THE CARBOXYL TERMINAL SEQUENCE OF SHEEP HEART PHOSPHOFRUCTOKINASE

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY AT MASSEY UNIVERSITY

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ABSTRACT

The aim of this project was to investigate the carboxyl terminal sequence of sheep heart phosphofructokinase. Existing methods for the preparation of the enzyme from sheep heart proved to be unsuitable because of the insoluble nature of the purified enzyme. Consequently it was necessary to develop a suitable purification scheme before sequencing work could be commenced. Purification strategies involving magnesium ion precipitation of phosphofructokinase, DEAE-cellulose chromatography and agarose chromatography were tried before a suitable method was found.

The carboxyl terminal sequence of phosphofructokinase was investigated by the tritium labelling method of Matsuo, by carboxypeptidase Y digestion and by isolation of the carboxyl terminal peptide generated by tryptic digestion. Digestion of phosphofructokinase by carboxypeptidase Y resulted in the release of leucine, isoleucine and phenylalanine. However attempts to characterise the carboxyl terminal residue by tritium labelling, and to isolate the carboxyl terminal peptide were unsuccessful. The carboxyl terminal sequence suggested by these results and the possible amidation of the carboxyl terminal residue are discussed.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP	Adenosine-5$^{'}$-diphosphate
AMP	Adenosine-5$^{'}$-monophosphate
ATP	Adenosine-5$^{'}$-triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3$^{'}$, 5$^{'}$-monophosphate
DCC	Diphenylcarbamyl chloride
DEAE	Diethylaminoethyl
EDTA	Disodium ethylenediaminetetracetate
F-1,6-bisP	Fructose-1,6-bisphosphate
F-6-P	Fructose-6-phosphate
NAD^{+}	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
PFK	Phosphofructokinase
POPOP	1,4-bis-2- (5-phenyloxazolyl) benzene
PPO	2,5-diphenyloxazole
SDS	Sodium dodecyl sulphate
TEMED	N,N,N$^{'}$,N$^{'}$-tetramethylene ethylene diamine
Tris	Tris (hydroxymethyl)amino methane

1. INTRODUCTION

Phosphofructokinase (E.C.2.7.11 ATP:D-fructose-6-phosphate 1-phosphotransferase) catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate.

$$F\text{-}6\text{-}P + MgATP^{-4} \longrightarrow F\text{-}1,6\text{-bisphosphate} + MgADP^{-3} + H^{+}$$

Phosphofructokinase (PFK) is an allosteric enzyme. The number of effectors and the sensitivity of the enzyme to them depends on the source of the enzyme. Generally the bacterial enzyme is influenced by a small number of metabolites, while the activity of the eucaryotic enzyme is affected by numerous compounds. Allosteric properties of the eucarvotic enzyme usually involve inhibition by high levels of ATP (1), and inhibition by citrate at inhibitory concentrations of ATP (2). Relief of ATP inhibition and activation of the enzyme are caused by inorganic phosphate (3), AMP (4), cAMP (5), ADP (3), F-6-P and F-1,6-bisP (3).

Because of its allosteric nature PFK is an important regulatory site of glycolