addition, to its role as a substrate, at high concentrations ATP exerts a strong inhibitory action on the enzyme (Vinuela et al. 1963; Salas et al. 1965a; Parmeggiani et al. 1966). PFK is also inhibited by citrate (Parmeggiani and Bowman, 1963; Garland et al. 1963; Passonneau and Lowry, 1963; Salas et al. 1965b; Underwood and Newsholme, 1965). While the yeast enzyme can utilise ATP, GTP, ITP or CTP as the phosphorylating agent, only ATP can bind at an allosteric site and inhibit the enzyme (Ramaiah et al. 1964; Atkinson and Walton, 1965).

Phosphofructokinase is activated by ADP or AMP; 3',5'-cyclic AMP for the liver fluke enzyme, and 5'-AMP for yeast, and both forms for the enzyme from various mammalian sources (Lardy and Parks, 1956; Passonneau and Lowry, 1962; Mansour and Mansour, 1962, Mansour, 1963)

From these activation/inhibition properties of PFK, it is apparent that, under aerobic conditions, when there is a plentiful supply of ATP, glycolysis will be inhibited; on the other hand, when the level of cellular ATP drops and ADP and AMP accumulate, the rate of glycolysis will increase to produce more ATP.

In terms of protein structure, rabbit muscle PFK has been the most widely studied. In the ultracentrifuge, at pH 8, both the native and dissolved crystalline enzyme exhibit a complex schlieren pattern; this consists of three inseparable boundaries with sedimentation rates of approximately 12 S, 19 S and 30 S (Ling et al. 1965; Parmeggiani et al. 1966; Paetkau and Lardy, 1967; Uyeda, 1969). A rapid reversible equilibrium exists between these different polymeric forms. The 12 S form (MW 320,000-360,000) represents the monomer

of polymerisation (Leonard and Walker, 1972) and is also the smallest fully active unit of the enzyme (Paetkau and Lardy, 1967). The precise nature of the polymerisation reaction is not known, however, Leonard and Walker (1972) suggest a closed hexamerisation may occur, while Aaronson and Frieden (1972) suggest that there are several forms of the active enzyme (MW 360,000) which differ in their mode of polymerisation.

In 0.8 M urea (Paetkau and Lardy, 1967), and at pH 6.0 (Aaronson and Frieden, 1972) the rabbit muscle enzyme dissociates to a 7 S form of molecular weight 160,000-180,000. Further dissociation occurs in 0.5 M acetic acid, in 1% dodecylsulphate and on maleylation to yield a 75,000-85,000 molecular weight form (Uyeda, 1969; Coffee et al. 1972). There is still some uncertainty as to the minimum molecular weight of the rabbit muscle enzyme. Leonard and Walker (1972) and Coffee et al. (1973) report values of between 75,000 and 85,000 in 6 M guanidine hydrochloride, while Paetkau et al. (1968) report values of 47,000 and 23,000 in 5.5 M and 6 M guanidine hydrochloride respectively.

Peptide mapping experiments by Paetkau et al. (1968), and experiments involving the isolation of carboxymethylcysteine-containing peptides (Coffee et al. 1973), support the existence of identical subunits of the 80,000 molecular weight species of rabbit muscle PFK.

While extensive studies have been carried out on rabbit muscle PFK, the structure of the sheep heart enzyme is less well-defined. At pH 8, the native sheep heart enzyme sedimented as two schlieren boundaries with sedimentation rates of 8.2 and 41 S, while the dissolved crystalline enzyme sedimented as a trailing 25.4 S peak (Mansour et al. 1966). At pH 6.5, the enzyme was converted to a 7.5 S form, the molecular weight of which was determined as 160,000 by gel filtration (Mansour and Ahlfors, 1968). In 5 M guanidine hydrochloride Mansour and Ahlfors observed a species with an  $s_{20w}$  value of 2.75 S and estimated its molecular weight as 32,000. They therefore proposed that the 7 S form of sheep heart PFK was made up of six subunits. This work reports further investigation of the sedimentation pattern of the purified enzyme and its subunit structure..

The biological activity and function of a protein is determined in the first instance by its amino acid sequence. A knowledge of an enzyme's primary structure is therefore essential for a full understanding of how an enzyme acts. Because of its central role in the control of the energy balance of the cell, and because no data was available on its primary structure, it was decided to investigate the amino acid sequence of phosphofructokinase. It is hoped that this information will act as a contribution to a more complete understanding of the structure and mechanism of action of the enzyme.

In this investigation, the amino acid sequence of tryptic peptides of PFK were determined using the Dansyl-Edman procedure, and in some cases, mass spectrometry was used.

## 2. METHODS

### 2.1 Materials

Ethyl alcohol, used in the purification of the enzyme, was twice distilled. Acetic acid was redistilled from ninhydrin. Pyridine was refluxed with potassium hydroxide and distilled from ninhydrin. Dimethyl sulphoxide was refluxed with calcium hydride and distilled at 45°C under vacuum (0.2 mm); this solvent was then stored over calcium hydride. Phenylisothiocyanate, trifluoroacetic acid and butyl acetate were treated as described by Edman and Begg (1967). Iodoacetate was recrystallised from ethanol. Maleic anhydride was resublimed, under vacuum, in a cold-finger apparatus. Urea was passed through an Amberlite IRA-400 column to remove cyanate. Iodoacetic acid-2-C<sup>14</sup> (specific activity 34 mCi/mm) was obtained from Amersham.

### 2.2 Enzyme purification and crystallisation

\* Phosphofructokinase was isolated from sheep heart using the procedure of Mansour (Mansour et al. 1966; Lorenson and Mansour, 1969). All operations were performed at 4°C or less.

1.5 Kg of frozen sheep hearts were minced and homogenised in 4 l of 10 mM Tris/HCl (pH 8.0), 2 mM EDTA. The suspension was centrifuged at 8,000 rpm in a GS3 rotor for 20 minutes and the supernatant discarded. The pellet was re-homogenised in 3 l of 20 mM Tris/HCl (pH 8.0), 50 mM MgSO<sub>4</sub>, 5 mM 2-mercaptoethanol, 50 μM ATP. The enzyme was extracted into this solution by stirring at 37°C for 25 minutes. The suspension was then centrifuged, as described above, and the pellet discarded.

The supernatant (Fr.1) was placed in a methanol bath at -2°C and ethanol (pre-cooled in liquid air) was added slowly via a jacketed column (cooled with ethanol/dry ice). When the ethanol concentration reached 8%, the mixture was allowed to stir for a further 10 minutes, and was then centrifuged. The pellet was discarded and the alcohol increased to 15%. The mixture was re-centrifuged and the supernatant discarded.

The pellet was homogenised in 70 ml of 10 mM phosphate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol, 0.1 mM ATP and 10  $\mu$ M fructose-1,6-diphosphate and centrifuged at 22,000 rpm in a SS-34 rotor for 30 minutes. The pellet was re-extracted with the same volume of buffer and re-centrifuged.

The combined supernatants (Fr.2) were applied to a 9 x 20 cm column of DEAE-cellulose. The column was developed with a linear gradient of 750 ml of 0.2 M Tris/HCl (pH 8.6) and 750 ml of 0.9 M Tris/HCl (pH 8.6); in addition, both of these solutions contained 5 mM 2-mercaptoethanol, 0.1 mM ATP and 10  $\mu$ M fructose-1,6-diphosphate. 25 ml fractions were collected and tubes containing enzymatic activity were pooled (Fr.3).

Saturated ammonium sulphate was slowly added to fraction 3 with continual stirring. When the solution reached 42% saturation it was centrifuged. The supernatant was then brought to 60% saturation, centrifuged, and the supernatant discarded.

The pellet containing the 'purified enzyme' was dissolved in 50 mM phosphate buffer (pH 8.0), containing 10 mM 2-mercaptoethanol, 0.1 mM ATP and 10  $\mu$ M fructose-1,6-diphosphate. This was dialysed against a solution of 50 mM phosphate buffer (pH 8.0), 1 mM dithioerythritol, 0.1 mM ATP, 10  $\mu$ M fructose-1,6-diphosphate and 1 mM EDTA (Fr.4). Most enzyme preparations were, however, directly crystallised by dialysis against the above solution, which was, in addition, 30% saturated with ammonium sulphate. After between two and four hours of dialysis, the solution was removed from the sack and allowed to crystallise at 2°C. The crystals were redissolved in the crystallising medium, less ammonium sulphate, by heating in a water bath at 60°C for three minutes.

Table 1 shows the yield and specific activity of the enzyme at the specified fractionation stages. The values quoted are for a preparation involving 1.5 Kg of heart. The enzyme assay system is described in Appendix 1. The protein estimations were performed by the Lowry method.

Table 1

Enzyme purification

Stage	Volume (ml)	Activity (units/ml)	Total units (units)	Protein (mg/ml)	Specific activity (units/mg.)	Yield %
Fr.1	2,740	11.9	32,700	4.7	2.54	100
Fr.2	130	152	19,600	14.6	10.4	61
Fr.3	310	45	14,000	0.47	95	43
Fr.4	13	1,140	14,800	7.6	150	45

Crystals large enough for x-ray analysis were prepared by dialysing the purified enzyme against a solution of 50 mM phosphate pH 8.0, 1 mM dithioerythritol, 0.1 mM ATP, 10  $\mu$ M fructose-1,6-diphosphate, 1 mM EDTA, and containing 26% saturated ammonium sulphate. After standing at 2°C for four days, individual crystals appeared on the walls of the dialysis sack. At this stage the ammonium sulphate concentration of the dialysing medium was increased to 26.8% saturation and over the subsequent seven days this was increased to 28.4%.

## 2.3 Polyacrylamide gel electrophoresis

### Basic gels

Gel electrophoresis, at pH 8.5, was based on the procedure of Davis (1964). The practice of using sample and spacer gels was not employed (Hjerten et al. 1965); instead the protein was applied on the gel surface in 30% sucrose. The following solutions were used for gel preparation:

- (a) 30 g acrylamide and 0.8 g N,N'-methylenebisacrylamide made up to 100 ml with distilled water;
- (b) 1.6% dimethylaminopropionitrile in Tris/glycine;
- (c) 0.48% ammonium persulphate.

7.5% gels were prepared by mixing equal volumes of solutions (a), (b), (c) and distilled water. Gels were cast in 7 x 0.5 cm glass tubes and run in an Acrylophor Apparatus. Both buffer chambers were filled with pH 8.5 Tris/glycine buffer (3 g Tris and 14.4 g glycine per l). Bromophenol-blue was used as an internal marker during electrophoresis. Gels were stained and fixed in 1% amido-black in 7% acetic acid. Destaining was performed electrophoretically.

### Sodium dodecyl sulphate gels

Dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (1969). The 10% gel formulation was used. Proteins were prepared for electrophoresis by boiling for 10 minutes in 10 mM phosphate buffer, containing 0.1% sodium dodecyl sulphate and 0.1% 2-mercaptoethanol.

## 2.4 Ultracentrifugation

Both sedimentation and diffusion coefficients were measured in a Spinco Model E ultracentrifuge with mechanical speed control (Chervenka, 1969). All runs were performed at 20°C in an ANB rotor using schlieren optics. Samples were prepared for analysis by dialysis against the specified buffer. Results were recorded on Ilford G 30 photographic plates, which were developed in red light.

### 2.4.1 Sedimentation analysis

A 12 mm Double Sector Cell was used in most sedimentation runs. When two samples were run simultaneously, a 12 mm Single Sector Cell was used, together with a similar cell containing a +1° wedge window; in this case the lugs were removed from the rotor to provide the reference edge.

Measurements were made on enlargements of the developed plates, using a photographic enlarger. The magnification factor was calculated by dividing the measured distance between the reference edges by the actual distance, 1,594 cm (determined in a travelling microscope). The position of maximum displacement of a boundary was measured from the inner reference edge. After division by the magnification factor, this value was added to 5.72 cm to give a value  $r$ , which represents the radial distance from the centre of rotation to the point of maximum displacement of the boundary. Generally 10 frames were exposed per run at pre-set time intervals. A graph of  $\log r$  was plotted as a function of time,  $t$  (in min), and the gradient  $\frac{d \log r}{dt}$  of the line was measured. The sedimentation coefficient ( $s$ ) was then calculated from equation (2), where  $\omega$  represents the angular velocity.

$$s = \frac{1}{\omega^2 r} \cdot \frac{dr}{dt \text{ (sec)}} \quad (1)$$

$$\Leftrightarrow s = \frac{2.303}{60 \omega^2} \cdot \frac{d \log r}{dt \text{ (min)}} \quad (2)$$

The sedimentation coefficient, in units of  $\text{sec}^{-1}$ , was converted to Svedberg Units (S) by multiplication by  $10^{13}$  sec.

### 2.4.2 Diffusion analysis

All runs were performed in a 12 mm cup-type synthetic boundary cell at 12,590 rpm. A 0.4 ml protein sample, in 0.1 M phosphate buffer pH 8.0, was placed in the cell centre-piece while 0.2 ml of the same buffer (from the dialysis medium) was introduced into the cup. As soon as the vacuum chamber was closed, the rotor was started and the vacuum pumps turned on. The rotor was allowed to coast at 3,000 rpm while the chamber pressure dropped to  $0.5 \mu$ . At this point, the speed was increased; at 8,000 rpm the buffer layered on top of the protein solution and at 12,590 rpm, readings were commenced. Five to ten frames were exposed per run at eight minute intervals. The bar angle was kept constant at  $75^\circ$ .

Measurements on the plates were made with the aid of a photographic enlarger. The diffusion coefficient was calculated from equation (3), where A represents the area of the schlieren boundary and  $(dc/dr)_{\max}$  the maximum displacement of the boundary.

$$D = \frac{A^2}{(dc/dr)_{\max}^2} \cdot \frac{1}{4\pi t_{(\min)}^{60}} \quad (3)$$

As the boundaries were symmetrical, peak areas were calculated as the product of height times width-at-half-height. After division of the area by the magnification factor, a graph of

$$\frac{A^2}{(dc/dr)_{\max}^2}$$

was plotted against time and the gradient determined. Equation (3) was then employed to calculate the diffusion coefficient in units of  $\text{cm}^2/\text{sec}$ .

### 2.4.3 Corrected coefficients

Sedimentation and diffusion coefficients were corrected to  $s_{20w}$  and  $D_{20w}$  values using equations (4) and (5) respectively.

$$s_{20w} = s \frac{\eta_t}{\eta_{20}} \cdot \frac{\eta_{sol}}{\eta_w} \cdot \frac{1 - \bar{v} \rho_{20w}}{1 - \bar{v} \rho_t sol} \quad (4)$$

$$D_{20w} = D \frac{293}{293+t} \cdot \frac{\eta_t}{\eta_{20}} \cdot \frac{\eta_{sol}}{\eta_w} \quad (5)$$

- $s_{20w}$  = Sedimentation rate in water at 20°C  
 $s$  = Observed sedimentation rate in buffer.  
 $\eta_t$  = Viscosity of water at temperature of run (t).  
 $\eta_{20}$  = Viscosity of water at 20°C.  
 $\eta_{sol}$  = Viscosity of sample at known temperature (t').  
 $\eta_w$  = Viscosity of water at known temperature (t').  
 $\rho_{20w}$  = Density of water at 20°C.  
 $\rho_t sol$  = Density of solution at temperature of run (t).  
 $\bar{v}$  = Partial specific volume.  
 $D_{20w}$  = Diffusion coefficient in water at 20°C.  
 $D$  = Observed diffusion coefficient.  
 $t$  = Temperature of run, in °C.

Viscosity measurements were carried out in a capillary viscometer immersed in a constant temperature water bath. The viscometer was calibrated with water and readings repeated on the sample until three consecutive values were obtained, which agreed to within  $\pm 0.05$  sec.

Densities were measured in a precalibrated 1000  $\mu$ l pipette (volume = 1.013 ml). At least three separate weighings were made per sample; the average of these values was used to calculate the sample density.

The term  $(\eta_t/\eta_{20})$ , in equations 3 and 4, had a value of unity as all runs were performed at 20°C. A value of 0.728 ml/gm was assumed for the partial specific volume (Paetkau and Lardy, 1967). In order to avoid the requirement of a large amount of sample, viscosity and density measurements were carried out on the solvent instead of the protein solution; this introduces only a small error (Chervenka, 1969).

#### 2.4.4 Molecular weight determinations

Molecular weights were calculated from sedimentation and diffusion data, using the Svedberg equation (6), where R is the universal gas constant ( $8.315 \times 10^7$ ) and T is the absolute temperature.

$$MW = \frac{RT_s}{D(1 - \bar{v}\rho)} \quad (6)$$

## 2.5 Carboxymethylation

Phosphofructokinase was either dissolved in, or dialysed against, 0.1 M Tris/HCl, pH 8.0 containing 8 M urea and 10 mM dithioerythritol (Nelson et al. 1965). A known protein concentration, of between 10 and 20 mg/ml was used. The reduction was allowed to proceed under nitrogen for 12 hours at room temperature; a 2.5-fold excess of iodoacetic acid was then added. This excess was over the total free thiol groups present. The carboxymethylation was allowed to proceed in the dark, under nitrogen, for 45 minutes. The reaction was terminated by the addition of 2-mercaptoethanol. The solution was then dialysed against distilled water, which resulted in precipitation of the protein.

When 2-C<sup>14</sup> iodoacetic acid was used for carboxymethylation, the protein was dissolved in 0.1 M Tris/HCl, pH 8.0, containing 8 M urea. To this was added an equi-molar amount of dithioerythritol to protein thiol groups. After reduction, a 1.2 molar excess of 2-C<sup>14</sup> iodoacetic acid (specific activity 1  $\mu$ Ci/mg) was added to the reaction mixture. After 30 minutes reaction, a further 1.2 molar excess of cold iodoacetic acid was added and the reaction continued for a further 15 minutes. This was to ensure complete carboxymethylation of the protein.

Amino acid analyses of the enzyme, carboxymethylated by the first procedure, indicated a 95-98% conversion of cysteine to S-carboxymethylcysteine. The second method, using 2-C<sup>14</sup> iodacetate, led to an 80-90% reaction.

## 2.6 Maleylation

Maleylation of the primary amino groups of the enzyme was carried out in 0.1 M borate buffer, pH 8.8, in a 'pH-Stat' at room temperature (Butler et al. 1969). Sufficient solid maleic anhydride (fine sublimed crystals) was added to

provide a 25-fold molar excess over protein lysine residues. On completion of the reaction, the protein was dialysed against 0.1 M phosphate buffer, pH 8.0.

## 2.7 Tryptic digestion of enzyme

Twice-crystallised trypsin was treated with TPCK (N-tosylphenylalanine chloromethyl ketone) in order to inhibit chymotryptic activity (Wang and Carpenter, 1965). Digestion with trypsin was based on the methods outlined by Smyth (1967).

S-carboxymethyl-phosphofructokinase (Approximately 10 mg/ml) was dialysed against distilled water and the pH of the suspension adjusted to 8.3 with dilute ammonia. Trypsin, equivalent to 0.5% (w/w) of the phosphofructokinase, was added and the digestion allowed to proceed at 37°C in a 'pH-stat'. After about 5 minutes the turbid suspension cleared and after 60 minutes digestion was complete. At this point, a further 0.5% of trypsin was added; although there was virtually no further uptake of ammonium hydroxide, the reaction was allowed to proceed for a further 30 minutes. The solution was then freeze-dried.

## 2.8 Peptide mapping

Thirty mg of peptide material was dissolved in 20 mM ammonium hydroxide and applied as a 1 x 30 cm band across the middle of a sheet of Whatman No.1 chromatography paper (46 x 57 cm). One  $\mu$ l of an internal fluorescent marker solution, containing DNS-Arg and DNS-OH (1 mg/ml), was spotted at 2 cm intervals along the band of peptide material. Ten  $\mu$ l of external R and T markers were also applied to the paper. (The R solution contained Lys, Arg, Leu, Val, Met, Pro, Phe and Tyr; while the T solution contained His, Gly, Ala, Ser, Ile, Thr, Glu and Asp; all at a concentration of 5 mM in 10% isopropanol.) The paper was moistened with pH 6.5 buffer (pyridine/acetic acid/water, 100 : 4 : 900), and excess buffer blotted off. Electrophoresis was carried out at pH 6.5 (Ryle and Sanger, 1955) in a Michl-type apparatus (Michl, 1951) at 3 kV for 45 minutes. After electrophoresis, the paper was viewed under U.V. light to locate the dansyl markers, and edge

markers were stained with ninhydrin to determine how far the peptides had run. At pH 6.5, basic peptides move to the cathode (up) and acidic peptides move to the anode (down). Neutral peptides move up as a band 1-2 cm from the origin due to endosmotic flow. This 29 cm-long band of neutral peptides was cut out and sewn onto a second sheet of Whatman No. 1 paper, 5 cm from the bottom. The backing strip was cut out and dansyl and R and U<sub>1</sub> markers were applied as described above. The neutral peptides were separated in the first dimension by electrophoresis at pH 2.1 at 3 kV for 55 minutes. The buffer used was acetic acid : water : formic acid (4 : 45 : 1). The dried sheet was again examined under U.V. light and edge markers stained with ninhydrin.

The sheet containing the neutral peptides was cut into six, 4 cm wide vertical strips, each of which was sewn onto a fresh sheet of paper, 12 cm from the top. The peptides on the strips were compressed into a fine line using 2% acetic acid. Internal and external markers were applied to the papers which were then subjected to descending chromatography (in butanol/ acetic acid/ water : 4 : 1 : 5 : upper phase) for 16 hours. The lower phase of the solvent was placed in the bottom of the tank for rapid equilibration.

Similarly, six, 4 cm wide vertical strips, containing the acidic and basic peptides were cut out and sewn, in pairs, onto six new sheets of paper. Chromatography was again used to achieve separation of these peptides in the second dimension.

Individual maps were then stained with ninhydrin (Heilmann et al. 1957) and chlorine o-tolidine (Dawson et al. 1969) for the detection of peptides. Further maps were stained with Sakaguchi reagent (Eastley, 1965), Pauly reagent (Dawson et al. 1969), Ehrlich's reagent (Smith, 1953) and 1-nitroso-2-naphthol, for the detection of arginine-, histidine-, tryptophan-, and tyrosine-containing peptides respectively.

## 2.9 Preparative electrophoresis

Peptides purified by preparative electrophoresis were generally run on Whatman 3 MM paper, using a maximum loading

rate of 50 n mol/cm. When lesser amounts of peptide were available, samples were run on Whatman No.1 paper with a maximum loading of 15 n mol/cm. In both cases, electrophoresis was conducted as described under peptide mapping; the same internal and external markers were applied to the paper. Peptides were located by staining edge marker strips with ninhydrin. Bands of peptide material were cut out and eluted using M/50 ammonium hydroxide.

## 2.10 C<sup>14</sup> Detection

Peptides containing C<sup>14</sup> were located by a combination of autoradiography and liquid scintillation counting. Autoradiographs were exposed for a period of six weeks. Results were recorded on Kodak RP Royal X-Omat film. Triton fluid was used for scintillation counting in a Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillation fluid was prepared by dissolving 3 g of PPO and 0.1 g POPOP in 600 ml toluene and adding 300 ml of Triton X-100.

## 2.11 Amino acid analysis

Analyses were performed on a Beckman 120C amino acid analyser. During the course of this investigation, the instrument was converted to a single column automated Beckman-Locarte system. Analyses on the whole protein were conducted using 0.2 mg samples of enzyme, while for peptides, one-twentieth of the purified sample was generally used. Peptides were hydrolysed in 8 x 0.7 cm pyrex tubes using 200  $\mu$ l of constant boiling point hydrochloric acid. The tubes were sealed under vacuum (0.02 mm) and hydrolysed at 110°C for about 15 hours.

The analysis values for threonine and serine, recorded under results, have been increased by 5% and 10% respectively. This was to allow for decomposition of these amino acids on acid hydrolysis.

## 2.12 N-terminal analysis (peptides)

N-terminal analysis was performed according to the method of Gray (1972). Between 0.1 and 1.0 nm of peptide was dried under vacuum in a 0.6 x 3.0 cm Duram Tube and redissolved in

10  $\mu$ l 0.2 M sodium bicarbonate. This solution was re-dried to remove traces of ammonia, and redissolved in 10  $\mu$ l of distilled water. Ten  $\mu$ l of dansyl chloride (2.5 ng/ $\mu$ l in acetone) was added and the tubes sealed with parafilm, and incubated at 45°C for 30 minutes. After drying down, 50  $\mu$ l of 6 N HCl was added, the tubes were sealed under vacuum and the peptides were hydrolysed at 110°C for between 4 and 16 hours. The tubes were opened and dried over sodium hydroxide.

The residue was extracted with 5  $\mu$ l of 95% ethanol. Equal amounts of the extract were spotted on each side of a 7.5 x 7.5 cm polyamide plate (Woods and Wang, 1967). One of these sample spots had 0.5  $\mu$ l of a marker solution superimposed on it. The marker contained dansyl derivatives of Phe, Ile, Pro, Gly, Glu, Ser and Arg, each at a concentration of 0.1 mg/ml in ethanol.

Polyamide plates were subjected to ascending chromatography in 1.5% formic acid for 10 minutes. After 15 minutes drying, the plates were chromatographed for a further 15 minutes in the second dimension using benzene/acetic acid (9:1). The plates were then inspected under a U.V. lamp (366 m $\mu$ ). At this stage, an N-terminal of either Phe, Leu, Ile, Pro, Lys, Gly or Tyr can be identified uniquely.

Chromatograms were re-run for 10 minutes in the second dimension in a solvent of ethyl acetate/methanol/acetic acid (20 : 1 : 1) and reinspected. This solvent allows identification of an N-terminal of either Ala, Glu, Asp, C $\alpha$ -Cys, Met, Ser or Thr.

Plates were rerun for 7 minutes in the second dimension in a solvent of acetic acid/pyridine/ethanol/water (40 : 22.5 : 25 : 2,500). This allows separation of DNS-His from DNS-Arg and DNS- $\epsilon$ -Lys.

When necessary, chromatography was repeated for 50 minutes in the second dimension in 0.5 M sodium phosphate/ethanol (3 : 1); this affords separation of DNS-Arg from DNS- $\epsilon$ -Lys (Hartley, 1970).

After use, the polyamide plates were washed in a solution of water/acetone/880 ammonia (500 : 500 : 25). With care, each plate could be reused about 30 times.

### 2.13 N-terminal sequence analysis

The 'Zoom' - Dansyl - Edman procedure of Gray (Gray and Smith, 1970) was used to investigate the N-terminal sequence of the first four or five residues in a peptide.

When the sequence of the first four residues was required, 2-10 nm of the peptide was placed in each of three 0.6 x 6 cm tubes (the first residue having been predetermined by the N-terminal analysis). After freeze-drying, 25  $\mu$ l of distilled water and 25  $\mu$ l of PITC was added to each of the tubes, which were then flushed with oxygen-free nitrogen, capped, and incubated at 45°C for 60 minutes. After drying over concentrated sulphuric acid, at 60°C for 30 minutes, 50  $\mu$ l of TFA was added to each sample. The tubes were again flushed with nitrogen, capped, and incubated at 45°C for 30 minutes. The cleaved peptides were then dried over sodium hydroxide.

One tube was set aside at this stage. The above process was repeated on the remaining two samples, at which point the second tube was removed. The cycle was repeated for a third time on the third tube. At this stage 25  $\mu$ l of distilled water was added to the three tubes, which were then extracted four times with butyl acetate. N-terminal analysis was performed directly on the aqueous phase from each tube. Analysis of the first tube yields the second residue of the peptide's sequence; analysis of the second tube reveals the third residue, while the third tube provides the fourth residue.

### 2.14 N-terminal analysis (protein)

The N-terminal of the enzyme was labelled, in 8 M urea, using dansyl chloride (Gray, 1967; and Gros and Labouesse, 1969). One mg of protein was dissolved in 1 ml of 8 M urea in 0.1 M sodium bicarbonate. To this, was added 1 ml of dansyl chloride (25 mg/ml, in acetone). The reaction

mixture was incubated at 37°C for 4 hours and then dialysed against distilled water. The precipitated protein was transferred to a hydrolysis tube (1 x 7.5 cm) and freeze-dried. Five hundred  $\mu$ l of 6 M hydrochloric acid was added; the tubes were sealed under vacuum and hydrolysed at 110°C for 16 hours.

After drying, the hydrolysate was extracted twice with 50  $\mu$ l of water-saturated ethyl acetate (this allows removal of dansyl sulphonic acid, which otherwise would interfere with the subsequent chromatography). The extract was dried and redissolved in 95% ethanol before spotting on a 15 x 15 cm polyamide plate. Chromatography was run as described previously.

The residue, which was left after ethyl acetate extraction, was dissolved in 95% ethanol and chromatographed as described above. (In addition to DNS-OH, DNS-NH<sub>2</sub> and DNS- $\epsilon$ -Lys, the residue could contain DNS-His or DNS-Arg).

## 2.15 Amino acid sequence determination

### 2.15.1 Dansyl-Edman technique

The Dansyl-Edman Technique (Gray, 1972) was used to determine the amino acid sequence of most peptides. Between 2 and 20 nm of peptide was used per residue.

The peptide was freeze-dried in a 6 x 1 cm screw-top tube and redissolved in 150  $\mu$ l of water. One hundred and fifty  $\mu$ l of FITC (5%, in pyridine) was added. The tube was flushed with oxygen-free nitrogen, capped, and incubated at 45°C for 90 minutes. In order to remove solvent, excess FITC and volatile by-products, the mixture was dried at 60°C for 40 minutes over concentrated sulphuric acid. After adding 200  $\mu$ l of TFA, the tube was again flushed with nitrogen, capped, and incubated at 45°C for 30 minutes. Excess TFA was removed over sodium hydroxide flakes. The dried residue was dissolved in 200  $\mu$ l of water and extracted three times with 1 ml washings of butyl acetate. After vigorous shaking, the two layers were separated in a clinical centrifuge. The upper organic phase, containing the 2-anilino-thiazolinone derivative of the N-terminal amino acid, was discarded at each washing step.

The aqueous phase (containing the original peptide, minus the N-terminal residue) was dried down and dissolved in 150  $\mu$ l of water. A sample of between 5 and 20  $\mu$ l was removed at this point and subjected to N-terminal analysis to identify the second residue in the sequence. This cycle of operations was repeated until the entire sequence of the peptide was obtained.

### 2.15.2 Mass spectrometry

Between 0.2 and 0.4 mg of peptide was freeze-dried in a ground glass tube. The sample was N-acetylated by adding 0.5 ml of acetic anhydride and 0.5 ml of distilled water. The reaction mixture was shaken for 40 minutes at room temperature before being exhaustively dried down and dissolved in one drop of dry dimethyl sulphoxide (DMSO)(Kent, 1970).

The base used for permethylation (Morris and Williams, 1971) was prepared by adding 2 ml of dry DMSO to 0.1 g of sodium hydride (prewashed with dry hexane). The tube was flushed with oxygen-free nitrogen, stoppered lightly, and incubated at 60°C for 10 minutes. A second batch of sodium hydride was added and the incubation continued for a further 10 minutes before the mixture was centrifuged.

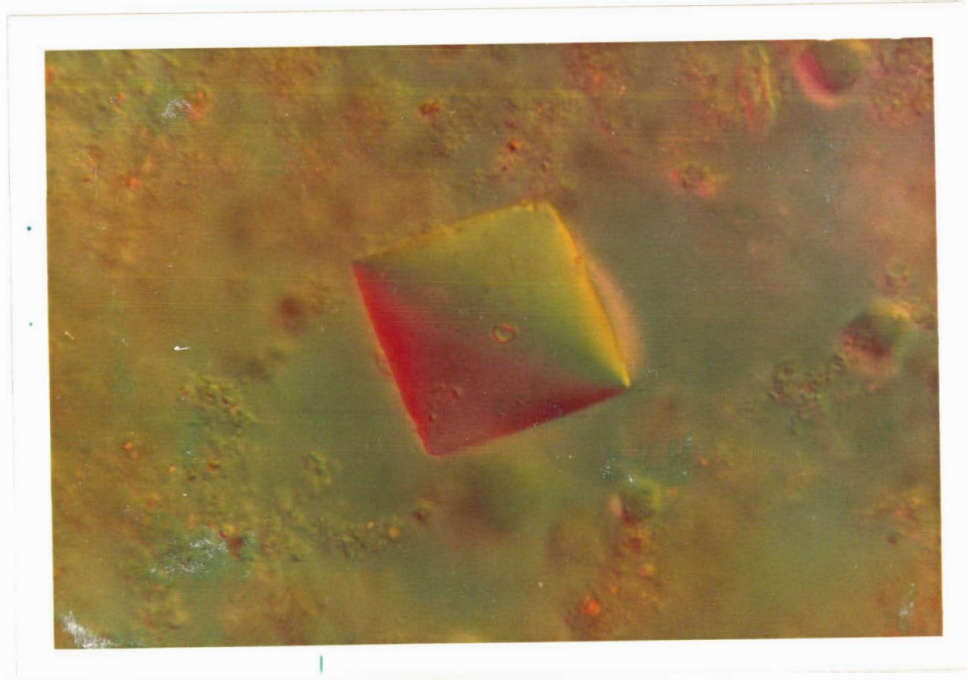
One ml of Methyl iodide was added to the tube containing the N-acetylated peptide; immediately after this, 1 ml of base was added. The permethylation was allowed to proceed for 20 seconds before being stopped by the addition of water. The product was isolated by extraction with 1 ml of chloroform. The chloroform extract was washed three times with water and evaporated in vacuo. The yellow oil obtained was dissolved in one drop of chloroform and applied to the direct insertion probe of a MS 902 Mass Spectrometer.

### 2.16 Digestion of peptides

Large tryptic peptides requiring further cleavage prior to amino acid sequence determination, were treated as described below.

The peptide samples were freeze-dried and dissolved in between 0.5 and 1 ml of 0.2 M ammonium bicarbonate. To this solution was added approximately 1% (w/w) of either Thermolysin, Chymotrypsin, or Subtilisin. The digest was carried out over-night at 37°C. The solution was freeze-dried and the extent of digestion was assessed by analytical electrophoresis. The peptide digested with pepsin was treated in a similar manner, except that the incubation was carried out in 2% formic acid.

A



B

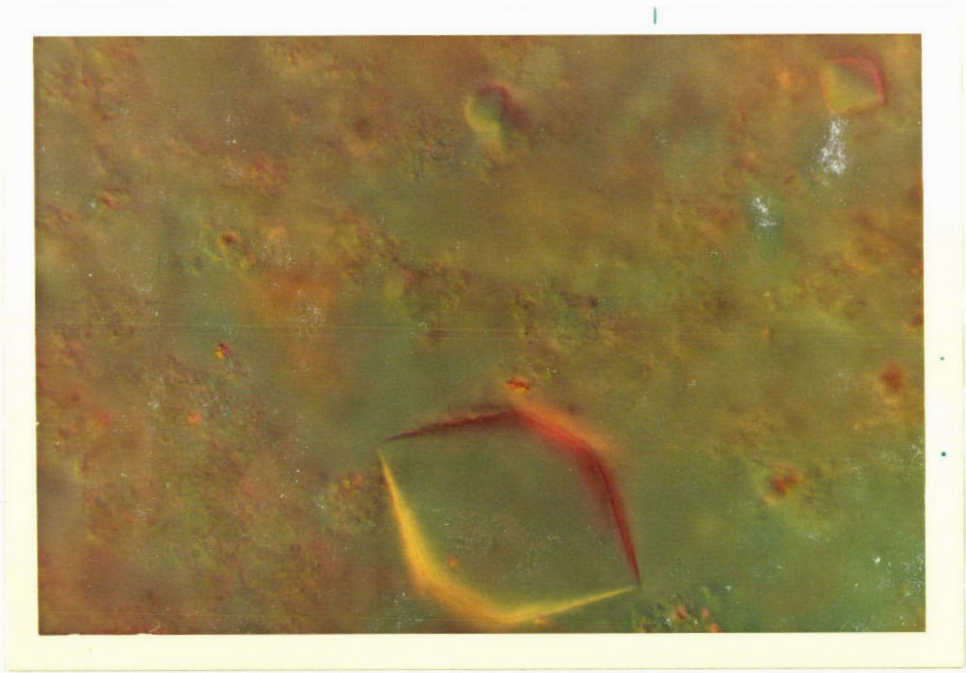


FIGURE 1 CRYSTALLINE PHOSPHOFRUCTOKINASE

## 3. RESULTS (1)

### 3.1 Crystals

Crystallisation of the enzyme by the method described on page 9 led to the formation of crystals of between 2 and 30  $\mu$ . Figure 1A and B show the photographs of phosphofructokinase crystals taken in a Zeiss interference microscope fitted with a Hamauski attachment. The views shown represent two planes observed by rotating the crystals through approximately  $90^\circ$ . When viewed on one surface, the crystals appeared as regular hexagons, and when rotated at right angles, a rhombic plane was observed, implying that the enzyme crystallises as hexagonal bipyramids.

The size of these crystals made them unsuitable for x-ray analysis; however, a method was developed (P. 11) for growing 500  $\mu$  crystals. These crystals were mounted in sealed capillary tubes. When subjected to x-ray analysis, axial lengths of 600, 250 and 220  $\text{\AA}$  were observed in still photographs. The crystals were of low symmetry, possibly monoclinic. Despite uncertainty regarding the space group, these results suggest a probable weight of protein of greater than  $10^6$  daltons per asymmetric unit. This large asymmetric unit could account for the very weak diffraction by the crystals, and is consistent with sedimentation studies on the dissolved crystalline enzyme.

### 3.2 Gel electrophoresis

On gel electrophoresis (7.5 gel at pH 8.5) the purified enzyme did not enter the running gel, but formed a band on the surface. A similar result was obtained when 5.6% gels were used; this situation is similar to observations on rabbit muscle PFK (Faetkau et al. 1968). The dissolved crystalline enzyme migrated as a band with mobilities between 0.2 and 0.4 in 3.5% gels at pH 8.5; a similar result has since been reported for chicken liver PFK (Kono, 1973).

On maleylation, both the purified and dissolved crystalline enzyme ran as a single band in pH 8.5 gels; this band had mobilities of 0.18, 0.4 and 0.8 in 12.5%, 7.5% and 3.5% gels

respectively. A single band was also obtained when electrophoresis was carried out in sodium dodecylsulphate (below).

### 3.3 Sedimentation studies on the purified enzyme

The purified enzyme was dialysed against 50 mM phosphate buffer pH 8.0, containing 1 mM dithioerythritol, 0.1 mM ATP, 10  $\mu$ M fructose 1,6-diphosphate and 1 mM EDTA, and subjected to sedimentation analyses at 42040 rpm. Figure 2 shows the schlieren pattern of the purified enzyme in a double sector cell at 20°C and at a protein concentration of 14 mg/ml. Initially, two boundaries were observed; however, as sedimentation proceeded, a small shoulder was detected in the trailing edge of the high molecular weight boundary. From a graph of log r as a function of time, the sedimentation coefficients of the three boundaries were determined. The buffer had a viscosity of 1.011 relative to water, and its density was 1.007 g/cc at 20°C. Using this data in conjunction with equation (4) (P.14),  $s_{20w}$  values of 5.6, 19.3 and 30.8 S were obtained for the low, medium and high molecular weight components. A schlieren pattern of similar shape to that shown in Fig.2 has been reported for sheep heart PFK (Mansour et al. 1966), however, in this instance, at a protein concentration of 3.7 mg/ml, sedimentation rates of 8.2 and 41 S were reported for the major boundaries.

In order to investigate the concentration dependence of the sedimentation coefficients, six additional sedimentation experiments were performed on dilutions of the above solution. Data on these determinations is shown in Table 2.

Two distinct high (30 S) and low (7 S) molecular weight peaks were observed at all concentrations; however, because of the small amount present, it was not possible to measure the sedimentation coefficient of the medium (19 S) molecular weight species at lower protein concentration. Figure 3 shows a plot of the sedimentation rates of the 7 and 30 S forms as a function of protein concentration. The asymmetric 30 S boundary showed a marked concentration dependence attaining a maximum value of 32 S between 7 and 11 mg/ml. The shape and the concentration dependence of this boundary

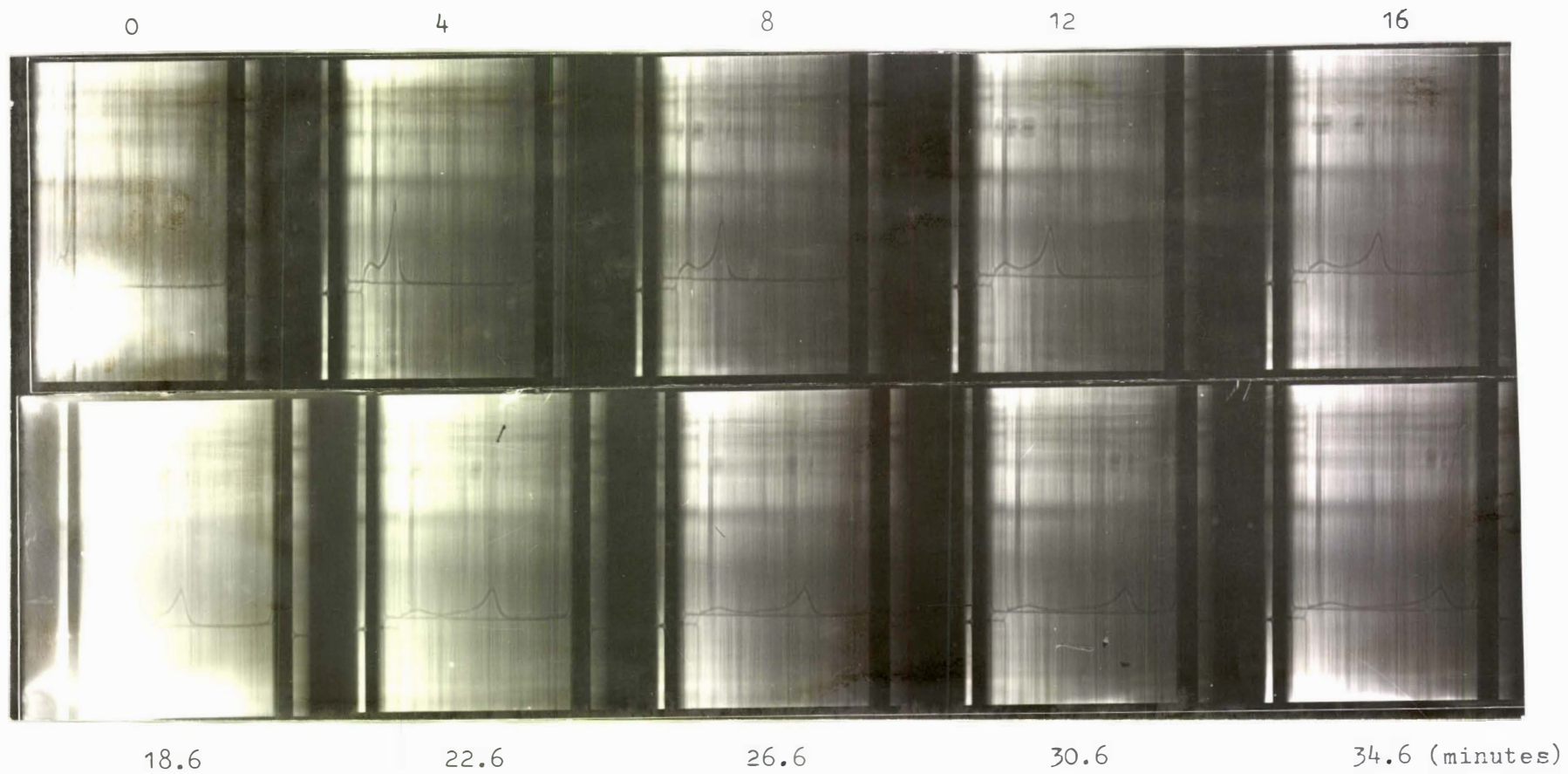


FIGURE 2 SEDIMENTATION PATTERN OF PURIFIED PFK

Table 2

Data for determination of sedimentation rates of the purified enzyme.

High molecular weight boundary

(P) (mg/ml)	$\frac{d \log r}{dt}$	s	$s_{20w}$
14.5	$1.49 \times 10^{-3}$	29.6	30.8
10.87	$1.56 \times 10^{-3}$	30.9	32.1
7.25	$1.55 \times 10^{-3}$	30.8	32.0
3.62	$1.49 \times 10^{-3}$	29.5	30.7
2.18	$1.41 \times 10^{-3}$	28.0	29.1
1.45	$1.33 \times 10^{-3}$	26.5	27.5
0.78	$1.28 \times 10^{-3}$	24.7	25.7

Low molecular weight boundary

14.5	$2.7 \times 10^{-4}$	5.4	5.6
10.87	$2.7 \times 10^{-4}$	5.4	5.6
7.25	$2.8 \times 10^{-4}$	5.5	5.7
3.62	$3.3 \times 10^{-4}$	6.5	6.7
2.18	$3.4 \times 10^{-4}$	6.7	7.0
1.45	$3.1 \times 10^{-4}$	6.1	6.3

Medium molecular weight boundary

14.5	$9.4 \times 10^{-4}$	18.6	19.3
10.87	$9.3 \times 10^{-4}$	18.4	19.1

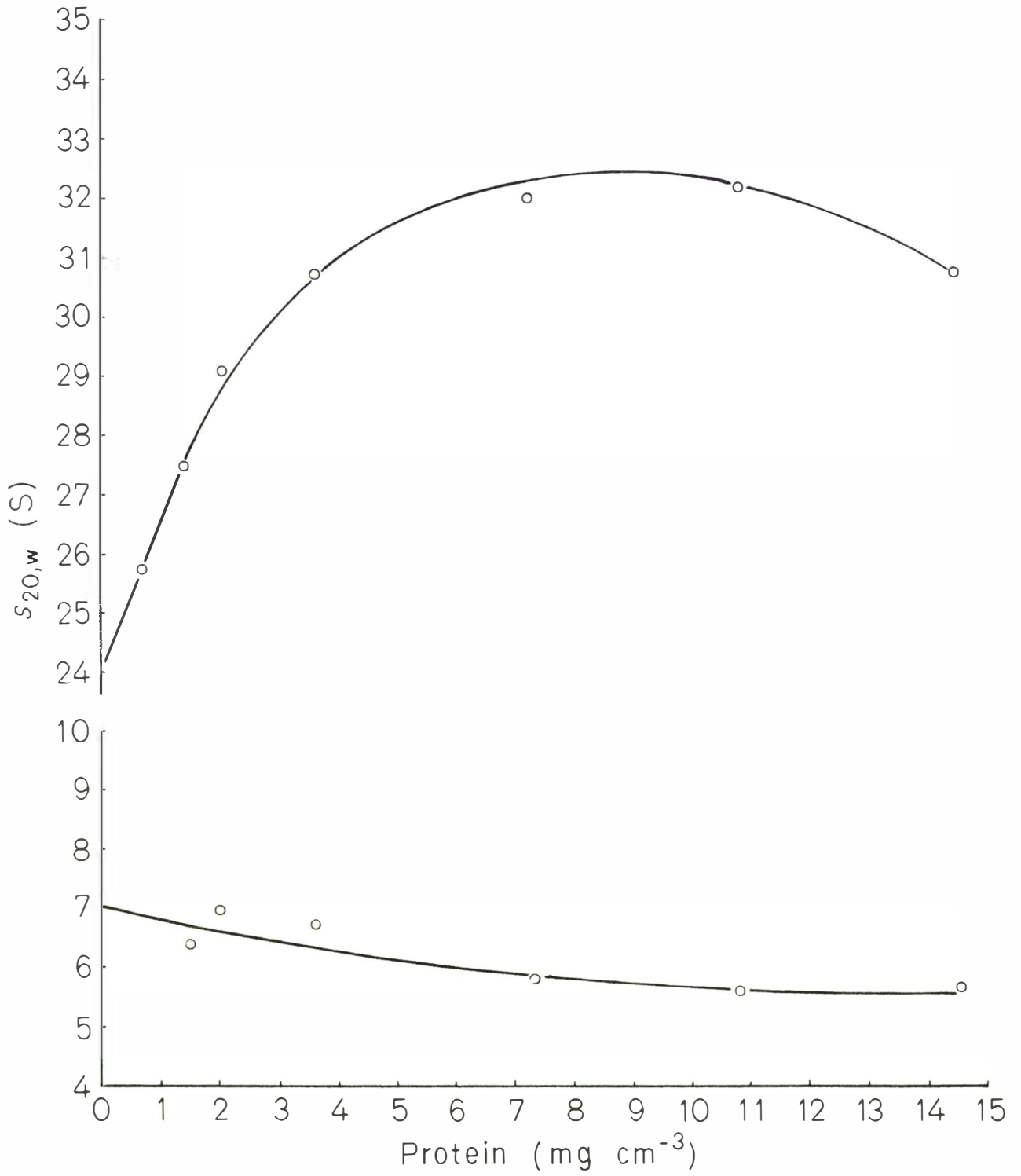


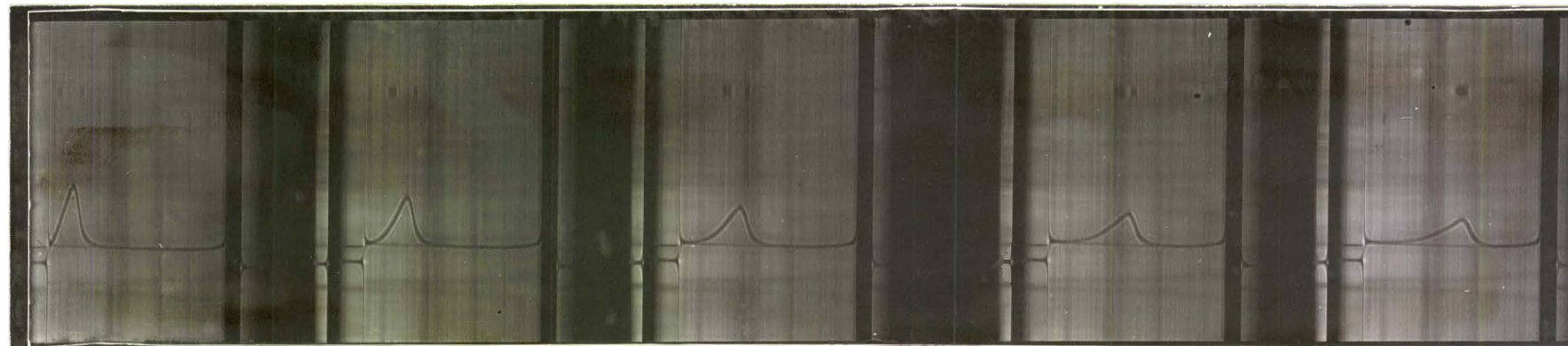
Figure 3 Concentration dependence of the sedimentation coefficient of purified PFK.

are characteristic of a polymer in rapid reversible equilibrium with lower molecular weight forms (Nichol et al. 1964; \* Bethune and Grillo, 1967). The sedimentation coefficient of the 7 S boundary decreased with decreasing concentration, and as far as could be judged, the relative area under this boundary remained constant as concentration decreased.

#### 3.4 Sedimentation studies on the dissolved crystalline enzyme

Three different preparations of the dissolved crystalline enzyme were subjected to sedimentation analysis and produced similar results. The crystals were dissolved in, and dialysed against, a solution of 50 mM phosphate buffer pH 8.0, 1 mM dithioerythritol, 0.1 mM ATP, 10  $\mu$ M fructose-1,6-diphosphate and 1 mM EDTA, for six hours prior to analysis. The schlieren pattern of the dissolved crystalline enzyme at 8 mg/ml and a rotor speed of 47,660 rpm is shown in Figure 4. The trailing boundary had a  $s_{20w}$  value of 31.6 S, and its shape was indicative of a rapidly polymerising system. Analyses on a second preparation of crystals, at 42,040 rpm and the same protein concentration, indicated a similar boundary, with a  $s_{20w}$  value of 31.4 S. Results on a third preparation, at 2 mg/ml and 59,780, indicated an asymmetric boundary with an  $s_{20w}$  value of 27.0 S. The sedimentation pattern of this preparation was reinvestigated after storage of the enzyme at 2° for 7 days; there was no change in either the shape or sedimentation rate of the boundary.

When a fourth preparation of dissolved crystalline enzyme was investigated, under conditions apparently identical to those described above, a different sedimentation pattern was observed, Figure 5 shows a tracing of this pattern at 42,040 rpm and a concentration of 6 mg/ml. (The glass plates clouded with time, and were unsuitable for reproduction.) Initially, three schlieren boundaries were observed with  $s_{20w}$  values of 27 S, 51 S and 53 S (peaks 1, 2 and 3 respectively). After 12 minutes, the 27 S and 53 S peaks disappeared, leaving a long trailing 51 S boundary in reversible equilibrium with lower molecular forms. From the relationship  $s_2/s_1 = (MW_2/MW_1)^{3/2}$ , the molecular weight ratios of the 27 S to



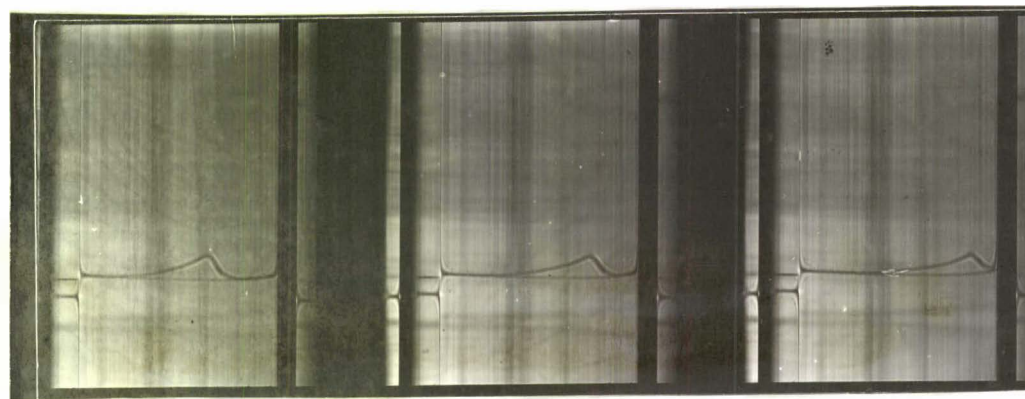
0

4

8

12

16



20

24

28 (minutes)

FIGURE 4 SEDIMENTATION PATTERN OF DISSOLVED CRYSTALLINE PFK

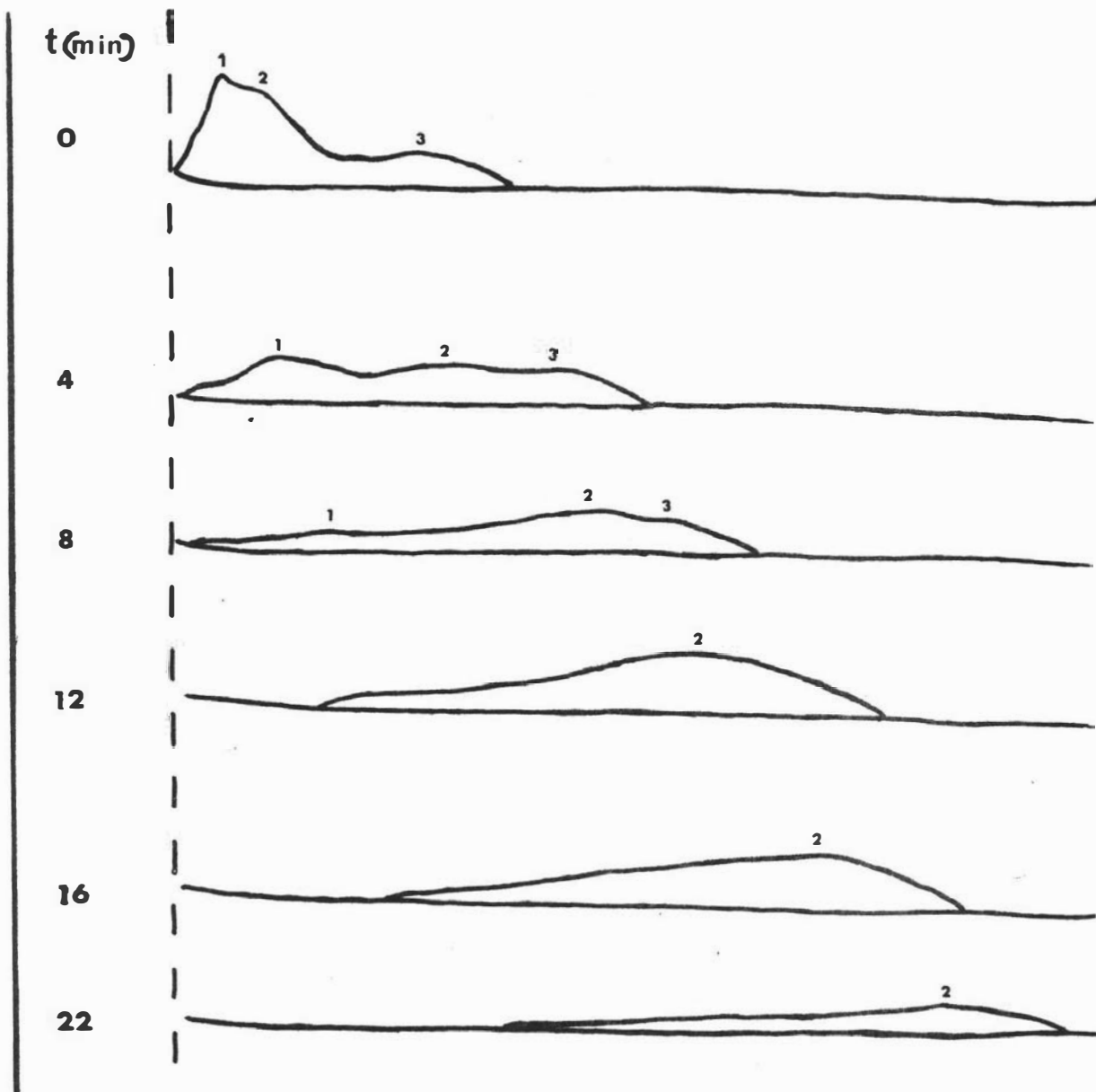


FIGURE 5

SCHLIEREN PATTERN OF DISSOLVED CRYSTALLINE PFK

( 4TH PREPARATION )

to the 53 S species was estimated as 1 : 2.7. In this case it appears that the enzyme may be undergoing a further polymerisation involving trimerisation of the 30 S form.

### 3.5 Molecular weight determinations under dissociating conditions

When the enzyme was maleylated and dialysed against 0.1 M phosphate buffer pH 8.0 prior to sedimentation and diffusion analyses, a single symmetrical boundary was obtained at all concentrations investigated. Values of the sedimentation and diffusion coefficient ( $D$ ), at various concentrations, are shown in Table 3. The speed used for sedimentation analysis was 59,780 rpm. The buffer had a viscosity relative to water of 1.029 and a density of 1.022 g/cc at 20°C.

Table 3

Sedimentation and diffusion coefficients  
of Maleyl-PFK.

F (mg/ml)	$s_{20w}$	$D_{20w}$ ( $\text{cm}^2\text{sec}^{-1}$ )
2.9	3.82	$3.55 \times 10^{-7}$
3.8	3.84	$3.85 \times 10^{-7}$
5.0	3.72	$3.90 \times 10^{-7}$
6.7	3.81	$3.76 \times 10^{-7}$
8.0	3.80	-
10.0	3.64	-
13.0	3.81	$3.54 \times 10^{-7}$

Figure 6 shows a plot of the sedimentation rate of maleyl-phospho-fructokinase as a function of protein concentration. From this graph the value of the sedimentation rate at infinite dilution ( $s_{20w}^0$ ) was obtained as 3.80 S. Similarly, the  $D_{20w}^0$  value was determined as  $3.75 \times 10^{-7} \text{ cm}^2\text{sec}^{-1}$ . Using these values, together with a partial specific volume of 0.728 cc/gm, and the density of water at 20°C, in the Svedberg equation ( $MW = RT s_{20w}^0 / D_{20w}^0 (1 - \bar{v}\rho)$ ) the molecular weight of maleyl-PFK was determined as 90,000.

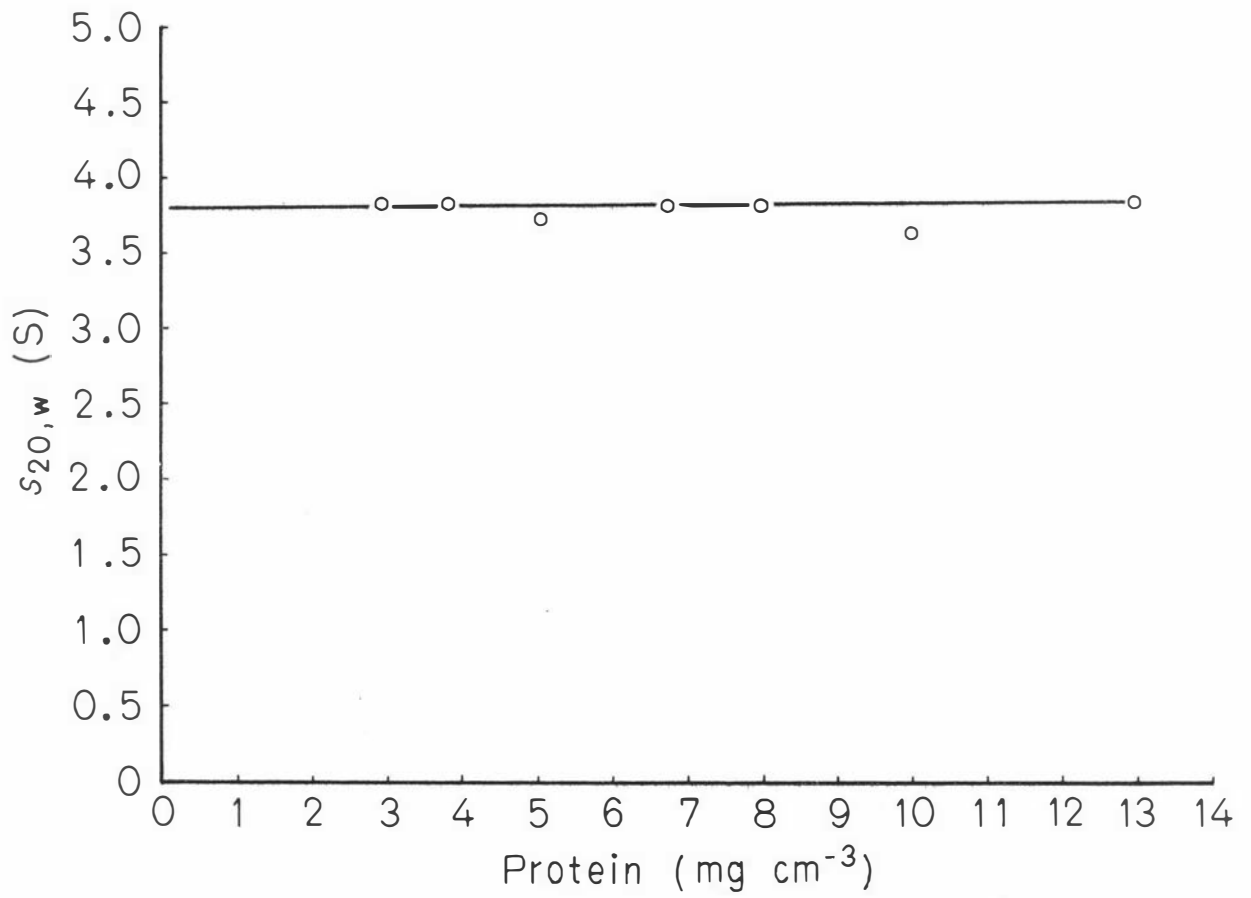


Figure 6 Concentration dependence of the sedimentation coefficient of Maleyl-PFK.

When the native enzyme (concentration = 3 mg/ml) was dialysed against 0.1 M phosphate buffer pH 8.0, containing 7.5 M urea and 10 mM dithioerythritol, and subjected to sedimentation analysis (59,780 rpm), a single 1.9 S boundary was observed. The solvent had a relative viscosity of 1.422 and a density of 1.1132 g/cc; using these figures, the  $s_{20w}$  value of the urea-dissociated enzyme was determined as 3.9 S.

BSA (MW 67,000), ovalbumin (MW 45,000), chymotrypsinogen (MW 25,000) and PFK were boiled in 10 mM phosphate buffer pH 7.0, containing 0.1% sodium dodecylsulphate and 0.1% 2-mercaptoethanol, and subjected to dodecylsulphate gel electrophoresis (Weber and Osborn, 1969). Log MW was plotted as a function of mobility with respect to bromophenol-blue (Fig.7). Three PFK samples each migrated as a single band with mobilities of 0.12, 0.12 and 0.14. These mobilities indicated a molecular weight of between 80,000 and 86,000 for the dissociated enzyme.

S-Carboxymethyl-phosphofructokinase was maleylated in 0.1 M borate buffer containing 7.5 M urea, and dialysed against 0.1 M phosphate buffer pH 8.0. On sedimentation analysis at 59,780 rpm, carboxymethyl-maleyl-PFK sedimented as a single symmetrical boundary with  $s_{20w}$  values of 2.25, 2.12 and 2.00 S at protein concentrations of 1.5, 3.0 and 4.5 mg/ml respectively. The synthetic boundary observed in diffusion experiments was gaussian, and diffusion coefficients of  $5.1$ ,  $4.9$  and  $4.5 \times 10^{-7}$  cm<sup>2</sup>/sec respectively were calculated at the above concentrations. Using the Svedberg equation, apparent molecular weights of 39,000, 39,000 and 40,000 were obtained for carboxymethyl-maleyl-PFK. This form of the enzyme migrated as a single band of mobility 1.0 on gel electrophoresis at pH 8.5 in a 5.6% gel.

### 3.6 N-terminal analysis

No N-terminal residue was detected for sheep heart PFK, although when N-terminal analysis was performed on myoglobin under the same condition, DNS-Val and DNS-Val-Leu were detected. The N-terminal sequence of sperm whale myoglobin is Val-Leu -, and the detection of DNS-Val-Leu on N-terminal analysis is a consequence of the resistance of Val-Leu bonds

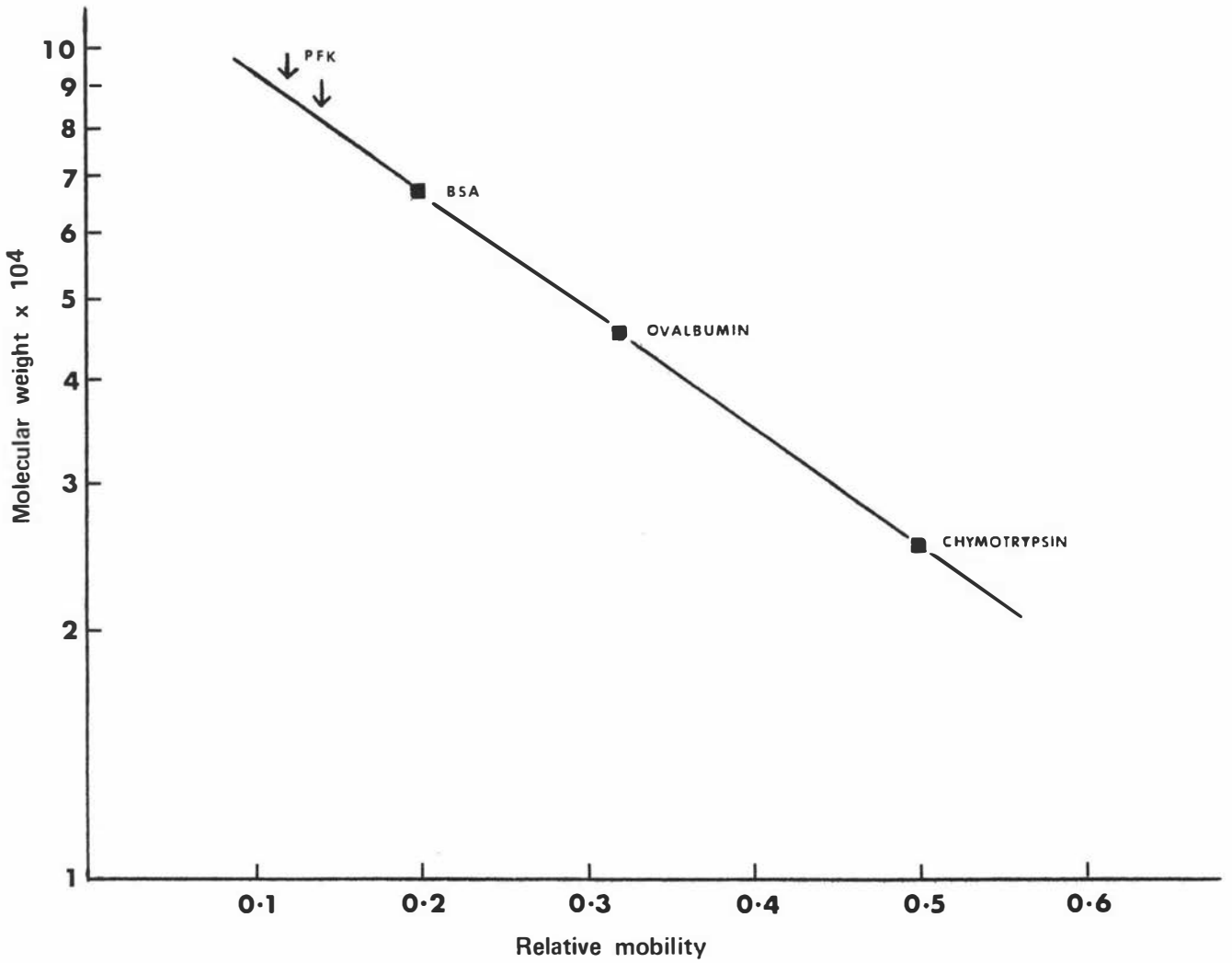


Figure 7 Graph for the determination of molecular weight of PFK by gel electrophoresis.

to acid hydrolysis. The failure to detect an N-terminal residue in sheep heart LFK is paralleled in rabbit muscle LFK,

3.7 Peptide maps

β-carboxymethyl-phosphofructokinase was digested with trypsin. Thirty mg of peptide material was subjected to peptide mapping (page 16). Composite maps showing the results of six detection procedures are presented in Figure 8A and B. Electrophoretic mobilities at pH 6.5 are expressed relative to aspartic acid, while at pH 2.1, mobilities are relative to dansyl-arginine. Chromatographic mobilities in the second dimension are relative to dansyl-arginine. Figure 8A shows the separated acidic and basic peptides, while figure 8B shows a map of the neutral peptides. Spots on the maps represent peptides detected with ninhydrin. The numeral 1 represents peptides detected with chlorine-O-tolidine; 2 represents peptides which stained with Sakaguchi reagent; 3 represents peptides which stained with Fauly Reagent; 4 represents peptides which stained with Ehrlichs Reagent; and 5 represents peptides detected with 1-nitroso-2-naphthol.

A total of 50 peptides were detected with ninhydrin; 44 of these also reacted with chlorine-O-tolidine. Twenty-seven peptides contained arginine, 9 contained histidine, 5 contained tryptophan and 5 contained tyrosine.

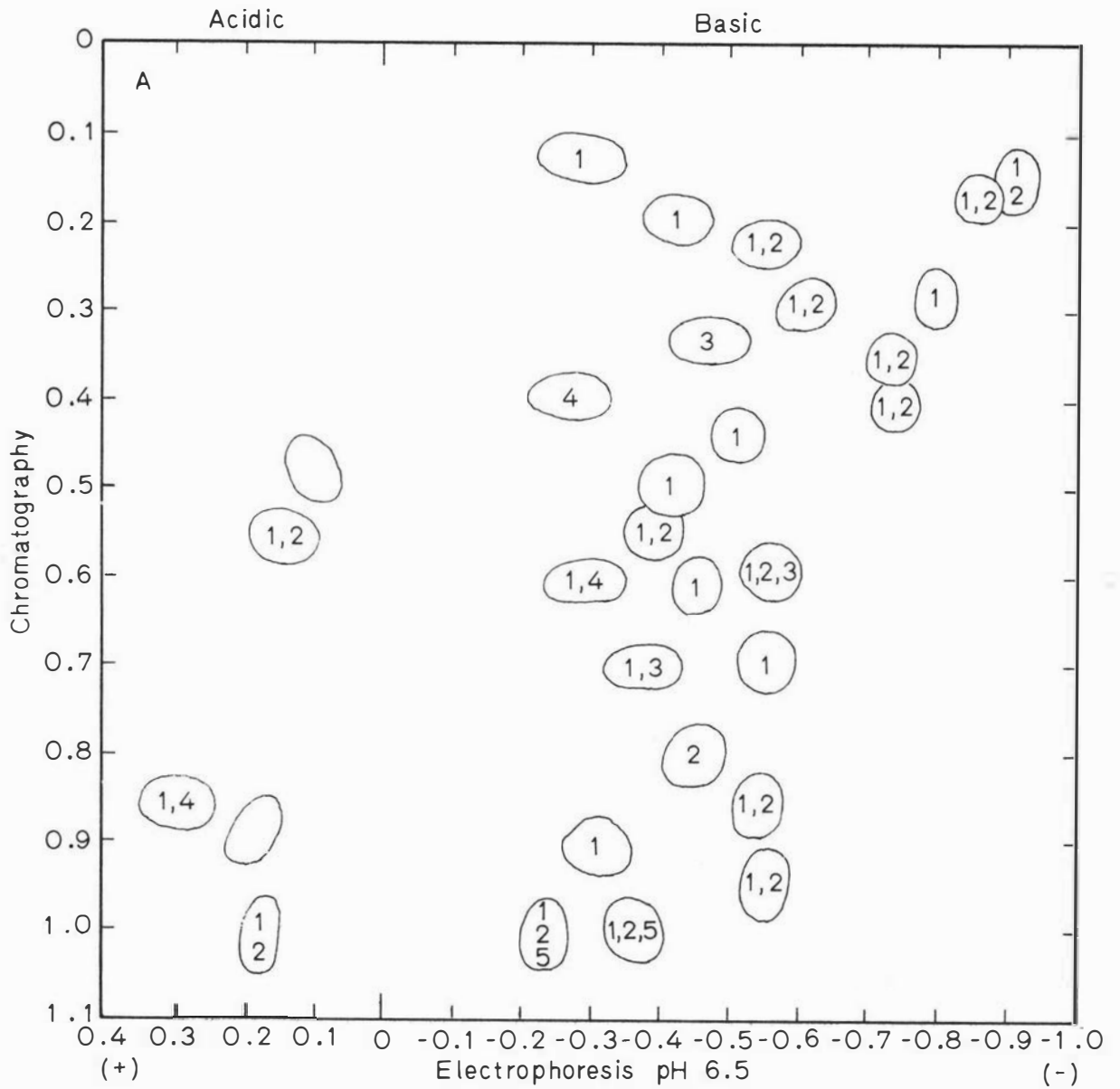


Figure 8A Peptide map of acidic and basic tryptic peptides of carboxymethyl-PFK.  
(see text for key)

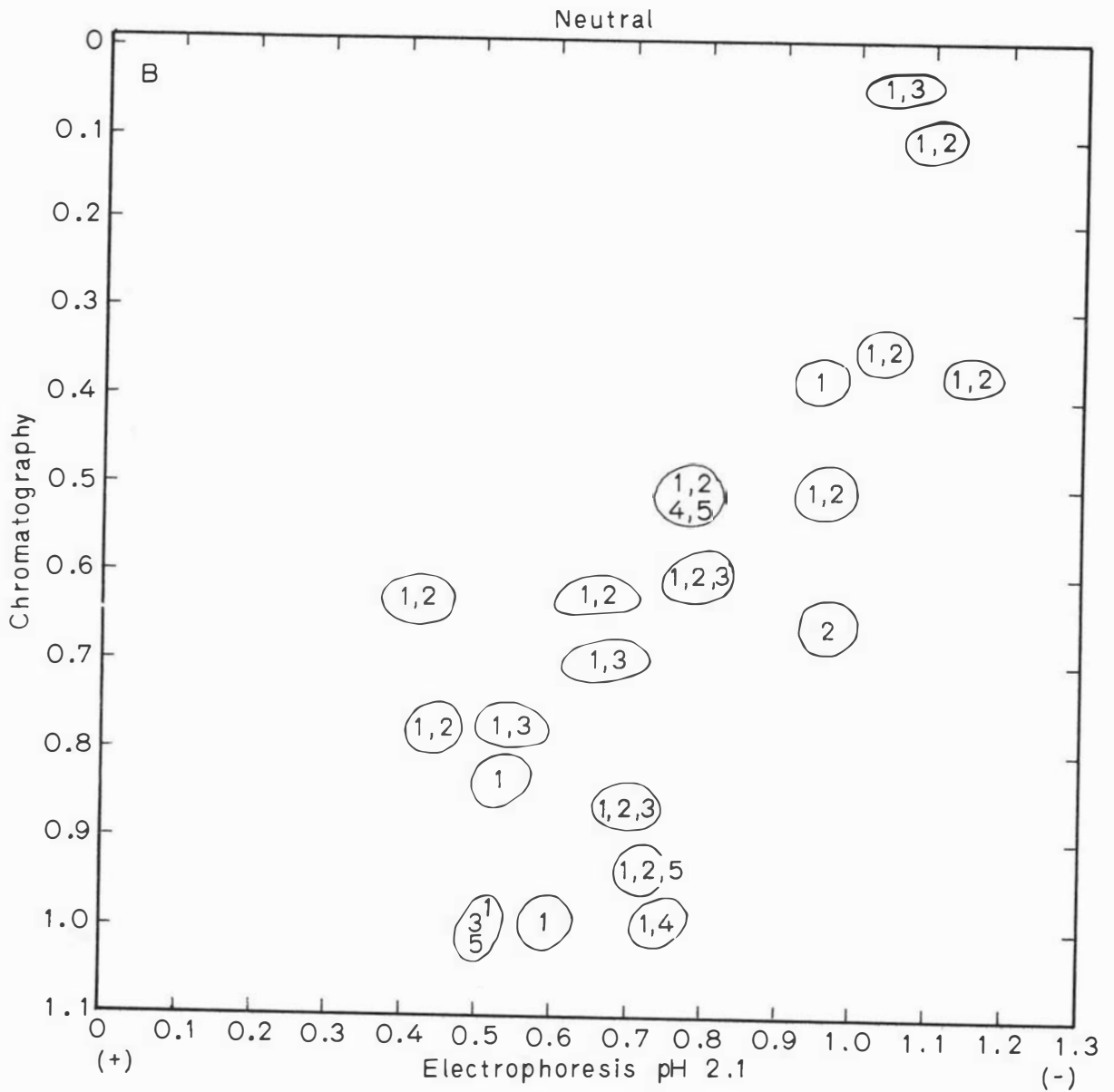


Figure 8B Peptide map of neutral tryptic peptides of carboxymethyl-PFK.  
(see text for key)

#### 4. RESULTS (2)

##### 4.1 Peptide separation, characterisation and amino acid sequence determination

Five hundred mg of carboxymethyl-phosphofructokinase (of which about a tenth was carboxymethylated using 2-C<sup>14</sup> iodoacetic acid) was digested with trypsin. The initially turbid dispersion rapidly cleared, and after two hours, digestion was complete. At this point, the pH of the reaction mixture was dropped to 4.0 and the acid-insoluble material removed by centrifugation. Both the supernatant and the pellet were freeze-dried.

The acid soluble tryptic peptides were dissolved in 7 ml of 0.2 M ammonium bicarbonate and applied to a 2.5 x 97 cm column of Sephadex G 50. The column was developed with 0.2 M ammonium bicarbonate using upward flow at 14.0 ml/hour. Thirty minute fractions were collected. Figure 9 shows the elution profile of peptides; optical densities were measured at 280 and 230 nm. Based on these results and on electrophoretic analysis of tubes 48 to 83, the following tubes were pooled and freeze-dried for further fractionation.

Tubes (58-76), designated fraction A;

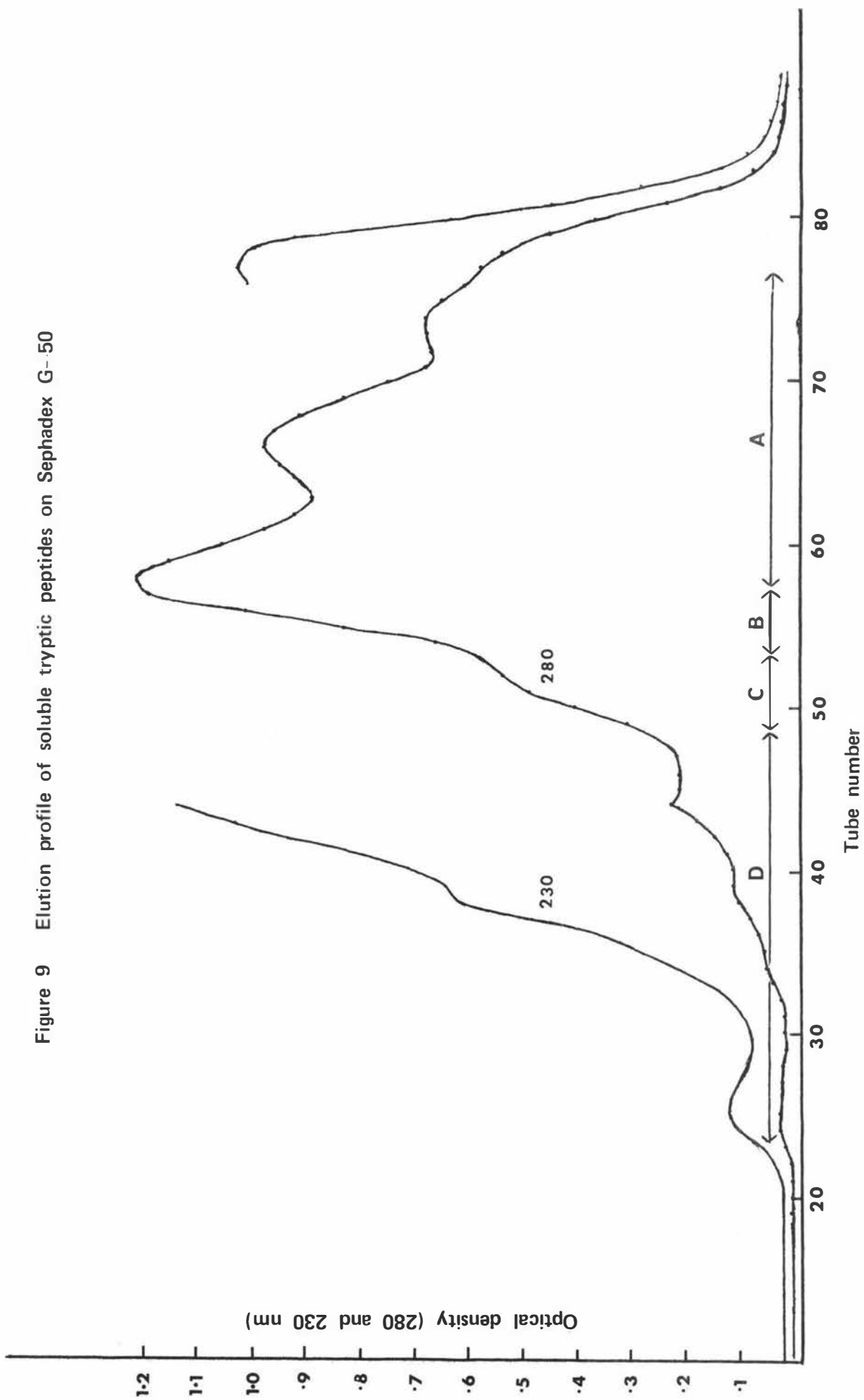
Tubes (54-57), designated fraction B;

Tubes (49-53), designated fraction C;

Tubes (23-48), designated fraction D;

Electrophoretic analysis indicated that the tubes 64 to 70 contained free arginine and lysine, implying that fraction A contains peptides which have been totally included in the Sephadex. Although Figure 9 indicates UV absorption occurring past tube No.76, electrophoresis and N-terminal analysis showed that this absorption was not due to peptide material.

Figure 9 Elution profile of soluble tryptic peptides on Sephadex G-50



## 4.2 Fraction A

Fraction A, representing peptides totally included in Sephadex G 50, was dissolved in 2 ml of 0.2 M pyridine/acetate buffer pH 3.0. The pH was adjusted to 2.5 with concentrated hydrochloric acid and the sample was applied, under nitrogen pressure, to a 0.9 x 50 cm column of Dowex 50 x 8 (22 spheres).

Peptides were eluted with 30 ml of 0.2 M pyridine/acetate buffer pH 3.0, followed by a gradient consisting of 200 ml of 0.2 M pyridine/acetate pH 3.0 and 400 ml of 2 M pyridine/acetate pH 5.0. The column was then developed with a gradient of 30 ml 2.0 M pyridine/acetate pH 5.0 and 30 ml of 3.5 M pyridine/acetate pH 5.6. Any peptides remaining absorbed on the column were removed by washing with 50 ml of the pH 5.6 buffer. The flow rate was maintained at 20 ml/hr using a Beckman Accu-Flo pump, and the column temperature was kept at 55°C using a circulating water pump. Fractions of about 5.3 ml were collected.

\* The column effluent was monitored by electrophoresis at pH 6.5 and pH 2.1. A 0.3 ml sample from each tube was freeze-dried, and dissolved in 20  $\mu$ l of M/50 ammonium hydroxide. Ten  $\mu$ l from consecutive samples were applied to each of two sheets of Whatman No.1 paper as a one - cm band. Electrophoresis was carried out as previously described. One set of elution profiles was stained with ninhydrin and the other with Sachaguchi Reagent. The column was also monitored for radioactivity by taking 5  $\mu$ l from each tube and spotting on Whatman No.1 paper, which was then subjected to autoradiography. Radioactive peaks occurred in tubes 43 and 58. In order to ascertain which peptides in these tubes were radioactive, the ninhydrin-positive spots from the above maps were cut out and counted in a scintillation counter. This indicated that peptides A 58 N 1.50, A(43-44).3 and A(43-44) N1.58 were radioactive (see below for numbering).

Figure 10A and B shows the superimposed results of the two staining procedures together with the radioautography results. Figure 10A displays the position of acidic and basic peptides

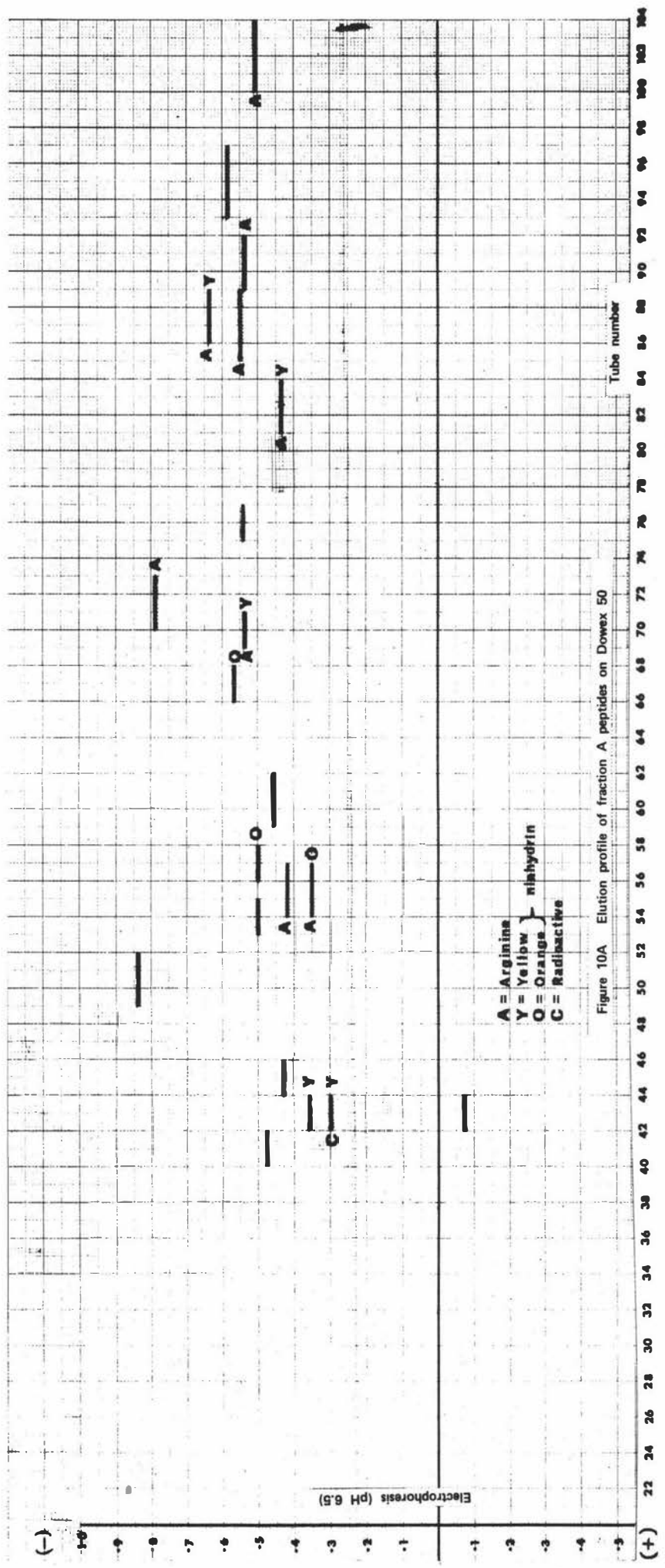


Figure 10A Elution profile of fraction A peptides on Dowex 50

A = Arginine  
 Y = Yellow ninhydrin  
 O = Orange ninhydrin  
 C = Radioactive

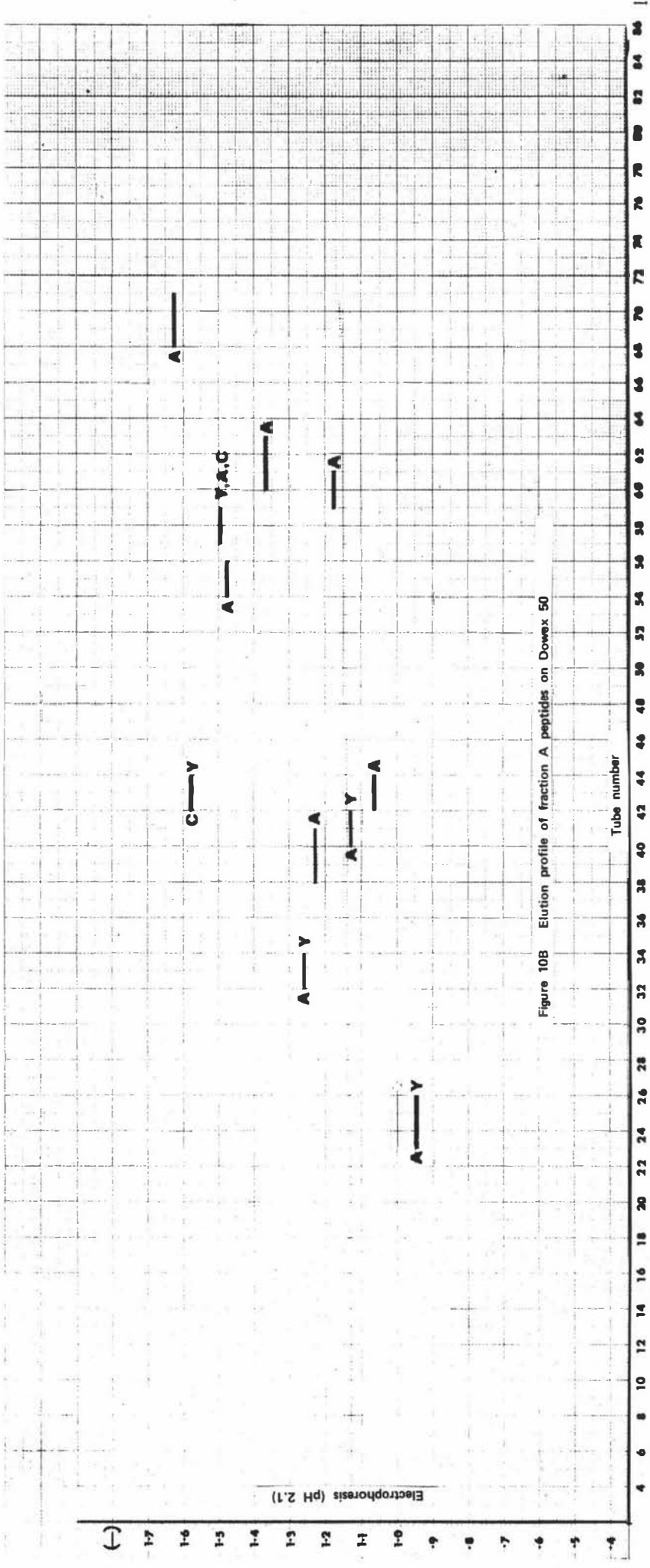


Figure 10B Elution profile of fraction A peptides on Dowex 50

with respect to their electrophoretic mobilities at pH 6.5 and their tube number. Figure B displays the same for the neutral peptides, except that the electrophoresis was carried out at pH 2.1. Although tube numbers do not start at one, no peptides were contained in the fractions which are not shown; further, no peptides were located beyond tube number 102. All the peptides from fraction A can be uniquely numbered by quoting their Dowex tube number, their electrophoretic mobility, and whether neutral or charged at pH 6.5. Thus the code, A(55-56) .35, represents a peptide which has been totally included in Sephadex (represented by A); has been further purified from a pooled fraction of tubes 55 and 56 inclusive, and has an electrophoretic mobility of 0.35 at pH 6.5. Similarly, the peptide numbered A(55-56).42 represents a peptide occurring in the same Sephadex and Dowex fractions, but with a mobility of 0.42 and pH 6.5. Peptide number A (55-56) N1.48 also occurs in the same Sephadex and Dowex fractions, but is neutral at pH 6.5 (N) and has a mobility of 1.48 at pH 2.1.

The method of further purification of peptides, numbered in this manner, is described below, together with other relevant data on each peptide, its amino acid analysis and sequence.

The following comments apply to peptides in this and all other fractions.

1. Unless stated to the contrary, all purified peptides were finally dissolved in 500  $\mu$ l of M/50 ammonium hydroxide.
2. Figures quoted under amino acid analyses are in n mol and the sample size represents one-twentieth of the total purified peptide.
3. The implied molecular weight was calculated from the peptide's electrophoretic mobility using Offord Diagrams (Offord, 1966).
4. The residue molecular weight was calculated from the amino acid sequence of the peptide.

5. Four letters (Y, O, R and S) are used to characterise each peptide's reaction with ninhydrin:

Y, indicates a yellow spot. Peptides with an N-terminal glycine, asparagine or carboxymethyl-cysteine give a yellow colour;

O, indicates that the peptide stained orange. N-terminal serine residues give this colour;

R, this stands for the reddish-violet colour usually associated with the ninhydrin reaction;

S, this refers to a peptide which reacts only slowly to give the red-violet colour.

Peptides with N-terminal leucine, isoleucine or valine react in this manner.

6. Unless otherwise stated, quoted amino acid sequences were determined by the Dansyl-Edman Method. The symbol N/A, in brackets after a sequence, indicates that it was deduced from N-terminal analyses and amino acid analyses.

7. The number of acidic residues present in a peptide as the amide, was calculated, either from its pH 6.5 mobility and residue weight, or its pH 6.5 and pH 2.1 mobility.

8. The abbreviation Ep (6.5) stands for a peptide's electrophoretic mobility at pH 6.5. This mobility is expressed relative to the distance between aspartic acid and the true origin (the position occupied by the neutral amino acids).

9. The abbreviation Ep (2.1) stands for a peptide's electrophoretic mobility at pH 2.1. Here mobility is expressed relative to the distance moved by serine from the true origin (the position occupied by dansyl-sulphonic acid).

PEPTIDE A(87-88) .64

Further purification: Prep. electrophoresis pH 6.5; eluted  
M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
Mobility: Ep (6.5) 0.64. Implied MW = 260.  
Ninhydrin: Y.  
Arginine: +  
N-terminal: Gly.  
Analysis: Arg (45), Gly (50).  
SEQUENCE: Gly-Arg. (N/A).  
Residue MW: 231.

PEPTIDE A(55-56) .42

Further purification: Prep. electrophoresis pH 6.5; eluted  
M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
Mobility: Ep (6.5) 0.42. Implied MW = 480.  
Ninhydrin: R.  
Arginine: +.  
N-terminal: Ala.  
Analysis: Arg (80), Ala (77), Val (155).  
SEQUENCE: Ala-Val-Val-Arg. (N/A).  
Residue MW: 443.  
This sequence was confirmed by mass spectrometry.

PEPTIDE A (55-56) .35

Further purification: Prep. electrophoresis pH 6.5; eluted  
M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
Mobility: Ep (6.5) 0.35. Implied MW = 610.  
Ninhydrin: O.  
Arginine: +  
N-terminal: Ser.  
Analysis: Arg (80), Thr (81), Ser (80), Val (91).  
SEQUENCE: Ser-Thr-Val-Arg.  
Residue MW: 453.

PEPTIDE A(55-56) HI.48

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1;  
 eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0; Ep (2.1) 1.48. Implied MW = 360.  
 Ninhydrin: R.  
 Arginine: +.  
 N-terminal: Glu.  
 Analysis: Arg (80), Glu (79), Gly (80).  
 No.amides: 0.  
 SEQUENCE: Glu-Gly-Arg. (N/A).  
 Residue MW: 360.

PEPTIDE A 67 .57

Further purification: Prep electrophoresis pH 6.5; eluted  
 M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0.57. Implied MW = 310.  
 Ninhydrin: 0.  
 Arginine: -.  
 N-terminal: Ser.  
 Analysis: Lys (86), Ser (70).  
 SEQUENCE: Ser-Lys. (N/A).  
 Residue MW: 233.

PEPTIDE A 54 .5

Further purification: Pre. electrophoresis pH 6.5; eluted  
 M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0.50. Implied MW = 370.  
 Ninhydrin: S.  
 Arginine: -.  
 N-terminal: Ile.  
 Analysis: Lys (74), Pro (79), Ile (68).  
 SEQUENCE: Ile-Pro-Lys. (N/A).  
 Residue MW: 356.

PEPTIDE A (83-84) .43

Further purification: Prep. electrophoresis pH 6.5; eluted  
 M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0.43. Implied MW = 460.  
 Ninhydrin: Y.

Arginine: +.  
 N-terminal: Asn.  
 Analysis: Arg (63), Asp (64).  
 No.amides: 1.  
 SEQUENCE: Asn-Arg. (N/A).  
 Residue MW: 286.

PEPTIDE A (43-44) .36

Further purification: Prep. electrophoresis pH 6.5, eluted  
 M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0.36. Implied MW = 600.  
 Ninhydrin: Y.  
 Arginine: -.  
 N-terminal: Asn.  
 Analysis: Lys (91), Asp (76), Thr (78), Ala (87), Phe (75).  
 No.amides: 1.  
 SEQUENCE: Asn-Phe-Ala-Thr-Lys.  
 Residue MW: 574.

The above sequence, which was determined by the Dansyl Edman procedure, was confirmed by mass spectrometry. The masses of the observed peaks and their structure is described below:

M/e	Structure
119	$\begin{array}{c} \text{Me} \\   \\ \text{Ac} - \text{Asn} \\   \\ \text{Me}_2 \end{array}$
360	$\begin{array}{c} \text{Me} \quad \text{Me} \\   \quad   \\ \text{Ac} - \text{Asn} - \text{Phe} \\   \\ \text{Me}_2 \end{array}$
445	$\begin{array}{c} \text{Me} \quad \text{Me} \quad \text{Me} \\   \quad   \quad   \\ \text{Ac} - \text{Asn} - \text{Phe} - \text{Ala} \\   \\ \text{Me}_2 \end{array}$
574	$\begin{array}{c} \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \\   \quad   \quad   \quad   \\ \text{Ac} - \text{Asn} - \text{Phe} - \text{Ala} - \text{Thr} \\   \quad \quad \quad   \\ \text{Me}_2 \quad \quad \quad \text{Me} \end{array}$
803	$\begin{array}{c} \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \\   \quad   \quad   \quad   \quad   \\ \text{Ac} - \text{Asn} - \text{Phe} - \text{Ala} - \text{Thr} - \text{Lys} - \text{OMe} \\   \quad \quad \quad   \quad \quad \quad   \quad \quad \quad   \\ \text{Me}_2 \quad \quad \quad \text{Me} \quad \text{Me} \quad \text{Ac} \end{array}$

Other peaks occurred at: Me = 171, corresponding to loss of CO from 119; at M/e = 542, corresponding to loss of MeOH from 574; and at 471, corresponding to  $\overset{\dagger}{O}=\overset{\dagger}{C}$ -Ala-Thr-Lys-OMe. (Masses of the amino acid fragments obtained on mass spectrometry of peptides have been described by Morris and Williams (1971)).

PEPTIDE A 57 .5

Further purification: Prep electrophoresis pH 6.5, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.5. Implied MW = 370.

Ninhydrin: O.

Arginine: -.

N-terminal: Ser.

Analysis: Lys (93), Ser (92), Gly (102).

SEQUENCE: Ser-Gly-Lys. (N/A).

Residue MW: 290.

PEPTIDE A (70-71)N 1.63

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0; Ep (2.1) 1.63. Implied MW = 290.

Ninhydrin: R.

Arginine: +.

N-terminal: Glu.

Analysis: Arg (196), Glu (191).

No.amides: O.

SEQUENCE: Glu-Arg. (N/A)

Residue MW: 303.

PEPTIDE A (76-77) .54

Further purification: Prep. electrophoresis pH 6.5, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) .54. Implied MW = 330.

Ninhydrin: S.

Arginine: -.

N-terminal: Leu.

Analysis: Lys (208), Leu (200).

SEQUENCE: Leu-Lys. (N/A).

Residue MW: 259.

## PEPTIDE A (43-44) N 1.07

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50  $\text{NH}_4\text{OH}$  redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .  
 Mobility: Ep (6.5) 0; Ep (2.1) 1.07. Implied MW = 730.  
 Ninhydrin: R.  
 Arginine: +.  
 N-terminal: Leu.  
 Analysis: Arg (116), Asp (125), Thr (120), Gly (130),  
 Leu (118), Tyr (112).  
 No.amides: 0.

N-terminal analyses on this peptide indicated the presence of DNS-Leu and DNS-O-Tyr. No second residue was detected, though DNS-O-Tyr was present. The third residue showed up as DNS-Bis-Tyr. As the Dansyl-Edman hydrolysis tubes were sealed under vacuum, this implies that the second residue was Trp. The peptide was previously known to contain a tryptophan residue as it reacted positively with Ehrlich Reagent.

SEQUENCE: Leu-Trp-Tyr-Asp-Thr-Gly-Arg.

Residue MW: 909.

## PEPTIDE A (41-42) N 1.13

Further purification: Prep. electrophoresis pH 6.5 and 2.1, eluted M/50  $\text{NH}_4\text{OH}$  redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .  
 Mobility: Ep (6.5) 0; Ep (2.1) 1.1. Implied MW = 670.  
 Ninhydrin: Y.  
 Arginine: +.  
 N-terminal: Gly.  
 Analysis: Arg (74), Asp (62), Ser (63), Gly (72), Ile (75),  
 Phe (69).  
 No.amides: 0.  
 SEQUENCE: Gly-Ile-Phe-Asp-Ser-Arg.  
 Residue MW: 693.

## PEPTIDE A 39 N 1.23

Further purified: Prep. electrophoresis pH 6.5 and 2.1, eluted M/50  $\text{NH}_4\text{OH}$  redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .  
 Mobility: Ep (6.5) 0; Ep (2.1) 1.23. Implied MW = 540.  
 Ninhydrin: S.

Arginine: +.

N-terminal: Leu.

Analysis: Arg (41), Thr (41), Ser (44), Glu (48), Leu (44).

No.amides: 0.

SEQUENCE: Leu-Ser-Glu-Thr-Arg.

Residue: 604.

PEPTIDE A (60-63) .46

Further purification: Prep. electrophoresis pH 6.5, eluted  
1/50 NH<sub>4</sub>OH redissolved in 0.5 ml 1/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.46. Implied MW = 420.

Ninhydrin: R.

Arginine: -.

N-terminal: Ala.

Analysis: Lys (70), His (63), Asp (70), Ala (120), Val (72),  
Leu (68).

No.amides: 1.

SEQUENCE: Ala-Ala-His-Asn-Leu-Val-Lys.

Residue MW: 752.

The reason the actual molecular weight is greater than the value implied from the electrophoretic mobility, is because the peptide contains a histidine residue, which confers on it an additional 'half charge' at pH 6.5. Further, if this peptide contained aspartic acid as opposed to asparagine, it should have a mobility of 0.16 at pH 6.5. As the peptide has a mobility of 0.46 at this pH, this implies that the aspartic residue is present as asparagine.

The second alanine residue in this peptide showed up only weakly on Dansyl Edman treatment. Figure 11 shows the fragment ions that were observed on mass spectrometry, together with a rationalisation of the derivation of the ions. The molecular ion was not detected, as the peptide underwent an internal elimination at the asparagine residue.

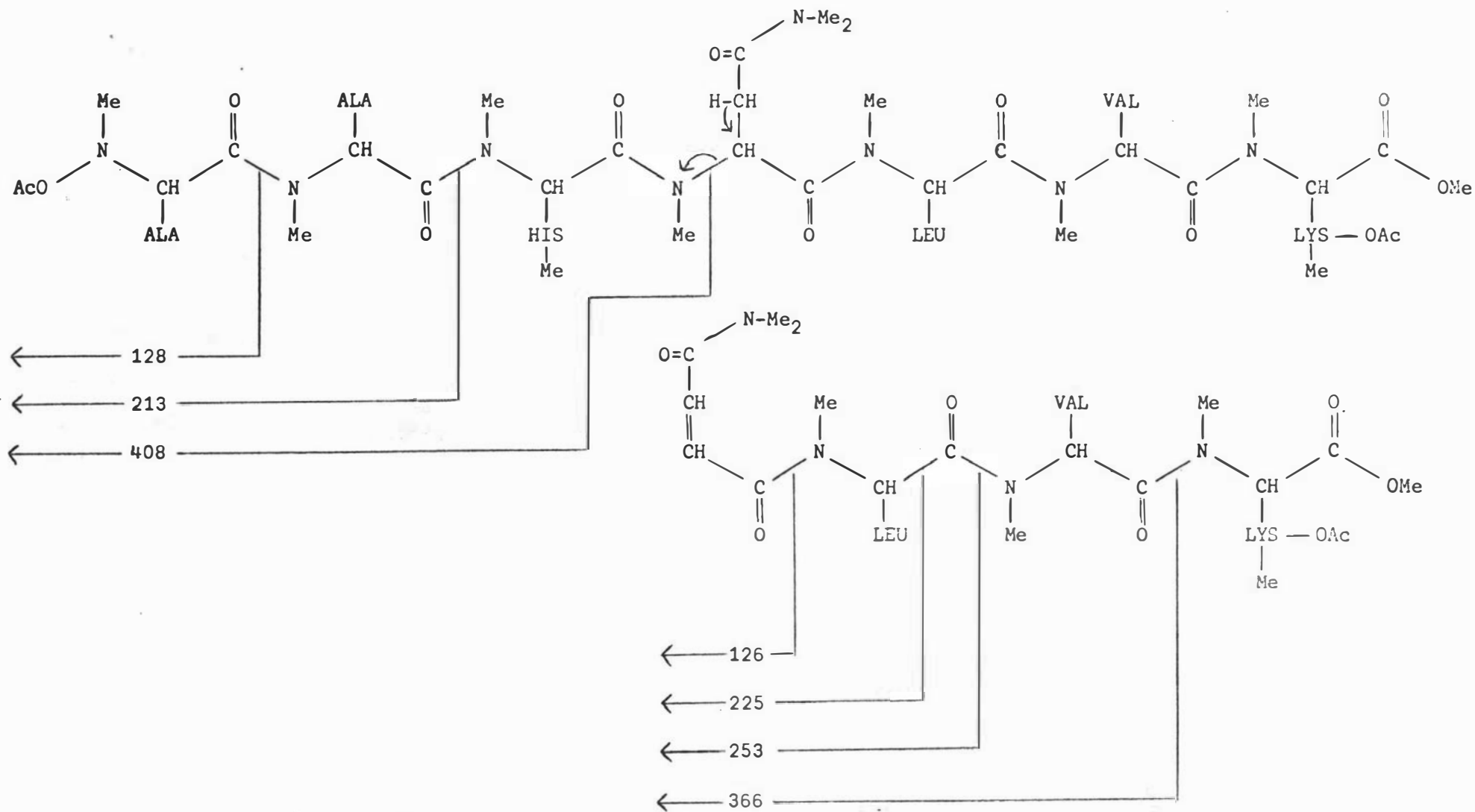


FIG. 11 FRAGMENTS OBSERVED ON MASS SPECTROMETRY OF PEPTIDE A[60,63].46

PEPTIDE A (41-42) .48

Further purification: Prep. electrophoresis pH 6.5, eluted  
M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.48. Implied MW = 380.

Ninhydrin: R.

Arginine: -.

N-terminal: Thr.

Analysis: Lys (55), Thr (95), Val (52).

SEQUENCE: Thr-Thr-Val-Lys.

Residue MW: 347.

PEPTIDE A (101-105) .51

Further purification: Prep. electrophoresis pH 6.5; eluted  
M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.51. Implied MW = 360.

Ninhydrin: S.

Arginine: +.

N-terminal: Leu.

Analysis: Arg (180), Leu (172).

SEQUENCE: Leu-Arg (N/A).

Residue MW: 287.

PEPTIDE A 33 N 1.26

Further purification: Prep. electrophoresis pH 6.5 and  
pH 2.1, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0; Ep (2.1) 1.26. Implied MW = 500.

Ninhydrin: Y.

Arginine: +.

N-terminal: Asp.

Analysis: Arg (95), Asp (96), Thr (93), Val (97).

No.amides: 0.

SEQUENCE: Asp-Val-Thr-Arg.

Residue MW: 489.

## PEPTIDE A (70-71) .54

Further purification: Prep. electrophoresis pH 6.5, eluted  
M/50  $\text{NH}_4\text{OH}$ , redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .

Mobility: Ep (6.5) .54. Implied MW = 330.

Ninhydrin: Y.

Arginine: +.

N-terminal: Gly.

Analysis: Arg (55), Ser (55), Gly (52).

SEQUENCE: Gly-Ser-Arg.

Residue MW: 318.

## PEPTIDE A 58 N 1.50

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1,  
eluted M/50  $\text{NH}_4\text{OH}$  redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .

Mobility: Ep (6.5) 0; Ep (2.1) 1.50.

Ninhydrin: Y.

Arginine: +.

N-terminal: Cys.

Radioactivity: 150 cpm per 10  $\mu\text{l}$ .

Analysis: Arg (22), Lys (21), Asp/CM-Cys (40), Phe (20).

Specific activity: 18 cpm per nm.

SEQUENCE: Cys-Lys-Asp-Phe-Arg.

Residue MW: 725.

No.amides: 0.

The cysteine residue at position one was detected as DNS-Carboxymethyl-cysteine. This peptide was neutral at pH 6.5 and as it contains both Arg and Lys, it must also contain two 'free-acid' side chains. One of these is provided by the CM-Cys residue and the other by the Asp residue, which has to be present as aspartic acid as opposed to asparagine.

The peptide will have a charge of +3 at pH 2.1; this, together with a mobility of 1.50, implies a molecular weight of 750, which is in good agreement with the residue weight of 725.

This peptide contains an internal lysine residue, which is unusual for a tryptic peptide. The rate of tryptic cleavage is, however, decreased when the basic residue is next to an

acidic side chain. Here there are two acidic residues adjacent to the lysine, resulting in partial cleavage of the -Lys-Asp- bond (see peptide A (43-44) N 1.58 below).

PEPTIDE A (43-44) N 1.58

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0; Ep (2.1) 1.58. Implied MW = 310.

Ninhydrin: Y.

Arginine: -.

N-terminal: Cys.

Radioactivity: 140 cpm/10  $\mu$ l.

SEQUENCE: Cys-Lys.

Residue MW: 327.

This peptide results from partial cleavage, by trypsin, of the -Lys-Asp- bond of peptide A 58 N 1.50.

PEPTIDE A (43-44) .3

Further purification: Prep. electrophoresis pH 6.5, eluted M/50 NH<sub>4</sub>O, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.24. Implied MW = 790.

Ninhydrin: Y.

Arginine: -.

N-terminal: No N-terminal detected.

Radioactivity: 85 cpm per 10  $\mu$ l.

Analysis: Lys (40), Gly (38), Ala (43). (See below also).

Specific activity: 5 cpm per nm.

SEQUENCE: X-Gly-Ala-Lys.

The N-terminal residue (X) of this peptide did not contain a blocked amino group, as it was possible to determine the sequence past the first residue. X was not tryptophan, as the peptide did not react with Ehrlich Reagent.

As mentioned above, this peptide was radioactive; as it contains no carboxymethyl-cysteine, the radioactivity must be coming from another carboxymethylated amino acid. In addition to the above amino acid analysis figures, the

peptide also contained 12 nm of methionine, 2 nm of homo-serine lactone and 14 nm of s-carboxymethyl-homocysteine. These compounds all represent breakdown products of carboxymethyl-methionine under acid hydrolysis.

It is concluded that the sequence of peptide A (43-44) .3 is Met-Gly-Ala-Lys. This gives a residue molecular weight of 464, which differs markedly from the implied value of 790 based on a mobility of 0.3. The lack of correlation between mobility and residue weight is probably a consequence of the presence of a sulphonium ion in this peptide.

PEPTIDE A (24-26) N 0.95

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0; Ep (2.1) 0.95. Implied MW = 950.

Ninhydrin: Y.

Arginine: +.

N-terminal: Gly.

Analysis: Arg (71), Asp (75), Thr (67), Ser (70), Pro (64), Gly (146), Ala (71), Phe (65).

No.amides: 0.

SEQUENCE: Gly-Gly-Thr-Pro-Ser-Ala-Phe-Asp-Arg.

Residue MW: 906.

PEPTIDE A (87-88) .55

Further purification: Prep. electrophoresis pH 6.5 and prep. chromatography (BAW), eluted M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.

Mobility: Ep (6.5) 0.55; Ep (2.1) 1.56.

Ninhydrin: S.

Arginine: +.

N-terminal: Leu.

Analysis: Lys (50), Arg (51), Pro (49), Ile (48), Leu (95).

SEQUENCE: Leu-Arg-Pro-Ile-Leu-Lys.

Residue MW: 738.

During Dansyl-Edman analysis, the third residue was detected as a faint DNS-Pro spot after a 16 hour hydrolysis. When

the hydrolysis was run for 4 hours, two spots were detected; one was DNS-Pro (80%), and the other was tentatively identified as the dipeptide DNS-Pro-Ile (20%). The identity of this spot was confirmed when the fourth residue was detected as DNS-Ile.

Assuming a net charge of +2, the pH 6.5 mobility of 0.55 implies a molecular weight of 850. At pH 2.1, the peptide will carry a net charge of +3; this together with a mobility of 1.56, implies a molecular weight of 700. These values are in agreement with a residue weight of 738.

This peptide contains an internal arginine residue; this is due to the slow rate of tryptic cleavage of -Arg-Pro- and -Lys-Pro- bonds.

PEPTIDE A (90-92) .54

Further purification: Prep. electrophoresis pH 6.5, eluted M/50  $\text{NH}_4\text{OH}$ , redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .

Mobility: Ep (6.5) 0.54; Ep (2.1) 1.56.

Ninhydrin: S.

Arginine: +.

N-terminal: Leu. (DNS-Leu-Leu was also present).

Analysis: Lys (41), His (33), Arg (46), Pro (68), Ala (38), Val (34), Leu (71).

SEQUENCE: Leu-Leu-Ala-His-Val-Arg-Pro-Pro-Lys.

Residue MW: 1,029.

At pH 6.5, this peptide carries a net charge of +2.5, implying a molecular weight of 1,150. This value is consistent with a charge of +4, and a mobility of 1.56 at pH 2.1.

A (60-64) N 1.18

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50  $\text{NH}_4\text{OH}$ , redissolved in 0.5 ml M/50  $\text{NH}_4\text{OH}$ .

Mobility: Ep (6.5) 0; Ep (2.1) 1.18. Implied MW = 590.

Ninhydrin: R.

Arginine: +.

N-terminal: Glu.

Analysis: Arg (41), Ser (44), Glu (47), Tyr (42).  
 No. amides: 0.  
 SEQUENCE: Glu-Ser-Tyr-Arg.  
 Residue MW: 553.

A (45-46) .43

Further purification: Prep. electrophoresis pH 6.5, eluted  
 M/50 NH<sub>4</sub>OH, redissolved in 0.5 ml M/50 NH<sub>4</sub>OH.  
 Mobility: Ep (6.5) 0.43. Implied MW = 470.  
 Ninhydrin: R.  
 Arginine: -.  
 N-terminal: Leu.  
 Analysis: Lys (38), Gly (40), Val (31), Leu (33).  
 SEQUENCE: Leu-Gly-Val-Lys.  
 Residue MW: 415.

A (70-71) .78

This peptide was isolated by electrophoresis and was found to be free arginine.

A (50-51) .84.

This peptide was isolated by electrophoresis and was found to be free lysine.

PEPTIDE A (60-63) R 1.37

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50  $\text{NH}_4\text{OH}$ , redissolved in 0.5 ml M/50

$\text{NH}_4\text{OH}$

Mobility: Ep (6.5) 0; Ep (2.1) 1.27.

Ninhydrin: R.

Arginine: +.

N-terminal: Arg.

Analysis: Lys (27), Arg (26), Asp (27), Glu (28), Ala (27), Met (24), Phe (25).

No.amides: 0.

SEQUENCE: Arg-Phe-Asp-Glu-Ala-Met-Lys.

Residue MW: 895.

This peptide will carry a net charge of +3 at pH 2.1; its mobility therefore implies a molecular weight of 900.

PEPTIDE A (43-44) -.07

Further purification: Prep. electrophoresis pH 6.5 and pH 2.1, eluted M/50  $\text{NH}_4\text{OH}$ , redissolved 500 ml  $\text{NH}_4\text{OH}$ .

Mobility: Ep (6.5) -.07; Ep (2.1) 1.60.

Ninhydrin: Very faint reaction.

Arginine: -.

N-terminal: No residue detected, although DNS- $\xi$ -Lys observed.

Analysis: Lys Thr Glu Ala His

nm: 43 34 102 34 104

Ratio: 1.26 1.00 3.00 1.00 3.06 (With Thr/Ala =1).

Ratio: 1.00 0.79 2.37 0.79 2.42 (With Lys=1).

Two attempts were made to sequence this peptide by Dansyl-Edman treatment. Both of these proved unsuccessful;

however, DNS- $\epsilon$ -Lys was observed on each Dansyl-Edman cycle. It appears, therefore, that this peptide contains a blocked N-terminal residue, probably a pyroglutamic acid residue.

Two hundred and eighty nm of this peptide was N-acetylated, permethylated and prepared for mass spectrometry as described previously. No peptide signals were detected. This was possibly due to a quaternisation of some or all of the histidine residues, which would result in the exclusion of the charged peptide from the final chloroform phase, which was applied to the mass spectrometer.

Two sets of amino acid analysis ratios are shown above; the first set of ratios derived from assigning alanine a value of unity, appears to give a more reasonable result. Such a peptide would have a sequence/composition of Pyroglu-(Glx, Glx, His, His, His, Thr, Ala)-Lys. If one or more of the glutamic acid residues here were present as the amide, then this peptide would be basic at pH 6.5. As this peptide was observed to be acidic, it must contain either three free glutamic acid side chains or two free acid side chains and one pyroglutamyl residue.

The above proposed peptide would have a residue weight of 1,098, and a charge of +4 at pH 2.1. The pH 2.1 mobility of 1.60 is consistent with this data.

As it was not possible to determine the sequence of this peptide by Dansyl-Edman analysis or by mass spectrometry, the remaining peptide material was subjected to a 12 hour digestion with thermolysin. The digest was subjected to duplicate analytical electrophoresis at pH 6.5; one strip was stained with ninhydrin, and the other was stained for histidine. Two faint peptides were detected with ninhydrin; one was basic with a mobility of 0.67 and the other, which was acidic, also stained for histidine and had a mobility of -0.09. These peptides were then separated by preparative electrophoresis.

The basic peptide had an amino acid sequence of Ala-Lys and was in very low yield. Amino acid analysis on the acidic

peptide indicated ratios of: Ala (1.00), Thr (1.00), Glu (3.30), His (3.03) and Lys (1.16). Clearly, this peptide represents undigested starting material.

The recovered peptide material was subjected to digestion with pepsin; however, as judged by electrophoresis, no digestion took place. The remaining peptide material was digested with subtilisin, and although electrophoresis indicated that digestion had occurred, there was insufficient of the peptide left to allow preparative separation of the sub-peptides.

#### 4.3 Fraction B

Fraction B, representing Sephadex tubes (54-57) was dissolved in 1 ml of M/25 ammonium hydroxide. A 15  $\mu$ l sample was subjected to electrophoretic analysis at pH 6.5 in the first dimension and at pH 2.1 in the second dimension; staining with ninhydrin indicated the presence of 3 basic, 4 acidic and 1 neutral peptide in this fraction. Liquid scintillation counting showed that two of these peptides were radioactive. A second 15  $\mu$ l sample was electrophoresed as described above and stained with Sachaguchi Reagent; the combined results of these detection procedures is shown in Figure 12.

The separation obtained (Fig.12) suggested that this fraction would be amenable to further purification by preparative electrophoresis; it was therefore applied as a 35 cm band across a sheet of Whatman 3 MM paper and subjected to electrophoresis at pH 6.5. Edge marker strips were stained with ninhydrin. The separation achieved was the same as that for the first dimension of Fig.12. As in fraction A, peptides in this fraction are numbered according to their Sephadex fraction letter and their electrophoretic mobility at pH 6.5.

The basic peptides B .55, B .25 and B .05 were eluted directly from the preparative pH 6.5 electrophoresis run with M/25 ammonium hydroxide. The neutral peptide B N1.10 was treated similarly after additional electrophoresis at pH 2.1. The peptides were then freeze-dried and dissolved in 0.5 ml of M/25 ammonium hydroxide.

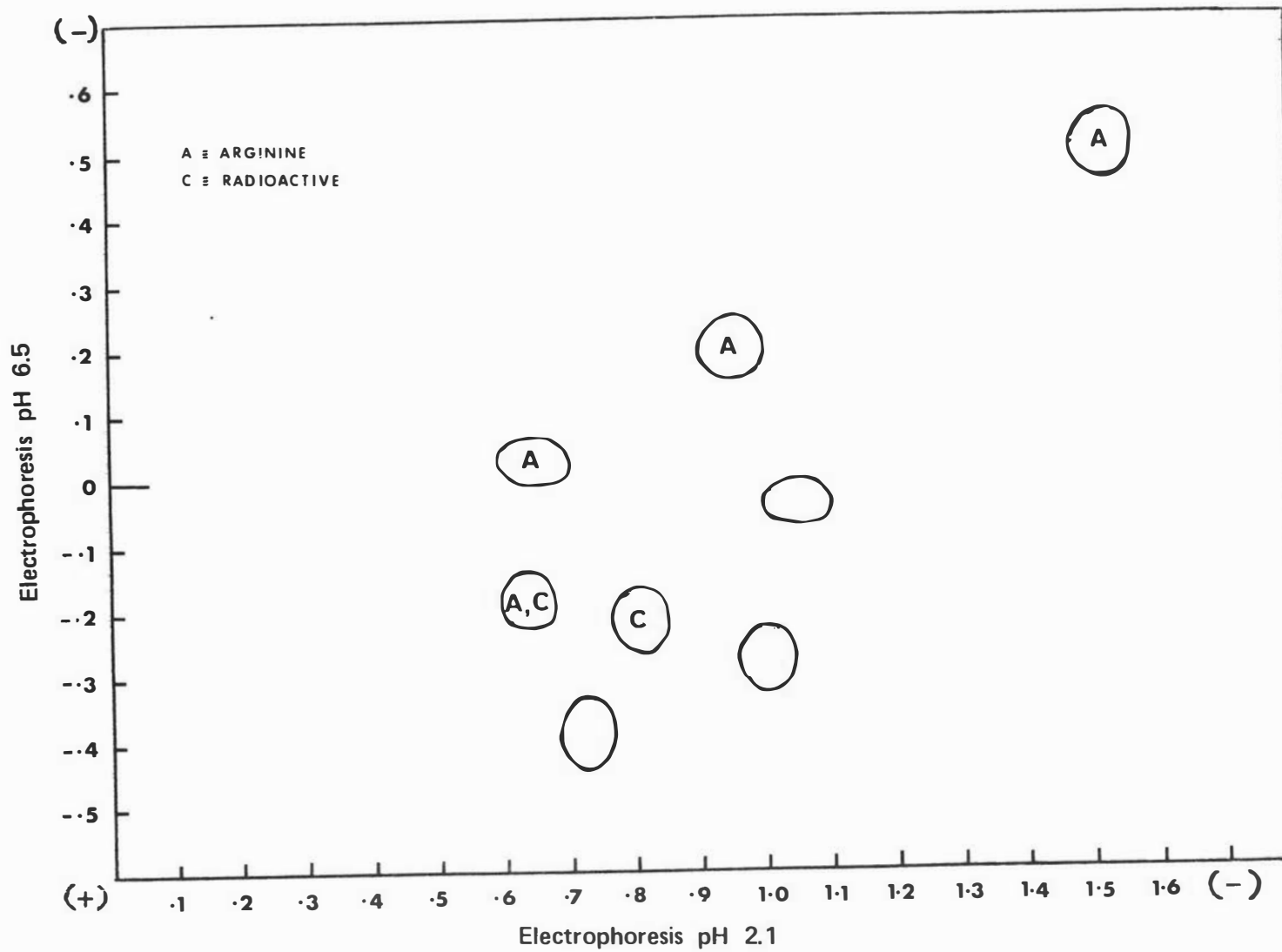


Figure 12 Peptide map of fraction B peptides

The preparative pH 6.5 band corresponding to the acidic peptide B -.44 was cut out and rerun preparatively at pH 2.1; as expected, a single peptide was obtained. The preparative pH 6.5 band, B -.33, was also rerun preparatively at pH 2.1; this gave rise to two peptides. The major component was nonradioactive and corresponded to peptide B -.33, while the minor radioactive component corresponded to peptide B -.26. When the preparative pH 6.5 strip corresponding to band B -.23 was rerun at pH 2.1, two radioactive peptides were obtained in equal amounts; these corresponded to peptides B -.23 and B -.26. Peptides B -.23, B -.33 and B -.44, and the two sources of B -.26 were then eluted directly from the preparative pH 2.1 runs and dissolved in 0.5 ml of N/25 ammonium hydroxide after freeze-drying.

#### PEPTIDE B .55

Isolation procedure: Sephadex and electrophoresis pH 6.5.

Mobility:  $E_p$  (6.5) 0.55.

Ninhydrin: S.

Arginine: +.

N-terminal: Leu.

Assuming that a basic tryptic peptide will carry a net charge of +1 at pH 6.5, the mobility (0.55) of this peptide suggested a molecular weight of 320, which implies that it should have been totally included in the G 50 and eluted in fraction A. The mobility of this peptide therefore suggested that it contains more than one basic residue.

Analysis:	Lys	His	Arg	Pro	Ala	Val	Leu
nm	15	8	10	23	11	9	24
Ratio	1.4	0.7	0.9	2.1	1.0	0.8	2.2
SEQUENCE:	Leu-Leu-Ala-His-Val-Arg-Pro-Pro-Lys.						

As predicted above, this peptide did indeed contain more than one basic residue. It in fact carries a charge of +2.5 at pH 6.5, implying a molecular weight of 1000, which is consistent with a residue weight of 1,029. The sequence of this peptide is the same as peptide A (90-92) .54.

PEPTIDE B .05

Isolation procedure: Sephadex and electrophoresis pH 6.5.

Mobility: Ep (6.5) 0.05.

Ninhydrin: Y.

Arginine: +.

N-terminal: Asp.

Analysis:	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Val	Met	Leu
nm	21	19	42	19	18	42	40	62	28	15	28
Ratio	1.0	1.0	2.1	1.0	0.9	2.1	2.0	3.1	1.4	0.8	1.4

In addition to the above analysis, lesser amounts of Ala (9 nm) and Tyr (8 nm) were present; possibly being derived from some slight contaminating peptide and from paper.

Based on the above molar ratios, it appeared that this peptide contained about 16 residues, making it too large for direct sequencing. It was therefore decided to attempt a thermolysin digestion. In order that the N-terminal thermolytic peptide might be readily identified, a 'Zoom' Edman analysis was carried out on the intact peptide. This showed an N-terminal sequence of Asn-Val-Leu-. This, however, meant that the peptide would be unsuitable for digestion, as this would lead to the production of an Asn-Val peptide together with another 14-residue peptide. It was therefore decided to commit this peptide to direct sequence analysis.

SEQUENCE: Asn-Val-Leu-Gly-His-Met-Glx-Glx-Gly-Gly-Ser-Pro-Thr-Pro-Asx-Arg.

On dansyl analysis, the second residue appeared as DNS-Val (60%) plus another dansyl spot, which was tentatively identified as DNS-Val-Leu (40%). The identity of the DNS-Val-Leu spot was confirmed when the third residue was determined as DNS-Leu. The presence of this dipeptide is a consequence of the resistance of the Val-Leu bond to acid hydrolysis.

This peptide has a residue weight of 1,695 and a mobility of 0.05 at pH 6.5; this implies a charge of +1/2 at this pH. The +1/2 charge is provided by the histidine residue at

position 5. The +1 charge on the arginine side-chain must therefore be cancelled out by a single free acidic side-chain. This implies that three out of the four acidic residues present occur as the amide. The N-terminal residue was asparagine, as the peptide stained yellow with ninhydrin.

PEPTIDE B .25

Amino acid analysis on this peptide indicated the presence of both arginine and lysine, it thus appeared that this tryptic peptide was impure, so it was further purified by preparative chromatography using butanol: acetic acid: water.

Isolation procedure: Sephadex, electrophoresis pH 6.5 and chromatography.

Mobility: Ep (6.5) 0.25.

Ninhydrin: R.

Arginine: +.

N-terminal: Lys.

Analysis:	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val
nm	32	23	28	46	22	33	55	48	63	20	30
Ratio	1.3	0.9	1.1	1.8	0.9	1.3	2.2	1.9	2.5	0.8	1.2

Analysis continued:	Met	Leu	Phe
nm	25	25	16
Ratio	1.0	1.0	0.6

After purification by chromatography, peptide B .25 contained both lysine and arginine (above). The purity of this peptide was investigated further by 'Zoom' - Edman analysis, which indicated an N-terminal sequence of Lys-Asx-Val-. The third residue here showed up as Val plus a Val-Leu dipeptide, which indicated a sequence of Lys-Asx-Val-Leu. As each of the 'Zoom' -Edman cycles yielded a single residue with little background, this peptide (B .25) was subjected to sequence analysis.

SEQUENCE: Lys-Asx-Val-Leu-Gly-His-Met-Glx-Glx-Gly-Gly-Ser-Pro-(?)-Pro-(?)-

Although it was not possible to determine the entire sequence of this peptide directly, it is clearly identical to peptide B B .05, (above), except that this peptide contains an additional lysine residue at the N-terminus. Peptide B .05 with an additional +1 charge would have a mobility of 0.25 at pH 6.5. This peptide does indeed have this mobility.

Peptide B .05 and B .25 are derived from a tryptic split occurring at a -Lys-Lys- or -Arg-Lys- sequence in the intact protein. If Trypsin first cleaves after the second basic group then peptide B .05 is obtained, plus a peptide with a -Lys-Lys or -Arg-Lys C-terminal sequence. A peptide containing such a sequence will be further degraded, yielding free lysine (free lysine has been detected; see peptide A (50-51) .84). If, on the other hand, the trypsin cleaves between the two basic groups first, then peptide B .25 will be obtained and will be resistant to further digestion.

PEPTIDE B -.26

This peptide was further purified by paper chromatography in butanol/acetic acid/water, and redissolved in 0.5 ml M/25 NH<sub>4</sub>OH.

Isolation procedure: Sephadex, electrophoresis pH 6.5, electrophoresis pH 2.1 and chromatography.

Mobility: Ep (6.5) -0.26. Ep (2.1) 0.84.

Ninhydrin: +.

Arginine: -.

N-terminal: Leu.

Radioactivity: 190 cpm per 10 µl.

Analysis: Analyser malfunctioned.

SEQUENCE: Leu-Pro-Leu-Met-Glx-Cys-Val-Glx-Val-Thr-Lys.

On Dansyl-Edman analysis, the second residue showed up weakly as DNS-Pro on a 16 hour hydrolysis. When this residue was repeated on a 4 hour hydrolysis, two dansyl spots were detected; DNS-Pro (30%) and DNS-Pro-Leu (70%). Initially, the 4th residue was detected as DNS-Met and DNS-Mes. This residue was repeated, sealing the hydrolyses tube under higher vacuum. This resulted in the detection

of DNS-Met as the sole derivative. The 6th residue was detected as DNS-CM-Cys. This residue was confirmed by subjecting the butyl acetate extract, containing the FITC derivative of this amino acid, to liquid scintillation counting. The dried down extract contained 2,720 cpm.

This peptide has a residue weight of 1,318. Assuming a charge of -1 at pH 6.5, a molecular weight of 1,000 would be expected. At pH 2.1, this peptide will have a net charge of +2, the mobility of 0.84 implies a molecular weight of 1,250. From these data, the peptide must contain two free acidic side-chains, as the carboxymethyl-cysteine residue will contribute one of these, one of the glutamic acid residues must therefore be present as glutamine.

#### PEPTIDE B -.33

Isolation procedure: Sephadex, electrophoresis pH 6.5 and  
electrophoresis pH 2.1

Mobility: Ep (6.5) -0.33; Ep (2.1) 1.04

Ninhydrin: S.

Arginine: -.

N-terminal: Ile.

Analysis	Lys	Thr	Glu	Ala	Ile
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nm	25	42	54	40	24
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Ratio	1.0	1.8	2.3	1.7	1.0
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SEQUENCE: Ile-Thr-Ala-Glu-Glu-Ala-Thr-Lys.

The pH 2.1 mobility of 1.04 implies a molecular weight of 800; this is consistent with a residue weight of 861 for this peptide. Therefore the pH mobility of -0.33 means that both acidic residues are present as the free acid.

During Dansyl-Edman analysis, high background levels built up on the polyamide plates after the second glutamic acid residue. In order to verify the above sequence, the remaining peptide material (90 nm) was subjected to digestion with thermolysin. The thermolytic digest was subjected to analytical electrophoresis at pH 6.5 and 2.1. Three ninhydrin positive spots were observed: one acidic with a mobility of -0.31; one basic with a mobility of 0.55; and one neutral with a mobility of 1.0.

The neutral peptide stained slowly with ninhydrin, indicating an Ile N-terminus. This corresponds to the expected thermolytic peptide Ile-Thr, which would be neutral and have a mobility of 1.04 at pH 2.1.

The observed basic peptide (mobility 0.55) corresponds to the expected basic peptide Ala-Thr-Lys, which would have a mobility of 0.55. The observed acidic peptide, with mobility -0.31, stained slowly with ninhydrin, indicating an N-terminal Ile residue. The mobility of this peptide suggests a molecular weight of 770, implying that this material represents undigested peptide B -.33. On preparative electrophoresis of the digested material, this peptide was the only one recovered in large enough amounts to subject to further analyses. N-terminal analysis indicated the presence of DNS-Ile and DNS-Lys, confirming the hypothesis that this peptide represented undigested starting material.

#### PEPTIDE B-.23

This peptide was further purified by paper chromatography in butanol/acetic acid/water, eluted, and redissolved in 0.5 ml M/25 NH<sub>4</sub>OH.

Isolation procedure: Sephadex, electrophoresis pH 6.5, electrophoresis pH 2.1 and chromatography.

Mobility: Ep (6.5) -.23; Ep (2.1) 0.78.

Ninhydrin: +.

Arginine: +.

N-terminal: Two spots were detected; one corresponding to DNS-Ile and the other to DNS-Ile-Phe.

Radioactivity: 205 cpm per 10  $\mu$ l.

Analysis:	Arg	(CM-Cys)	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val
nm	.24		61	21	20	10	?	49	32	28
Ratio	1.0	1.0	1.5	0.9	0.8	0.4	?	2.0	1.3	1.2

Analysis (Contd.) Met Ile Leu Phe

nm 18 21 33 24

Ratio 0.7 0.9 1.4 1.0

Specific activity 21 cpm/nm.

SEQUENCE: Ile-Phe-Ala-Asx-Thr-Pro-Asx-Ser-Gly-Cys-Val-Leu-Gly-

The cysteine residue at position 10 was confirmed by counting the dried down butyl acetate extract containing the PITC derivative of this amino acid: this contained 5,017 cpm.

It was not possible to directly determine the sequence of residues past the glycine at position 13.

The pH 2.1 mobility of this peptide implies a molecular weight of 1,450; the pH 6.5 mobility therefore implies a net charge of -1 at pH 6.5. This means that one of the two Asx residues above is present in the peptide as the free acid.

At this stage, approximately 80 nm of peptide was remaining; this material was digested with chymotrypsin. Based on the above data, one would expect to observe three chymotryptic peptides; one neutral Ile-Ihe dipeptide, one radioactive acidic decapeptide with a charge of -2, and one arginine-containing peptide which would stain yellow with ninhydrin.

Duplicate analytical electrophoresis was run at pH 6.5 on the chymotryptic digest. One strip was stained with ninhydrin and the other with Sakaguchi Reagent. Three peptides were detected with ninhydrin; one basic (which stained yellow), one neutral, and one acidic. The basic peptide also stained positively for arginine and the acidic peptide was radioactive. These three peptides were then separated preparatively at pH 6.5 and eluted. Data on these peptides is described below.

#### Basic chymotryptic peptide.

Ninhydrin: yellow, implying Gly N-terminal.  
 Arginine: +, implying C-terminal peptide of B-.23.  
 Mobility: Ep (6.5) 0.53, Implies MW = 340.

This peptide was then hydrolysed with 6M hydrochloric acid. The hydrolysate was then dansylated and analysed on a polyamide sheet. Three fluorescent spots were observed, corresponding to DNS-Met, DNS-Gly and DNS-Arg. The sequence can therefore be deduced as Gly-Met-Arg, which has a residue weight of 362.

## Neutral chymotryptic peptide

This peptide was not investigated further, as it must represent the expected Ile-Phe N-terminal peptide.

## Acidic chymotryptic peptide

Ninhydrin: R.  
 Arginine: -.  
 CN Cys: +.  
 Mobility: Ep (6.5) -0.54. Implied MW = 880.  
 N-terminal: Ala.

This peptide represents the expected Ala-Asx-Thr-Pro-Asx-Ser-Gly-Cys-Val-Leu- peptide, which has an expected mobility of -0.49.

As discussed above, one of the Asx residues in this peptide is present as asparagine and the other as the free acid. In order to determine the position of the amide, the peptide material was divided into two equal amounts. One-half of the material was subjected to a one-stage 'Zoom'-Edman treatment and the other half to a two-stage treatment. This results in the production of two peptides: 1, Asx-Thr-Pro-Asx-Ser-Gly-Cys-Val-Leu, and 2; Thr-Pro-Asx-Ser-Gly-Cys-Val-Leu. At this point, these two peptides were subjected to direct electrophoretic analysis at pH 6.5 and stained with ninhydrin.

Peptide 1 had the expected pH 6.5 mobility of -0.55 and stained yellow with ninhydrin, indicating an Asn N-terminal.

If the second residue of the original chymotryptic peptide was Asp, one would expect peptide 2 to have a net charge of -1 and therefore a mobility of -0.31; if, on the other hand, the second residue was Asn-, then peptide 2 would have a net charge of -2 and therefore a mobility of -0.59. As peptide 2 had an observed pH 6.5 mobility of -0.56, it is concluded that the sequence of the original chymotryptic peptide is: Ala-Asn-Thr-Pro-Asp-Ser-Gly-Cys-Val-Leu.

From the chymotryptic data the entire sequence of peptide B -0.23 can be deduced as:

Ile-Phe-Ala-Asn-Thr-Pro-Asp-Ser-Gly-Cys-Val-Leu-Gly-Met-Arg.

## PEPTIDE B-.44

Isolation procedure: Sephadex, electrophoresis pH 6.5 and  
electrophoresis pH 2.1.

Mobility: Ep (6.5) 0.44; Ep (2.1) 0.78.

Ninhydrin: R.

Arginine: -.

N-terminal: No N-terminal was detected using dansyl chloride. This peptide was, however, fluorescent and gave a purple colour with Ehrlichs Reagent, indicating the presence of Tryptophan. As the hydrolysis tube was sealed under high vacuum and it was possible to sequence past the first residue, tryptophan was assigned to the N-terminal position.

Analysis:	Lys	Asp	Ser	Glu	Gly	Leu
nm	19	38	29	43	30	49
Ratio	1	2	1.5	2.2	1.6	2.6

SEQUENCE: Trp-Glx-Glx-Gly-Asx-Leu-Leu-Ser-Asx-Leu-Gly-Lys

After the PITC derivative of the first residue was cleaved with trifluoroacetic acid, the remaining peptide was tested with Ehrlichs Reagent. No reaction was observed, confirming tryptophan as the first residue.

The penultimate residue was missed on direct sequencing; in order to determine this residue, the peptide was digested with chymotrypsin and the basic subpeptide isolated by electrophoresis at pH 6.5. This peptide was hydrolysed with 6M HCl, dansylated and analysed on a polyamide sheet. Two spots were detected, DNS-Gly and DNS-Bis-Lys. This indicated that the penultimate residue was Gly.

The residue weight of this peptide (1,360) is consistent with a pH 2.1 mobility of 0.78 and an implied molecular weight of 1,450. The pH 6.5 mobility of -0.44 therefore implies a net charge of -2, which means that only one of the acidic residues is present as the amide.

## PEPTIDE B N 1.1

Isolation procedure: Sephadex, electrophoresis pH 6.5 and  
pH 2.1

Mobility: Ep (6.5) 0; Ep (2.1) 1.1.

Ninhydrin: +.

Arginine: -.

N-terminal: Val.

Analysis:	Lys	His	Asp	Glu	Gly	Ala	Val	Leu	Ile
nm	22	15	19	23	41	24	50	44	11
Ratio	1.0	0.7	0.9	1.0	2.0	1.1	2.3	2.0	0.5

SEQUENCE: Val-Leu-Val-Val-His-Asx-Gly-Ile-Glx-Gly-Leu-Ala-Lys.  
Residue MW: 1382.

The third residue in this sequence was detected partly as  
DNS-Val (50%) and partly as DNS-Val-Val (50%).

#### 4.4 Fraction C

Fraction C, representing Sephadex tubes (49-53) was dissolved  
in 0.5 ml of N/25 ammonium bicarbonate and subjected to  
analytical electrophoresis at pH 6.5. Staining with ninhydrin  
indicated that there were no basic peptides present. The  
acidic peptides, however, did not resolve and were detected as  
a streak on the paper. Analytical electrophoresis at pH 2.1  
did not resolve this fraction any better; a long streak was  
again observed. Chromatography in butanol/acetic acid/water  
indicated that this method was also unsuitable for a prepara-  
tive separation of these peptides.

Fraction C was freeze-dried and dissolved in 1 ml 25 mM Tris/  
HCl pH 8.0, 5 mM NaCl and applied to a 19 x 1.6 cm column of  
DE 32 ion exchange cellulose. The column was developed with  
a linear gradient of 100 ml 25 mM Tris/HCl, 5 mM NaCl and  
100 ml 25 mM Tris/HCl, 0.4 M NaCl. This was followed by a  
steeper gradient of 50 ml 25 mM Tris/HCl, 0.4 M NaCl and  
25 mM Tris/HCl, 1 M NaCl. The column effluent was monitored  
at 225 and 280 nm. Results indicated the presence of three  
partially resolved peaks. The resolution was not sufficient  
for a satisfactory purification of any peptide. Therefore,  
tubes containing peptide material were pooled, freeze-dried,  
and desalted on a 50 x 2 cm column of Sephadex G 25.

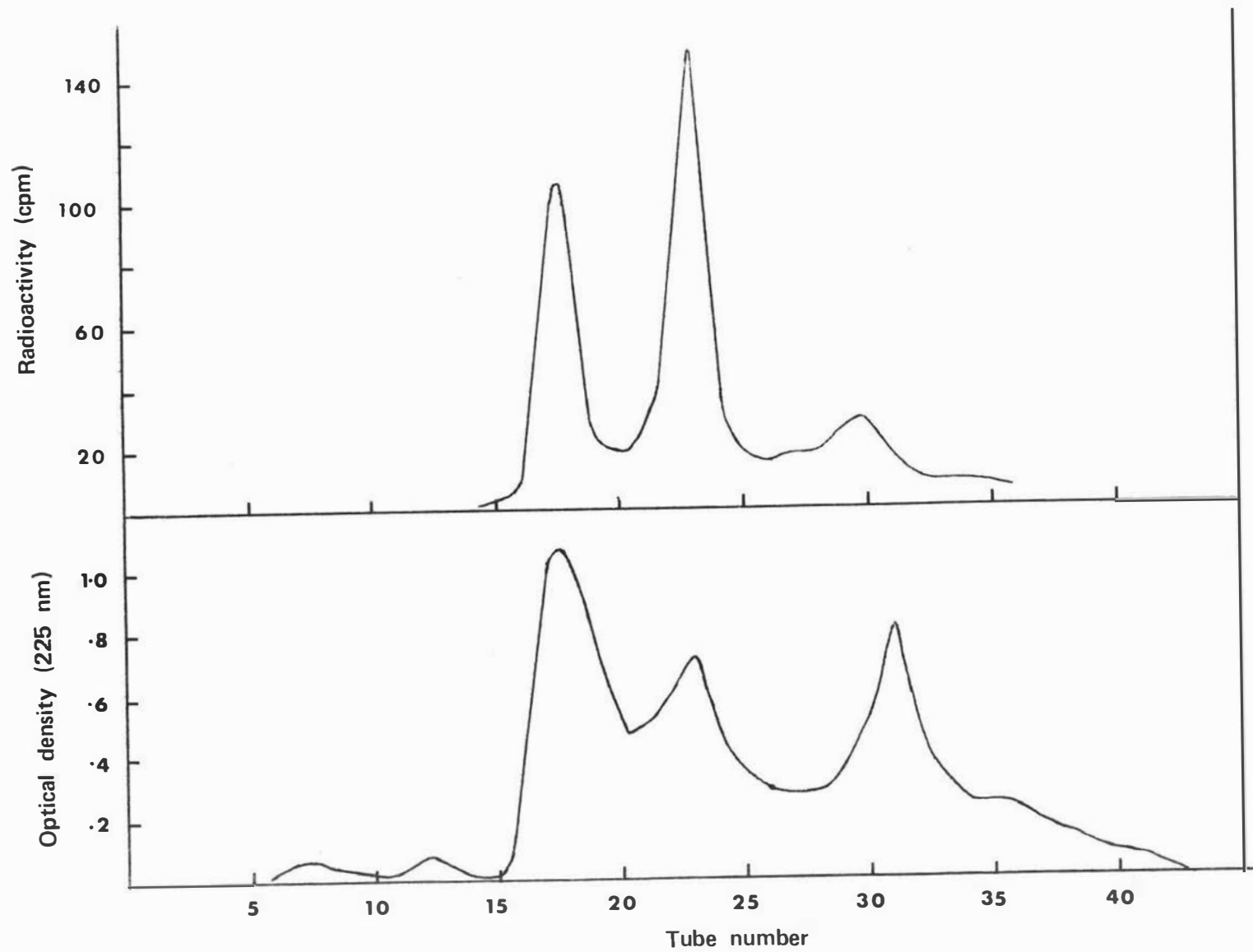


Figure 13 Peptide fraction C on DE 32 cellulose

Fraction C was again applied to a DE 32 column using a modified elution system of: 100 ml 25 mM Tris/HCl pH 8.0 5 mM NaCl; followed by a gradient of 150 ml 25 mM Tris/HCl, 5 mM NaCl and 150 ml 25 mM Tris/HCl, 0.2 M NaCl. Ten ml fractions were collected, tubes were read at 225 nm and a 50  $\mu$ l sample was taken for Liquid Scintillation counting. Results are shown in Fig.13. Based on these results, the following tubes were pooled, freeze-dried, and desalted on a 19 x 2 cm column of Sephadex G25.

- Tubes 17 and 18, designated C(17-18)
- Tubes 19 and 20, designated C (19-20)
- Tube 23 designated C 23
- Tubes 30, 31 and 32, designated C (30-32)

After desalting, these fractions were freeze-dried and each was dissolved in 500  $\mu$ l M/25 NH<sub>4</sub>OH.

Both amino acid analysis and 'Zoom'-Edman analysis indicated that C(19-20) contained no significant amount of peptide material.

PEPTIDE C(17-18)

'Zoom'-Edman analysis indicated that this peptide was about 70% pure and had an N-terminal sequence of Leu-Pro-Leu-. Analytical electrophoresis at pH 2.1 revealed a major radioactive spot at mobility 0.82. This peptide was therefore further purified by preparative electrophoresis at pH 2.1.

Isolation procedure: Sephadex, DE 32, and electrophoresis at pH 2.1.

Mobility: Ep (2.1) 0.82. Implied MW = 1,300.

Ninhydrin: S.

N-terminal: Leu.

Analysis:	Lys	CM	Cys	Thr	Glu	Pro	Gly	Val	Met	Ile	Leu
nm	13	16		19	32	14	9	27	13	10	27
Ratio	0.9	1.1		1.3	2.3	1.0	0.6	1.9	0.9	0.7	1.9

SEQUENCE: Leu-Pro-Leu-Met-Glx-Cys-Val-Glx-Val-Thr-Lys.

Residue MW: 1,318.

The second residue was detected as DNS-Pro (40%) and DNS-Pro-Leu (60%). This peptide is the same as peptide B -.26.

## PEPTIDE C (30-32)

On analytical electrophoresis at pH 2.4, this peptide was not located with ninhydrin. 'Zoom'-Edman analysis, however, indicated an N-terminal sequence Glu-Leu-Leu-Phe-. One-tenth of the sample (50  $\mu$ l) was taken for amino acid analysis.

Analysis:	His	Arg	Asp	Thr	Glu	Pro	Gly	Ala	Val	Leu	Phe
nm	116	113	240	255	778	?	91	116	167	303	180
Ratio	1.0	1.0	2.1	2.2	6.9	?	0.8	1.0	1.5	2.7	1.6

Four hundred nm of this peptide was taken for Dansyl-Edman treatment. The initial sequence was determined as: Glx-Leu-Leu-Phe-Glx-Pro-Val-Asx-Glx-Leu-. As it was not possible to determine the sequence past position 10, the remaining peptide material (approximately 450 nm) was digested with chymotrypsin.

Analytical electrophoresis of the digest revealed the presence of five chymotryptic peptides; one was basic, two acidic, and two neutral. These peptides were separated by preparative electrophoresis and are numbered (below) according to their electrophoretic mobilities. The prefix (CH) indicates the peptides' chymotryptic origin.

- (CH) N 1.0 This peptide was found to be the free Leu on N-terminal and amino acid analysis.
- (CH) N 0.75 This peptide was found to be free Phe on N-terminal and amino acid analysis.
- (CH) .27 Sequence; Glu-His-Arg.  
The residue weight of this peptide (440) and its pH 6.5 mobility of 0.27 implied that the glutamic acid residue was present as the free acid. As this peptide terminates with arginine, it represents the C-terminal sequence of the parent peptide.
- (CH) -.70 Sequence; Glu-Leu.
- (CH) -.56 The amino acid analysis ratios for this peptide are shown below:
- | Asp | Thr | Glu | Pro | Gly | Ala | Val | Leu | Phe |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1.1 | 1.1 | 3.3 | 0.8 | 0.9 | 0.8 | 0.9 | 0.8 | 1.0 |

It was only possible to determine the sequence of the first three residues in this peptide. The initial sequence was: Glx-Pro-Val-.

The scheme below shows the origin of these chymotryptic peptides in relation to the parent peptide.

C(30-32)	Glx-Leu-Leu-Phe-Glx-Pro-Val-Asx-Glx-Leu-
(CH) -.71	Glu-Leu
(CH) N 1.0	Leu
(CH) NO.75	Phe
(CH) -.56	Glx-Pro-Val-(Asx, Glx <sup>2</sup> , Thr, Ala, Gly, Leu, Phe).
(CH) .27	~ Glu-His-Arg

From the above data, the sequence/composition of peptide C(30-32) can be derived as:

Glu-Leu-Leu-Phe-Glx-Pro-Val-Asx-Glx-Leu-(Glx, Thr, Ala, Gly, Phe)~Glu-His-Arg.

Totalling the number of amino acids of each residue here, and comparing these values with those for the amino acid analysis of peptide C(30-32), indicated that a fragment of composition (Glx<sup>2</sup>, Asx, Thr) was missing from the above sequence. The missing amino acids are represented by the symbol ~.

#### PEPTIDE C 23

One-tenth of the peptide material was taken for amino acid analysis.

Analysis:	Lys	Arg	CM Cys*	Asp*	Thr	Ser	Glu	Pro	Gly
nm	5	25	130		90	58	33	0	170
Ratio	0.2	1.0	1.0	4.2	3.6	2.3	1.3	0	6.8

Analysis, continued:	Ala	Val	Met	Ile	Leu	Tyr	Phe
nm	58	38	0	58	75	0	32
Ratio	2.3	1.5	0	2.3	3.0	0	1.3

(\*: The CM-Cys and Asp peak did not resolve during analysis.)

This peptide was radioactive and had a specific activity of 20 cpm/nm based on arginine. From the results obtained on

63.  
peptides A 58 H 1.50 and B -.23, which contained only one CM-Cys residue and had specific activities of 18 and 21 cpm/nm respectively, it may be deduced that this peptide contains a single CM-Cys residue. Thus, 25 nm out of the 130 nm peak, observed for Asp, were attributable to CM-Cys.

Amino acid analyses showed 5 nano-moles of lysine. It was assumed that this was derived from a small amount of contaminating peptide material. In an effort to remove this contaminant and obtain a more precise analysis, the peptide was subjected to preparative electrophoresis at pH 2.1. On electrophoresis, the peptide ran as a long streak. It was not possible to recover this material from the paper.

#### 4.5 Fraction D

Fraction D, representing Sephadex tubes (23-48) was dissolved in 1 ml of 25 mM Tris/HCl pH 8.0, 5 mM NaCl and applied to a 20 x 1.6 cm column of DE 32 cellulose. The column was developed with 100 ml of the above buffer followed by a gradient of 150 ml 25 mM Tris/HCl, 5 mM NaCl and 150 ml 25 mM Tris/HCl, 0.2 M NaCl. This was followed by a gradient of 75 ml 25 mM Tris/HCl, 0.2 M NaCl and 75 ml 25 mM Tris/HCl, 0.4 M NaCl. The column was stripped with 50 ml 25 mM Tris/HCl, 1 M NaCl. Ten ml fractions were collected, the column effluent was monitored at 225 nm and 50  $\mu$ l samples from each tube were subjected to liquid scintillation counting.

Figure 14 shows the elution profile of this column; only tubes 25 and 26 contained a peptide which was in sufficient yield to be amenable to further investigation. These tubes were pooled, freeze-dried and desalted on a 19 x 2 column of Sephadex G 25.

#### PEPTIDE D (25-26)

'Zoom'-Edman analysis on this peptide indicated an N-terminal sequence of Leu-Gly-Val-. One-twentieth of the peptide material was taken for amino acid analysis.

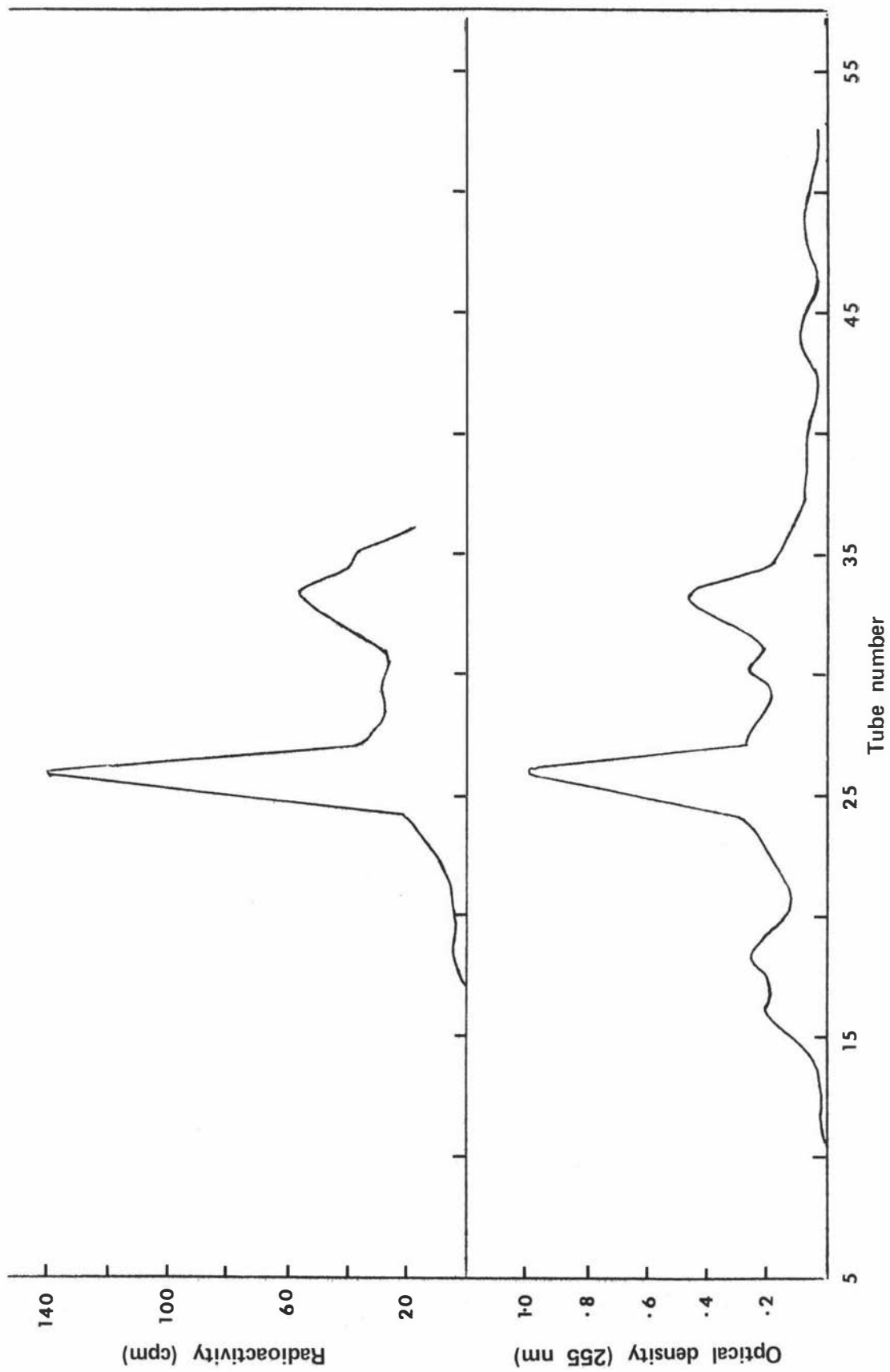
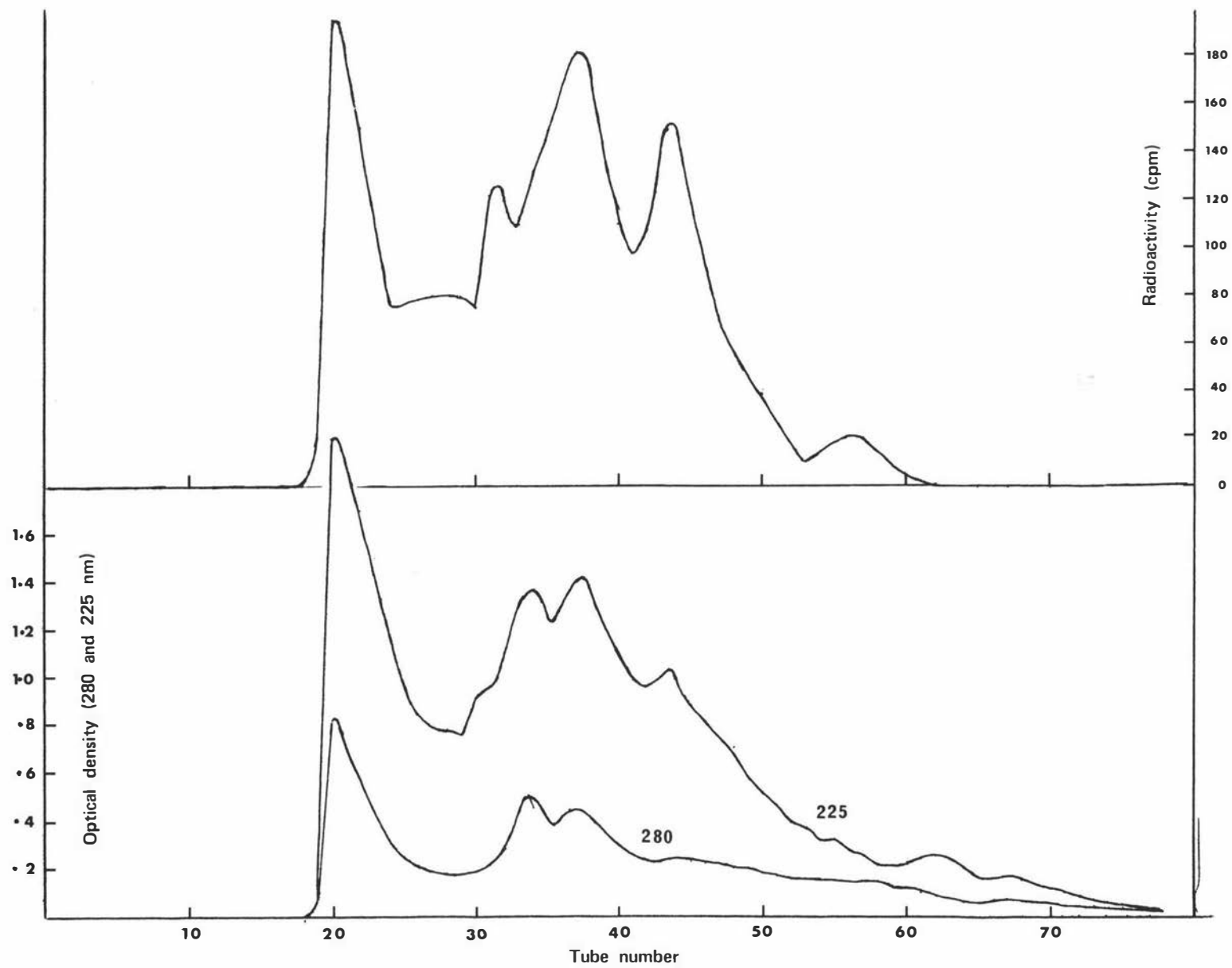


Figure 14 Peptide fraction D on DE 32 cellulose

Analysis:	Arg	Cm-Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu
nm	22	21	120	87	88	140	?	109	125	96	35	18	100
ratio	1.0	1.0	5.5	4.0	4.0	6.3	?	5.0	5.7	4.4	1.6	0.8	4.5

Two hundred and fifty nm of this peptide was subjected to Dansyl-Edman analysis; an initial sequence of Leu-Gly-Val-Ala-Val-met- was found.

Figure 15 Elution profile of insoluble peptides on Sephadex G-50



## 5. ACID-INSOLUBLE TRYPTIC PEPTIDES

The 'acid-insoluble' tryptic peptides (p31) were dissolved in 4.5 ml of 0.2 M ammonium bicarbonate and applied to a 2.5 x 93 cm column of Sephadex G 50. The column was developed with 0.2 M ammonium bicarbonate using upward flow at 13.5 ml/hour. Thirty-five-minute fractions were collected. The column effluent was monitored at 280 and 225 nm, and 50  $\mu$ l samples were taken for scintillation counting (Fig.15). The following tubes were pooled and freeze-dried for further purification.

- Tubes (53-60), designated fraction Z;
- Tubes (42-48), designated fraction Y;
- Tubes (28-40), designated fraction X;
- Tubes (20-27), designated fraction W.

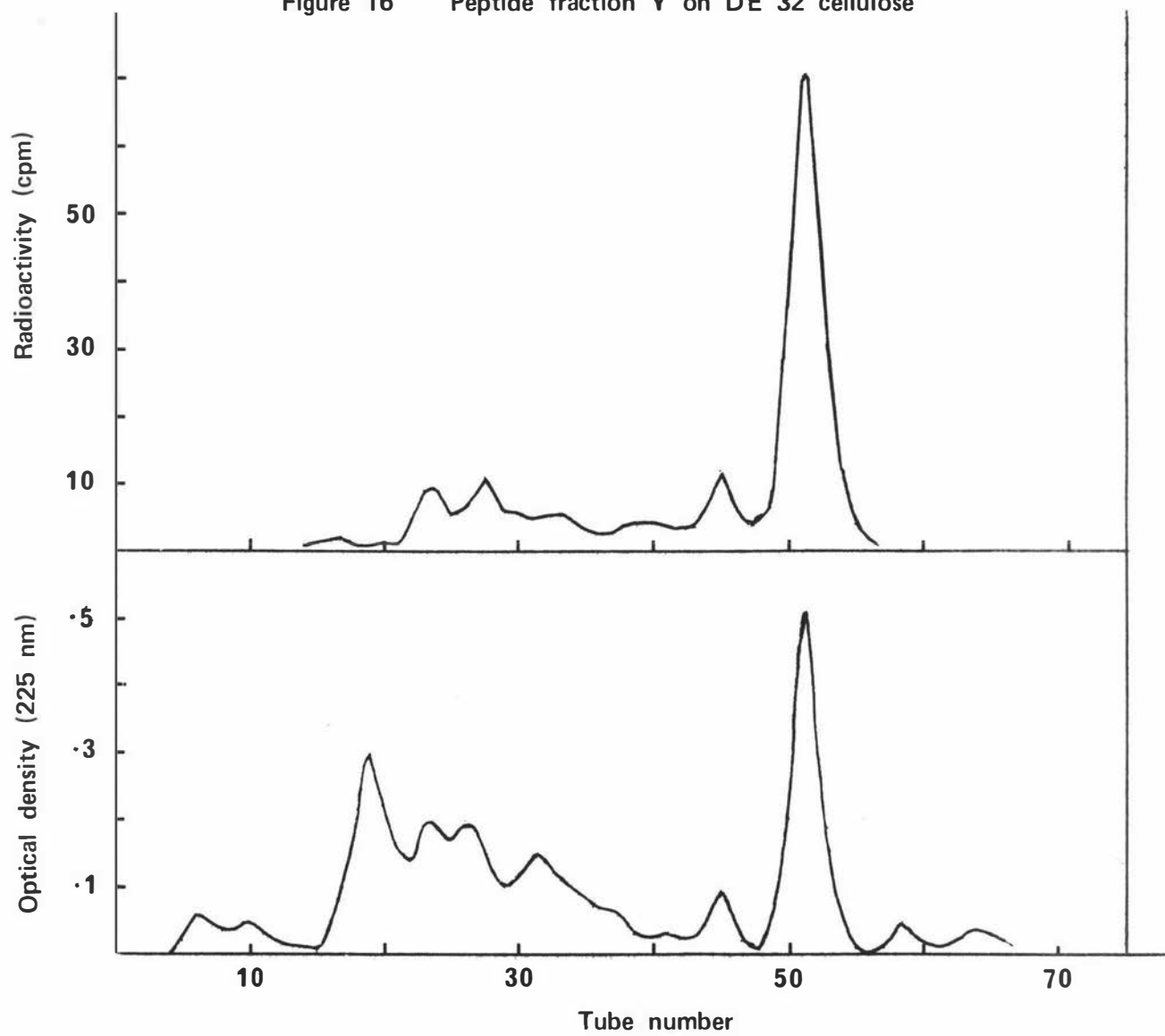
Fraction Z was subjected to preparative electrophoresis. The only significant component present was a small amount (approx. 200 nm) of free lysine.

### 5.1 Fraction Y

Fraction Y, representing Sephadex tubes (42-48) was dissolved in 2 ml of 25 mM Tris/HCl pH 8.0, 5 mM NaCl and applied to a 19 x 1.6 cm column of DE 32 cellulose. The column was developed with 100 ml of this solvent, followed by a linear gradient of 220 ml 25 mM Tris/HCl, 5 mM NaCl and 220 ml 25 mM Tris/HCl, 0.2 M NaCl. The column was then developed using a gradient of 80 ml of the 0.2 M buffer and 80 ml of a 0.4 M solution of buffer. Eighty ml of 1 M NaCl in Tris buffer was then applied to the column. Ten ml fractions were collected and read at 225 nm; 50  $\mu$ l samples were taken from tubes for scintillation counting (Fig.16).

Tubes 16-20 and 50-54 were pooled, freeze-dried, and desalted on Sephadex G 25. These are referred to as fractions Y (16-20) and Y (50-54). When one-twentieth of fraction Y (16-20) was subjected to amino acid analysis, peaks of only 5-10 nm were observed. Using the same sample size, fraction Y (50-54) yielded peaks of between 90 and 350 nm. It was

Figure 16 Peptide fraction Y on DE 32 cellulose



concluded from this that fraction Y (16-20) contained no significant amount of peptide material.

### PEPTIDE Y (50-54)

Both electrophoresis and N-terminal analysis indicated that this peptide was pure, so it was subjected to further analysis without additional purification.

Mobility: Ep (6.5) -0.55  
Ep (2.1) peptide did not move off origin.

Ninhydrin: Y.

Arginine: -.

N-terminal: Cys.

Radioactivity: 680 cpm/10  $\mu$ l.

Analysis:	Lys	CH	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ile	Leu	Tyr	Phe
nm	90	65	354	190	140	286	?	115	101	112	168	177	
Ratio	1.0	0.7	4.0	2.1	1.6	3.2	?	1.3	1.1	1.3	1.9	2.0	

Specific activity: 19 cpm/nm

SEQUENCE: Cys-Asx-Glx-Asx-Tyr-(?)-(?)-Asx-

One hundred  $\mu$ l of peptide was subjected to direct Dansyl-Edman Analysis with the above result. The Cys residue at position one was confirmed by counting the first butyl acetate extract, which contained 5,564 cpm. As it was not possible to determine the sequence of this peptide, past residue eight, 200  $\mu$ l was taken and digested with chymotrypsin.

Analytical electrophoresis of the digest revealed two major, yellow, acidic spots (one of which was radioactive) and one minor neutral spot. These peptides were separated by preparative electrophoresis, eluted, freeze-dried, and redissolved in 250  $\mu$ l of M/50 ammonium hydroxide. These chymotryptic peptides (CH) are numbered according to their electrophoretic mobility and discussed below.

(CH) -.66

Mobility: Ep (6.5) -0.66  
 Ninhydrin: Y  
 Radioactivity: +.  
 N-terminal: Cys.

Analysis: CM Cys/Asp (45), Glu (17), Tyr (16)  
 SEQUENCE: Cys-Asx-Glx-Asx-Tyr.  
 Residue MW: 601.

Assuming a net charge of -2 at pH 6.5, the mobility of this peptide implies a molecular weight of 650.

(CH) N 0.68

Mobility: Ep (6.5) 0; Ep (2.1) 0.60  
 Ninhydrin: R.  
 N-terminal: Thr (faint).  
 Analysis: Thr (5), Leu (7), Tyr (7).  
 SEQUENCE: Thr-Leu-Tyr.  
 Residue MW: 395

(CH) -.44

Mobility: Ep (6.5) -0.44  
 Ninhydrin: Y.  
 N-terminal: Ser.  
 Analysis: Lys (16), Asp (15), Thr (15),  
 Ser (16), Glu (33), Gly (17),  
 Phe (15)  
 SEQUENCE: Ser - Thr - Asx - Phe - Lys  
                   Glx    Glx    Gly

Clearly, this sample was a mixture of two peptides. The mobility of -0.44 implies that each peptide has a charge of -1. One peptide contains a Ser N-terminal and as only a single residue was detected, the other peptide must contain an N-terminal Trp. It can be deduced that Gly and Lys are present in the same peptide, as the other chymotryptic peptide has to contain a terminal aromatic residue (Phe). Further, the peptide containing the terminal -Gly-Lys sequence must also contain two free acid residues, in order to be acidic. Also, the peptide containing the C-terminal Phe residue has to contain one free acid residue to give it a charge of -1. The sequence of these peptides was therefore deduced as:

Trp-Thr-Asp-Phe.	1
Ser-Glu-Glu-Gly-Lys	2

The four chymotryptic peptides described above account for 17 residues. Amino acid analysis on the original peptide indicated that it contained 20 amino acids. In order to locate the missing residues, 150  $\mu$ l of peptide Y (50-54) was digested with thermolysin. Analytical electrophoresis on this digest indicated two acidic spots, one of which was yellow and radioactive, and one neutral spot. The thermolytic peptides (TH) were separated by preparative electrophoresis and dissolved in 250  $\mu$ l of M/50 ammonium hydroxide. These peptides are described below:

(TH) -.67

Mobility:	Ep (6.5) -0.67
Ninhydrin:	Y
Radioactivity:	+
N-terminal:	Cys.
SEQUENCE:	Cys-Asx-Glx-Asx-Tyr.
Residue MW:	601

This peptide is identical to the chymotryptic peptide (CH) -.67. It is surprising that the thermolysin did not cleave this peptide at the Tyr residue. The pH 6.5 mobility of -0.67, together with a residue weight of 601, implies that this peptide carries a net charge of -2, therefore it must contain two amides.

After the first two residues were removed, by treatment with FITC and TFA, the remaining peptide (Glx-Asx-Tyr) was subjected to electrophoresis at pH 6.5. A single spot was observed with a mobility of -0.48, implying a charge of -1 on this peptide. This means that the second Asx residue of peptide (TH) -.67 is present as asparagine. When the third residue was removed, the remaining peptide (Asx-Tyr) was again subjected to electrophoresis at pH 6.5. A single neutral, yellow spot was observed, implying a sequence of Asn-Tyr.

The sequence of peptide (TH) -.67 can be deduced as: Cys-Asn-Glu-Asn-Tyr.

(TH) -.33.

Mobility: Ep (6.5) -0.33.  
 Ninhydrin: S.  
 N-terminal: Leu.  
 Analysis: Lys (15), Ser (15), Glu (28),  
 Gly (16), Leu (14), Tyr (14)  
**SEQUENCE:** Leu-Tyr-Ser-Glu-Glu-Gly-Lys.  
 Residue MW: 824.

The residue weight, together with the pH 6.5 mobility, imply a charge of -1. This is consistent with observations on peptide (CH) -.44 (2). Further, this peptide contains an overlap between peptides (CH) N .60 and (CH) -.44.(2). Also, from the above sequence, the sequence of peptide (CH) -.44 (1) can be deduced as: Trp-Thr-Asp-Phe.

(TH) N 0.78

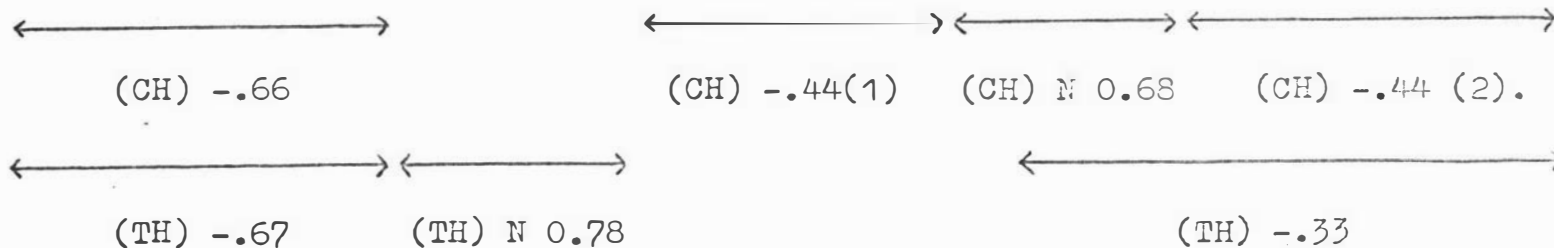
Mobility: Ep (6.5) 0; Ep (2.1) 0.78.  
 Implies MW = 440.  
 Ninhydrin: R.  
 N-terminal: Ile.  
 Analysis: Asp (8), Ile (8), Phe (7).  
**SEQUENCE:** Ile-Phe-Asn.  
 No. amides: 0.  
 Residue MW: 393.

From the above data on chymotryptic and thermolytic peptides, the sequence of the parent peptide, Y (50-54), can be derived as:

Cys-Asn-Glu-Asn-Tyr-Ile-Phe-Asn-Trp-Thr-Asp-Phe-Thr-Leu-Tyr-Ser-Glu-Glu-Gly-Lys. (A sequence overlapping scheme is shown on the next page.).

This sequence is consistent with the amino acid analysis on peptide Y (50-54). Further, the above sequence indicates that the peptide has a residue weight of 2,285, and a net charge of -4 at pH 6.5. It is therefore possible to predict that the peptide should have a mobility of -0.54 at pH 6.5; this is consistent with the observed mobility of -0.55.

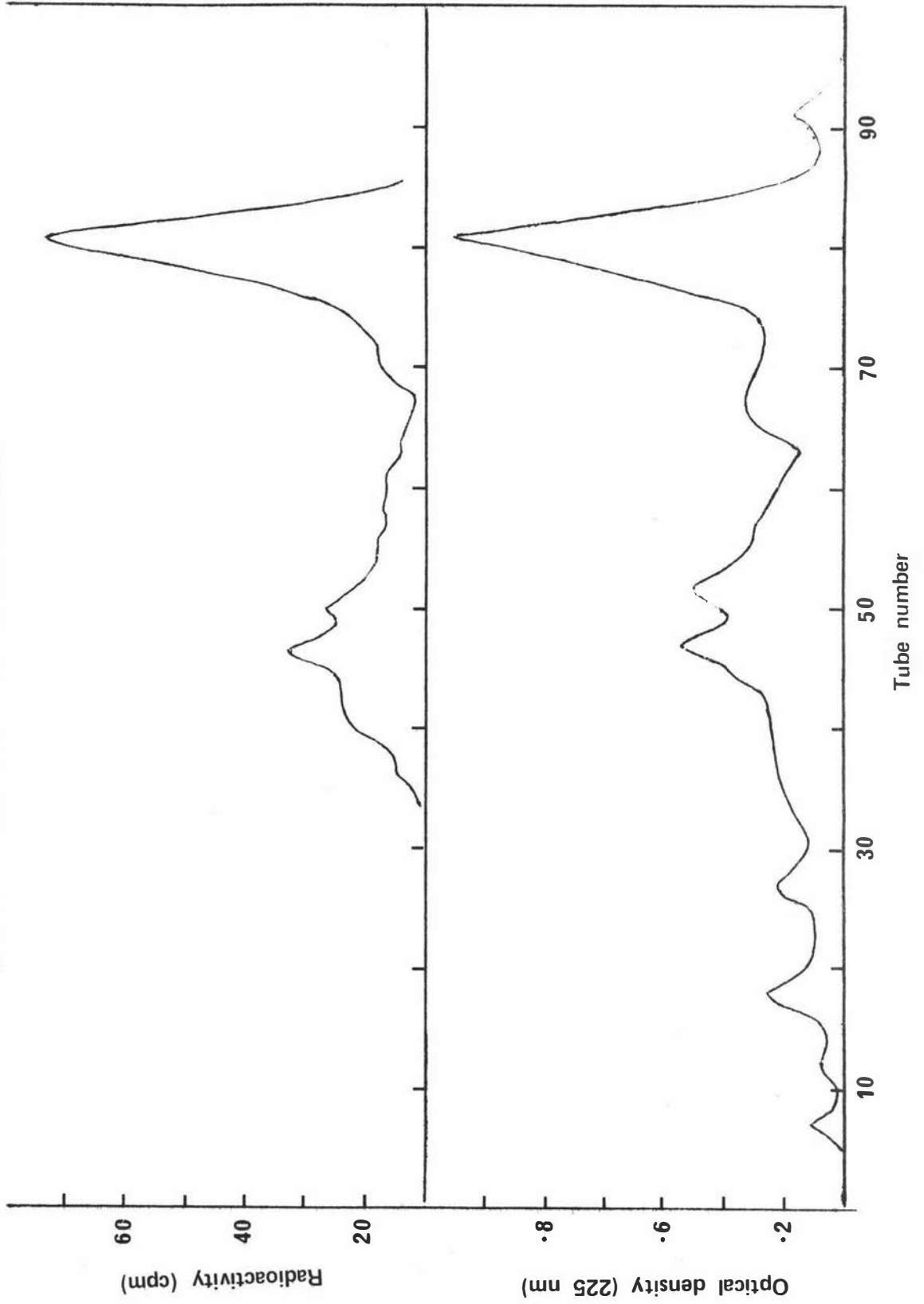
Cys-Asn-Glu-Asn-Tyr-Ile-Phe-Asn-Trp-Thr-Asp-Phe-Thr-Leu-Tyr-Ser-Glu-Glu-Gly-Lys



N-terminal sequence Y (50-54)

Scheme for overlapping of chymotryptic and thermolytic peptides of Y (50-54).

Figure 17 Peptide fraction X on DE 32 cellulose



## 5.2 Fraction X

Fraction X, representing Sephadex tubes (28-40) was applied to a DE 32 column. The column was eluted and monitored as described for fraction Y; 3.5 ml fractions were collected (Fig.17). Tubes 76-84 were pooled, freeze-dried, and desalted on Sephadex G25. Material in this fraction was designated peptide X (76-84).

### PEPTIDE X (76-84)

When this peptide was subjected to analytical electrophoresis at pH 6.5, it ran as a streak towards the anode. When subjected to N-terminal analysis, spots corresponding to DNS-His and DNS-O-Tyr were observed; however, DNS-Ser, -Leu, -Gly and -Ala were also present at a level of about 25%.

Analysis:	His	Arg	CMCys*	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val
nm	82	53		310	51	90	200	160	150	111	100
ratio	1.5	1.0	1	4.8	0.9	1.7	3.8	3.1	2.9	2.1	1.9

Analysis Contd.	Ile	Leu	Tyr	Phe	
nm		85	182	54	70
ratio		1.6	3.4	1.1	1.3

(\*This peptide had a specific activity of 19 cpm/nm, based on arginine; this value is consistent with one mole of CMCys per mole of peptide.)

Half of the peptide material was digested with thermolysin and half with chymotrypsin. The subpeptides were isolated by preparative electrophoresis. Data on these peptides, which are numbered according to their mobilities, is described in Table IV.

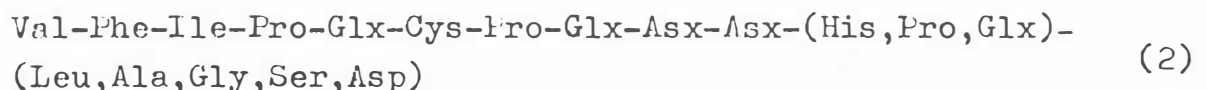
Peptides (TH)- .38 and (CH)- .34 represent overlapping sequences from the N-terminal of the parent peptide. In both peptides, no second residue was detected; though DNS-O-Tyr was observed. The second residue may have been tryptophan, although because the peptides reacted only faintly with Ehrlich reagent, the possibility that it might be an acid labile amino acid derivative cannot be excluded. There is evidence which suggests the presence of an additional acidic group in these

peptides, namely, the inconsistency of the pH 6.5 and pH 2.1 mobilities. A further unusual feature of these peptides is that the third residue was detected as a mixture of DNS-Gly and DNS-Bis-Tyr. As amino acid analysis indicated the presence of a single tyrosine residue, and as this was found to occupy the 4th position, glycine was assigned to position 3. It was not possible to determine the sequence of either peptide past the 4th residue. However, phenylalanine must occupy the C-terminal position of peptide (CH)-.34, as it is a chymotryptic peptide. The N-terminal sequence of the parent peptide (X (76-84)) is proposed as:



Peptides (TH)-.57 a and (TH)-.57 b were eluted as a single band on electrophoresis at pH 6.5; after electrophoresis at pH 2.1, the peptide appeared to run as two bands close together; these were eluted separately as peptides (TH)-.57a and b. Amino acid analysis on these peptides indicated that they were identical. However, at this stage, there was insufficient material for sequence determination.

The peptide (TH)-.78 appears to represent a shorter segment of peptide (TH)-.66; in fact the residues missing from peptide (TH)-.78 appear in peptide (TH)-.57. Thus the peptides (TH)-.78 and (TH)-.57 represent the products of partial thermolytic cleavage at the Leucine residue of peptide (TH)-.66. The sequence composition of this section of the parent peptide was determined as:



The sequence data on chymotryptic peptides (CH)-.63 and (CH)-.56 indicates that what was eluted as two peptides was in fact a single peptide which ran as a streak on electrophoresis.

The segments of sequence; (1) and (2) above; contain the majority of the residues comprising peptide X (76-84). However, amino acid analysis indicates that in addition to these residues, peptide X (76-84) contains an additional ten residues, viz: Arg, Thr, Ser, Glu, Ala, Val, Ile and 3 Leu.

TABLE IV

Amino acid analyses of chymotryptic (CH) and thermolytic (TH) peptides of peptide X (76-84).

PEPTIDE	Lys	His	Arg	Asp	Thr	Ser	Glu	Fro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Ep(6.5)	Ep(2.1)
(TH)-.38		1.0		0.9					2.0						0.9		-.38	1.12
(CH)-.34		1.0		0.8					2.1						1.0	1.3	-.34	
(TH)-.66		1.0		4.4		1.0	3.3	3.1	1.1	1.0	1.0		1.1	1.0		0.9	-.66	
(TH)-.78		1.0		3.8			3.5	3.2			0.8		1.0			0.9	-.78	
(CH)-.63		1.0		5.0		1.3	3.4	3.4	1.6	1.4	1.0		1.0	1.1		1.0	-.63	
(TH)-.57a				1.0		1.0			1.1	1.1					0.9		-.57	
(TH)-.57b				1.0		1.0			1.0	0.9					0.7		-.57	
(CH)-.56	Analyser Malfunctioned.																	

Sequence data on above peptides

(TH)-.38	His-Trp(?) <sup>Tyr</sup> -Gly-Tyr
(CH)-.34	His-Trp(?) <sup>Tyr</sup> -Gly-Tyr -
(TH)-.66	Val-Phe-Ile-Pro-Glx-Cys-Pro-Glx-Asx-Asx-
(TH)-.78	Val-Phe-Ile-Pro-Glx-Cys-Pro-Glx-Asx-
(CH)-.63	Val-Phe-Ile-Pro-
(CH)-.56	Val-Phe-Ile-Pro-Glx-Cys-Pro-Glu-Asx-Asx-

### 5.3 Fraction W

Fraction W, which probably represented undigested denatured starting material, was infected by bacteria and unusable for further analysis.

## 6. DISCUSSION

### 6.1 Polymerisation

The data on the sedimentation characteristics of the purified enzyme indicated that, at pH 8, sheep heart PFK exists as a polymer in rapid reversible equilibrium with lower MW forms. The schlieren pattern contained two peaks at 7 and 30 S and a minor shoulder at 19 S; this differs from the situation with rabbit muscle PFK, where the 30 S boundary contains prominent shoulders at 19 and 12 S (Ling et al., 1965; Pameggiani et al., 1966; Paetkau and Lardy, 1967; Uyeda, 1969). The difference in shape of the trailing 30 S boundary possibly reflects a difference in the equilibrium constant for the polymerisation reaction between the two sources of enzyme. Sheep heart PFK undergoes a concentration-dependent polymerisation. The concentration dependence of the sedimentation rate of the 30 S boundary observed here is virtually identical to that reported for rabbit muscle (Leonard and Walker, 1972). This would indicate that the polymer of the equilibrium reaction has the same molecular weight in both species. The major difference in the sedimentation pattern between the two enzymes is the absence of 7 S material in rabbit muscle at pH 8. This form of the enzyme does, however, exist in equilibrium with the 12 S form of rabbit muscle, at pH values between 6 and 8 (Aaronson and Frieden, 1972).

In contrast to the purified enzyme, the dissolved crystalline enzyme, sedimented as a single trailing 30 S boundary with no 7 S material (a similar result has been recently reported for the enzyme from chicken liver (Kono et al., 1973)). From this, it would appear that the 7 S species does not represent the monomer in the formation of the 30 S species of sheep heart. When the sedimentation pattern of the dissolved crystalline enzyme was reinvestigated after seven days, there was no change in the pattern; this is significant in that it also suggests that the 7 S form is not in equilibrium with the 30 S species. By analogy with rabbit muscle, it appears likely that the monomer of polymerisation of sheep heart PFK, at pH 8, is the 12 S form. This may appear to be an unjustified

statement, as no 12 S species was observed in the schlieren patterns; however, according to the Gilbert Theory, if the equilibrium constant for polymerisation is sufficiently high, then the monomer species may not be observed in a system undergoing rapid reversible equilibrium.

Ling et al., (1965) reported sedimentation coefficients of 13.2, 20.4 and 31.4 S for the three boundaries of rabbit muscle PFK; knowing the molecular weight of the 13 S boundary (360,000) and employing the approximation  $s_1/s_2 = (MW_1/MW_2)^{2/3}$  (Martin and Ains, 1961), they predicted molecular weights of 690,000 and 1,300,000 for the 20.4 and 31.4 S species respectively. Similarly, Paetkau and Lardy (1967), using the empirical relation  $MW = 5.4 \times 10^3 (S)^{1.66}$ , predicted molecular weights of 360,000, 770,000 and 1,600,000 for the three components in the schlieren pattern of rabbit muscle PFK. This led to the rationalisation of the observed sedimentation pattern in terms of a monomer (12 S), dimer ((19 S), tetramer (30 S) equilibrium; indeed, from the above equations and the data presented here, a system involving half monomer (7 S), (monomer) (12 S), dimer (19 S), tetramer (30 S) could be proposed for the native sheep heart enzyme. It should be pointed out, however, that the above-mentioned workers did not fully investigate the concentration-dependence of the sedimentation rates of the species involved, nor did they consider the implications of the Gilbert Theory, where, in a polymerising system undergoing rapid reversible equilibrium, the observed schlieren boundaries do not necessarily represent a particular molecular weight species (Gilbert, 1959; Bethune and Kegeles, 1961).

While this word of caution has been sounded regarding the speculation of molecular weights based on sedimentation coefficients alone, it is reasonable to deduce that the 30 S polymer represents a species with a molecular weight in the order of  $1.5 \times 10^6$ . This is consistent with x-ray analysis, which implied a protein weight of greater than  $10^6$  Daltons per asymmetric unit. This 30 S species does not represent the upper limit of polymerisation, as in one experiment at least, this form was observed in equilibrium with a 53 S species.

## 6.2 Subunit structure

Using gel filtration at pH 6, Mansour and Ahlfors (1968) demonstrated that the 7 S form of sheep heart PFK had a molecular weight of about 160,000; they also observed a 2.75 S species in a single sedimentation experiment in 5.5 M guanidine hydrochloride. Based on the latter observation, it was suggested that the 160,000 MW species was made up of six 32,000 MW subunits. This proposal is not supported by the data presented in this investigation. On maleylation, and on treatment with urea, the polydisperse enzyme dissociated to a single symmetrical 3.8 S boundary; using  $s_{20w}^0$  and  $D_{20w}^0$  values, a molecular weight of 90,000 was obtained for this form of the enzyme. A molecular weight of 80,000-86,000 was also obtained for the enzyme in 1% sodium dodecylsulphate. Further dissociation to 40,000 molecular weight form (2.3 S) was obtained when the carboxymethyl-enzyme was maleylated in 7.5 M urea. These findings are analogous to results obtained for rabbit muscle PFK, where molecular weights of 160,000-180,000 (Faetkau and Lardy, 1967), 80,000-90,000 (Faetkau et al., 1968; Uyeda, 1969) and 40,000-47,000 (Uyeda and Racker, 1965; Faetkau et al., 1968) have been reported.

Peptide mapping experiments were undertaken in a further investigation of subunit structure. Amino acid analysis of sheep heart PFK (Davis, unpublished results) indicated the presence of 46 moles of lysine and 52 moles of arginine per 85,000 g of recovered amino acids. If the 85,000 MW form represented a single polypeptide chain, or if it were composed of two dissimilar subunits, then one would expect to observe 99 tryptic peptides, 52 of which would contain arginine; on the other hand, if the 85,000 MW form contained two identical subunits, then 49 peptides, 26 of which contained arginine, would be expected. As 50 peptides were detected with ninhydrin, and 27 with Sachaguchi Reagent, it is concluded that the 85,000 - MW form is composed of two identical 40,000 - MW subunits. This conclusion is supported by the detection of only 9 peptides containing histidine, 5 containing tyrosine and 5 containing tryptophan.

From amino acid analysis, 20-, 16- and 14- peptides respectively would have been expected if the 85,000 MW species were composed of non-identical subunits. The number of peptides detected containing these residues is less than half the value expected for the situation with non-identical subunits; this situation can arise because these residues can occur more than once in a particular tryptic peptide.

Data presented by Coffee et al. (1973) on the subunit structure of rabbit muscle PFK supports the conclusions reached here for the sheep heart enzyme. While the smallest subunit they observed was the 85,000 MW species in 6 M guanidine hydrochloride, they concluded that it was composed of two identical subunits. This conclusion was based on the isolation of only 8 ( $C^{14}$ ) carboxymethyl-cysteine containing tryptic peptides; half the number expected if the 85,000 MW form represented a single polypeptide chain (amino acid analysis, discussed below, indicated that each of these peptides contained only one CM-Cys residue). Six M guanidine hydrochloride in 2-mercaptoethanol is a solvent reputed to dissociate proteins to single polypeptide chains, however, phosphoglucomutase has been shown to be resistant to dissociation by the solvent; a 62,000 MW species is observed instead of the two non-identical 31,000 MW subunits (Duckworth and Sanwal, 1972).

In a report just published on chicken liver PFK, Kono et al. (1973) provide evidence for a 100,000 MW protomer composed of two identical 50,000 MW subunits. This proposal was based on ultracentrifugation, peptide mapping and electron microscopy.

The proposed structure of two 40,000 MW subunits making up the 85,000 MW species of rabbit muscle and sheep heart PFK, appears to be at variance with substrate binding studies; Kemp and Krebs (1967) demonstrated that rabbit muscle PFK bound one mole of either AMP, ADP or fructose-6-phosphate, and 3 moles of ATP per 90,000 g of protein. However, half-site reactivity occurs in E.coli CTP synthetase (Levitzki et al. 1971), where an affinity label reacts with only one half of the susceptible sites of this enzyme. It is therefore

possible that AMP, ADP and fructose-6-phosphate bind to only one of the available 40,000 MW subunits of PFK. It has been demonstrated that there is one sulphhydryl group per 90,000 g of rabbit muscle PFK that reacts rapidly with 5,5'-dithiobis (2-nitrobenzoic acid) (Kemp and Forest, 1968); with identical subunits one might expect to observe two such sulphhydryl groups. This situation may, however, be analogous to glutamate dehydrogenase, which, although it contains six identical subunits, only three of these are reactive towards trinitrobenzene sulphonate (Coffee et al. 1971).

Maleylation of a protein effects dissociation by electrostatic repulsion. Maleic anhydride reacts specifically with primary amino groups, effectively changing the charge on the lysine residues from + 1 to -1. In the experiments described in this investigation, maleylation was conducted under two sets of conditions. In the first case, the native enzyme was maleylated in 0.1 M borate buffer pH 8.8; this resulted in the production of a 90,000 MW species. In the second set of experiments, a 40,000 MW species was observed when the carboxymethylated enzyme was maleylated in 7.5 M urea. Since carboxymethylation of the cysteine residues does not affect the molecular weight values (75,000) obtained in guanidine hydrochloride (Coffee et al, 1973), the possibility of disulphide bonds holding two identical chains together appears to be excluded. The increased dissociation could result from the fact that in 7.5 M urea, the enzyme is already dissociated to the 7 S form, thereby making the lysine residues more accessible to the maleic anhydride.

In terms of dissociation of a protein into subunits, dodecylsulphate has a two fold action; its 12-carbon chain disrupts hydrophobic interactions between subunits and the charged sulphate group causes dissociation through electrostatic repulsion. The number of detergent molecules bound to a protein is sufficiently large to swamp its intrinsic charge. The dodecylsulphate-protein complex has an extended rod structure; the length of the rod being a function of molecular weight. This makes it possible to rapidly determine subunit molecular weights by electrophoresis in dodecylsulphate; the rate of

migration, at a given gel concentration, is proportional to the length of the polypeptide chain.

Reagents such as 8 M urea and 6 M guanidine hydrochloride bring about dissociation by disruption of hydrogen bonds. The size of the dissociated protein can be conveniently measured by gel filtration or analytical ultracentrifugation.

It appears that the 80,000 MW form is composed of two 40,000 MW subunits held together by a very strong combination of nonspecific bonds. It further appears that the 180,000 MW form, the active 360,000 MW monomer and its various polymers are all held together by progressively weaker interactions. This is demonstrated by the increasingly vigorous conditions required to effect dissociation as the molecular weight decreases.

### 6.3 Primary Structure

Data on the amino acid sequence of tryptic peptides, obtained in this investigation, is summarised in table V.

It is pertinent to note that all the peptides whose complete sequences were established, contained arginine or lysine in the C-terminal position. This may appear a trivial observation, as the peptides were derived from a tryptic digestion of the enzyme; however, this does demonstrate that the TPCK-treated trypsin was free of all chymotryptic activity.

By inspection of some of the sequences, interesting observations can be made regarding the specificity of trypsin. For example, the peptide Leu-Arg-Pro-Ile-Leu-Lys and Leu-Leu-Ala-His-Val-Arg-Pro-Pro-Lys both contain an internal arginine residue; this reflects the slow rate of tryptic hydrolysis at Arg-Pro bonds. The isolation of a peptide of sequence Ile-Pro-Lys demonstrates that tryptic cleavage is not impaired when the proline residue is on the N-terminal side of the basic residue. The isolation of peptides of sequence CMCys-Lys and CMCys-Lys-Asp-Phe-Arg reflects the decreased rate of tryptic attack at basic residues flanked by two acidic side chains. The inability of trypsin to cleave

N-terminal basic residues is aptly demonstrated by the occurrence here of a peptide of sequence Arg-Phe-Asp-Glu-Ala-Met-Lys. A further subtlety in the mechanism of attack of trypsin at Arg-Lys sequences is provided by the isolation of two peptides of sequence:

Lys-Asn-Val-Leu-Gly-His-Met-Glx-Glx-Gly-Gly-Ser-Pro-Thr-Pro-Asx-Arg (1)

and a peptide of the same sequence, but lacking the N-terminal Lysine residue (2). These two peptides are derived from a sequence of -X-Y-Arg-Lys-Asn-Val-Leu-Gly-Arg in the parent enzyme. If the trypsin first cleaves between the Arg and Lys residues, then the peptide (1) is obtained, and is resistant to further cleavage; if, on the other hand, the first tryptic split occurs between the lysine and asparagine residue, then peptide (2) is obtained, plus a peptide of sequence -X-Y-Arg-Lys. This also explains the isolation of free lysine, as the latter peptide would be further hydrolysed to -X-Y-Arg and Lys.

Not only do rabbit muscle and sheep heart PFK polymerise to the same degree and have the same subunit structure, but early indications appeared to show a similar primary structure in that both proteins contained a blocked N-terminal residue. The similarity in primary structure of the two enzymes is borne out by comparison of the carboxy-methyl-cysteine containing peptides isolated here, and those isolated from rabbit muscle (Coffee et al., 1973). The rabbit muscle peptides R1, R2, R3, R4, R5, and R6 (Table VI) are clearly very similar to the sheep heart peptides S1, S2, S3, S4, S5, and S6. Peptides Ra and Rb have no complementary partner in sheep heart, and rabbit muscle contains no peptide corresponding to S7; this probably represents a species difference between the two enzymes.

Peptide R1 contains both arginine and lysine. Coffee et al. (1973) stated that 'this probably represents a Lys-Arg or Arg-Lys sequence at the C-terminus'. If by this they meant the C-terminus of the peptide, then as pointed out above, this situation could not arise, as the C-terminal residue would be cleaved by trypsin. If, on the other hand, they meant at the C-terminus of the protein, then this would be based on the assumption that peptide R1 lacked a C-terminal basic group and contained instead an internal Arg-Lys or Lys-Arg sequence. However, as pointed out previously, such a sequence would also be susceptible to

tryptic digestion, with the result that a peptide of composition R1 could not be isolated. The sheep heart peptide S1 has the same composition as R1 and the sequence of this peptide was elucidated as CMCys-Lys-Asp-Phe-Arg; there is no evidence that this represents the C-terminal sequence of the enzyme.

Forty-five tryptic peptides were purified to homogeneity and their amino acid sequence or sequence/composition established (Table V). This represents 43 unique peptides, as peptides A (90-92).54 and B .55, and B-.26 and C (17-18) are identical. Of these 43 peptides, there are three groups of overlapping peptides (the result of partial tryptic cleavage); peptide A (50-51) .84 plus peptide B .05 equals peptide B .25; peptide A (43-44) N 1.58 is derived from peptide A 58 N 1.5, and peptide A (70-71) .78 is derived from peptide A (60-63) N 1.37; for this reason, peptides B .25, A (43-44) N 1.58, and A (70-71) .78 have not been included in Table VII.

Table VII shows the amino acid composition of the various peptides, isolated in this investigation, in relation to the amino acid compositions of the original enzyme. From this table, it can be seen that, of the 406 amino acids in the 45,000 MW subunits, 350 residues have been accounted for in the isolated peptides.

It is perhaps pertinent at this juncture to make some comment on the state of play in the determination of the complete primary structure of phosphofructokinase. In this investigation 350 out of the 406 residues have been located in tryptic peptides. Repeating this work to locate the missing residues would be relatively unproductive in terms of a strategy for the determination of the complete amino acid sequence of the enzyme. The determination of overlaps necessitates the reisolation of known, as well as unknown segments of sequence. For this reason and because phosphofructokinase is difficult to isolate in large quantities, it would be more productive to subject the limited amount of enzyme available to other forms (non tryptic) of digestion.

Because of its high degree of specificity, and since PFK contains 10 methionine residues, cyanogen bromide would appear to be a most useful proteolytic agent in the determination of overlaps between the tryptic peptides. Unpublished results of Davis, however, indicated that the cyanogen bromide peptides were insoluble, and could only be dissolved successfully in a 1:1:1 mixture of phenol/acetic acid/water.

The insolubility of these peptides makes subsequent purification very difficult. It appears therefore, that it would be more fruitful to use some other approach to the overlap problem.

In the initial digestion of a protein, prior to the isolation and sequence determination of the resulting peptides, it is essential that a highly specific method of proteolysis be employed. In this respect, sequencing of the tryptic and cyanogen bromide peptides usually provides the first line of attack. Since the cyanogen bromide peptides are not amenable to purification, other less specific cleavage methods must be employed.

The most useful overlap data would probably be obtained from chymotryptic peptides of PFK. In general, chymotrypsin cleaves rapidly at aromatic residues. As the enzyme contains 8 tyrosine, 14 phenylalanine and 7 tryptophan residues, about 30 chymotryptic peptides might be expected. However, because leucine residues are also susceptible to attack, an additional 33 or more peptides could be produced. Because of the differential rate of chymotryptic attack at different sites, it is not possible to predict accurately the number of peptides that will be produced. As an initial step prior to the preparative digestion of PFK with chymotrypsin, an analytical digestion should be carried out and the extent of hydrolysis assessed by peptide mapping. A short digest would be expected to yield fewer peptides, whereas a prolonged digestion would produce more peptides, the result of increased cleavage at leucine residues.

Thermolysin digestion of PFK would be unsuitable at this stage, as this enzyme cleaves at phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine and even alanine residues. This would lead to the production of a large quantity of very small peptides. The large number of peptides produced would complicate the purification of the individual peptides, and the size of these peptides would make overlapping more uncertain. Similar comments would apply to the use, at this stage, of pepsin, subtilisin or partial acid cleavage.

One method that is of considerable use in overlap determination involves chemical modification of the basic residues to prevent tryptic attack. If the enzyme was maleylated and digested with trypsin, then only the arginine residues would be cleaved, resulting in the production of 27 peptides (perhaps 25, because of the two peptides containing Arg-Pro sequences). This small number of peptides should be

relatively easy to purify. The approach to the isolation of the maleyl-peptides could be to perform an initial purification on Sephadex G 50 in 0.2 M Ammonium bicarbonate (pH 8.5). It would then be desirable to unblock at least the lower molecular weight peptides so that they could be further fractionated by chromatography on Dowex 50 or by electrophoresis, where pH values below 6.5 are encountered.

Elucidation of the sequence of maleylated tryptic peptides would provide only the lysine overlaps and this would be of little use. The arginine overlaps could be obtained if this process was repeated after blocking the arginine residues with 1,2 cyclohexanedione. Ideally, the entire primary structure could be determined by establishing the sequence of the tryptic peptides of the enzyme, which had first been blocked with maleic anhydride, and then the sequence of the peptides of the enzyme which had been blocked with cyclohexanedione.

If this approach was used, then the sequence data on the tryptic peptides established in this investigation would be redundant, and the next step in the sequence strategy should attempt to make the maximum use of the data already accumulated. In this respect, the most fruitful approach would be to investigate the sequence of the peptides produced from a short chymotryptic digestion.

PFK has one of the most reactive thiol groups known for any enzyme (Kemp and Forest, 1968). One -SH group per 90,000 molecular weight reacts very rapidly with 5,5' dithiobis(2-nitrobenzoic acid); however, this reactivity is inhibited by the presence of Mg-ATP. Kemp suggested that the reactivity of this thiol group was an indication for the binding of Mg-ATP at the inhibitory site of the enzyme (Kemp, 1969). If this -SH group does indeed reside at the allosteric site, it would be of considerable interest to establish the amino acid sequence of this region of the enzyme. Experimentally, this would involve the following steps:

- (1) Reduction of the enzyme in 0.1 M Tris/HCl pH 8.0, containing  $\beta$ -mercaptoethanol and removal of excess reducing agent by exhaustive dialysis against 0.1 M Tris/HCl pH 8.0 under nitrogen;
- (2) The reaction of the reduced enzyme with  $C^{14}$  iodoacetate. If this reaction was performed using a 14-fold excess of enzyme over iodoacetate, then the rapidly reacting thiol group would be selectively labelled;
- (3) The remaining cysteine residues could then be carboxymethylated

in 8 M urea using unlabelled iodoacetate;

- (4) If the enzyme was now digested with trypsin, then the radioactive allosteric site peptide could be isolated and its sequence established.

Another unresolved problem in the determination of the primary structure of PFK is the identity of N- and C- terminal residues and peptides.

The decapeptide gramicidin S, which lacks detectable N and C terminal residues, has been shown to possess a cyclic structure. No precedent has, however, been established to indicate that enzymes are capable of possessing cyclic structures. Blocking groups are, however, often encountered at the N, and less frequently at the C terminus. Blocked N-termini are usually caused by the presence of an N-acyl or a pyroglutamyl group, while blocked C-termini are usually attributable to an amidated residue.

A peptide (A (43-44) -.07, p 47) with a blocked N terminus has been isolated in this investigation. From the amino acid composition of this peptide (Glu<sub>3</sub>, His<sub>3</sub>, Thr, Ala, Lys), it appears likely that the blocking group consists of a pyroglutamyl residue. This proposal is consistent with the observed electrophoretic mobilities of the peptide. It is possible however that the presence of this pyroglutamyl residue is an artifact produced during isolation.

It would be of interest to investigate the N-terminus of PFK using the pyrrolidonyl peptidase of Pseudomonas fluorescens. This enzyme specifically hydrolyses an N-terminal pyroglutamyl linkage (Doolittle and Armentrout, 1968).

With respect to the C-terminal peptide, no peptide which lacked a C-terminal arginine or lysine residue was isolated in this investigation. This leaves open the following possibilities:

- (1) That the C-terminal residue of the enzyme is arginine or lysine;
- (2) That the single basic residue contained in the incompletely sequenced peptides, C 23, D (25-26) or X (76-84) could be internal, in which case, one of these could represent the C-terminal peptide of the protein;
- (3) That the C-terminal peptide was lost during isolation.

If the C-terminus is indeed amidated, then most of the peptides whose complete sequences were established can be excluded as possible C-terminal peptides, because their electrophoretic mobilities are not consistent with the presence of an amidated C-terminus.

It is to be hoped that when both the primary and X-ray structure of PFK are eventually established, that this information will provide a model, which will rationalise the many kinetic studies which have been made on this enzyme.

TABLE V

Summary of amino acid sequence data obtained for sheep heart PFK

PEPTIDE	SEQUENCE	AMIDE
A(87-88).64	Gly-Arg *	
A(55-56).42	Ala-Val-Val-Arg ✓	
A(55-56).35	Ser-Thr-Val-Arg ✓	
A(55-56)N 1.48	Glu-Gly-Arg ✓	0
A 67 .54	Ser-Lys	
A 54 .5	Ile-Pro-Lys ✓	
A(83-84).43	Asn-Arg	1
A(43-44).36	Asn-Phe-Ala-Thr-Lys ✓	1
A 57 .5	Ser-Gly-Lys	
A(70-71)N 1.63	Glu-Arg ✓	0
A(76-77) .54	Leu-Lys ✓	
A(43-44)N 1.07	Leu-Trp-Tyr-Asp-Thr-Gly-Arg	0
A(41-42)N 1.13	Gly-Ile-Phe-Asp-Ser-Arg	0
A 39 N 1.23	Leu-Ser-Glu-Thr-Arg ✓	0
A(60-63).46	Ala-Ala-His-Asn-Leu-Val-Lys	1
A(41-42).48	Thr-Thr-Val-Lys ✓	
A(101-105).51	Leu-Arg	
A 33 N 1.26	Asp-Val-Thr-Arg	0
A (70-71) .54	Gly-Ser-Arg	
A 58 N 1.50	Cys-Lys-Asp-Phe-Arg	0
A(43-44)N 1.58	Cys-Lys	

TABLE V (Contd.)

PEPTIDE	SEQUENCE	ANIDE
A(43-44).3	Met-Gly-Ala-Lys	
A(24-26)N 0.95	Gly-Gly-Thr-Pro-Ser-Ala-Phe-Asp-Arg ✓	0
A(87-88).55	Leu-Arg-Pro-Ile-Leu-Lys -	
A(90-92).54	Leu-Leu-Ala-His-Val-Arg-Pro-Pro-Lys ✓	
A(60-63)N 1.18	Glu-Ser-Tyr-Arg ✓	0
A(45-46).43	Leu-Gly-Val-Lys	
A(70-71).78	Arg	
A(50-51).84	Lys	
A(60-63)N 1.37	Arg-Phe-Asp-Glu-Ala-Met-Lys ✓	0
A(43-44)-.07	Pyroglu -(2Glu,3His,Thr)-Ala-Lys	0
B .55	Leu-Leu-Ala-His-Val-Arg-Pro-Pro-Lys ✓	
B .05	Asn-Val-Leu-Gly-His-Met-Glx-Glx-Gly-Gly-Ser-Pro-Thr-Pro-Asx-Arg	3
B .25	Lys-Asn-Val-Leu-Gly-His-Met-Glx-Glx-Gly-Gly-Ser-Pro-✓	
B-.26	Leu-Pro-Leu-Met-Glx-Cys-Val-Glx-Val-Thr-Lys ✓	1
B-.33	Ile-Thr-Ala-Glu-Glu-Ala-Thr-Lys	0
B-.23	Ile-Phe-Ala-Asn-Thr-Pro-Asp-Ser-Gly-Cys-Val-Leu-Gly-Met-Arg ✓	1
B-.44	Trp-Glx-Glx-Gly-Asx-Leu-Leu-Ser-Asx-Leu-Gly-Lys ✓	1
B N 1.1	Val-Leu-Val-Val-His-Asx-Gly-Phe-Glx-Gly-Leu-Ala-Lys ✓	
C(17-18)	Leu-Pro-Leu-Met-Glx-Cys-Val-Glx-Val-Thr-Lys ✓	
C(30-32)	Glu-Leu-Leu-Phe-Glx-Pro-Val-Asx-Glx-Leu-(Glx,Thr,Ala,Gly,Phe)- (2Glx,Asx,Thr)-Glu-His-Arg ✓	?
C 23	(Arg,Cys,4Asx,4Thr,2Ser,Glu,7Gly,2Ala,Val,2Ile,3Tyr,Phe)	?

TABLE V (Contd.)

PEPTIDE	SEQUENCE	AMIDE
D(25-26)	Leu-Gly-Val-Ala-Val-Met-(Cys, 5Asx, 4Thr, 4Ser, 6Glx, 3Pro, 4Gly, 5Ala, 3Val, Met, 2Ile, 2Leu)-Arg	?
Y(50-54)	Cys-Asn-Glu-Asn-Tyr-Ile-Phe-Asn-Trp-Thr-Asp-Phe-Thr-Leu-Tyr-Ser-Glu-Glu-Gly-Lys	3
X(76-84)	His-Trp(?)-Gly-Tyr-(Asp, Gly)-Phe, Val-Phe-Ile-Pro-Glx-Cys-Pro-Glx-Asx-Asx-(His, Pro, Glx)-(Leu, Ala, Gly, Ser, Asp), 3Leu, Ile, Val, Ala, Glx, Ser, Thr, Arg	?

TABLE VI

Comparison of amino acid compositions of Cm-Cys-containing peptides of rabbit muscle (R) and sheep-heart (S) FFI.

RESIDUE	R.1	S.1	R.2	S.2	R.3	S.3	R.4	S.4	R.5	S.5	R.6	S.6	R.a	R.b	S.7
Lys	1.1	1.0	0.9	1.0											1.0
His											1.0	1.5			
Arg	1.4	1.0			1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	
Cm-Cys	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	0.9	1.0
Asp	1.6	1.0			3.0	2.0	5.0	5.5	4.2	4.2	5.9	4.3	1.7	2.1	4.0
Thr			1.0	1.0	1.6	1.0	4.1	4.0	4.2	3.6	2.0	0.9		1.0	2.0
Ser					1.6	1.0	3.8	4.0	1.9	2.3	2.2	1.7		1.0	1.0
Glu			2.0	2.0	0.9	0	5.5	6.3	0.7	1.3	4.1	3.8			3.0
Pro			1.0	1.0	1.5	1.0	2.9	?			2.0	3.2		1.1	
Gly					3.2	2.0	5.7	5.0	6.6	6.8	3.9	2.9	1.7	2.2	1.0
Ala					1.7	1.0	7.5	5.7	1.9	2.3	2.3	2.1		1.0	
Val			1.7	2.0	1.7	1.0	4.9	4.4	0.7	1.5	2.0	1.9		0.6	
Met			0.9	1.0	1.1	1.0	2.8	1.6						0.9	
Ile					1.0	1.0	0	0.8	1.8	2.3	2.2	1.6			1.0
Leu			2.0	2.0	1.7	1.0	5.0	4.5	2.7	3.0	3.9	3.4		0.7	1.0
Tyr											1.1	1.0			2.0
Phe	0.9	1.0			1.0	1.0			1.2	1.3	1.1	1.3			2.0
Trp															1.0

Code number of sheep-heart peptides in results section: S.1 = 1 53 N 1.50; S.2 = B -.26 and C (17-18); S.3 = B -.23; S.4 = D (25-25); S.5 = C23; S.6 = K (76-84); S.7 = Y (50-54).

TABLE VII

Amino acid composition of peptides in relation to composition of the original enzyme.

PEPTIDE	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp
A(87-88).64			1						1									
A(55-56).42			1							1		2						
A(55-56).35			1		1	1						1						
A(55-56)N 1.48			1				1		1									
A 67 .54	1					1												
A 54 .5	1							1						1				
A(83-84).43			1	1														
A(43-44).36	1			1	1					1								1
A 57 .5	1					1			1									
A(70-71)N 1.63			1				1											
A(76-77).54	1														1			
A(43-44)N 1.07			1	1	1				1						1	1		1
A(41-42)N 1.13			1	1		1			1				1				1	
A 39 N 1.23			1		1	1	1								1			
A(60-63).46	1	1		1						2		1			1			
A(41-42).48	1				2							1						
A(101-105).51			1												1			
A 33 N 1.26			1	1	1							1						
A(70-71).54			1			1			1									
A 58 N 1.5	1		1	1								1						1

TABLE VII (Contd.)

PEPTIDE	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp
A(43-44).3	1								1	1			1					
A(24-26)N 0.95			1	1	1	1		1	2	1								.1
A(87-88).55	1		1					1						1	2			
A(60-63)N 1.18			1			1	1										1	
A(45-46).43	1								1			1			1			
A(50-51).84	1																	
A(60-63)N 1.37	1		1	1			1			1			1					1
A(43-44)-.07	1	3			1		3			1								
B .55	1	1	1					2		1		1			2			
B .05		1	1	2	1	1	2	2	3			1	1		1			
B-.33	1				2		2			2				1				
B-.23			1	2	1	1		1	2	1	1	1	1	1	1	1		1
B-.44	1			2		1	2		2						3			1
B N 1.1	1	1		1			1		2	1		3			2		1	
C(17-18)	1				1		2	1			1	2	1		2			
C(30-32)		1	1	2	2		7	1	1	1		1			3			2
C 23			1	4	4	2	1		7	2	1	1		2	3			1
D(25-26)			1	5	4	4	6	?	5	6	1	5	2	2	3			
Y(50-54)	1			4	2	1	3		1		1			1	1	2	2	1
X(76-84)		2	1	5	1	2	4	3	2	2	1	2		2	4	1	1	1

TABLE VII (Contd.)

PEPTIDE	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp
Total residues located	20	10	24	36	27	20	38	13	35	22	7	24	7	12	33	5	13	4
No.residues per subunit*	23	10	26	37	27	23	43	15	37	33	7	31	10	22	33	8	14	7

\*Number of moles of each residue per 45,000 g of recovered amino acids (Davis, unpublished results).

## APPENDIX I

The following solutions were used when assaying for phosphofructokinase:

- Solution A, containing: 8 ml 0.5 M Tris/HCl, pH 8.0,  
0.2 ml 0.1 M magnesium chloride,  
2 ml 0.1% BSA,  
44 mg cysteine,  
6 ml distilled water.
- Solution B, containing: 0.2 ml 0.1 M fructose-6-phosphate,  
1 ml  $10^{-2}$  M ATP (pH 8.2)  
2 ml distilled water
- Solution C, containing: 20  $\mu$ l -glycerophosphate dehydrogenase/triosephosphate isomerase,  
10  $\mu$ l aldolase,  
0.47 ml  $10^{-2}$  M Tris/HCl pH 7.5.
- Solution D, containing: 1.4 mg  $\text{NADH}_2$  per ml of water.

Solutions A, B, C and D were made up fresh daily. The assay system was prepared by mixing: 0.22 ml of A, 0.08 ml of B, 0.05 ml D and 0.05 ml C in a cuvette immediately before use. To this solution was added 0.05 ml of PFK extract. The PFK sample was diluted to a suitable strength prior to assay using a solution containing: 2 ml  $10^{-1}$  2-mercaptoethanol, 2 ml 0.1% BSA, 0.2 ml 1 M Tris/HCl pH 8.0 and 15.8 ml of water.

### CORRIGENDA

Throughout the text, where a quantity of peptide or amino acid is quoted in nano moles, the symbol nm has been used: the correct SI symbol is however n mol.

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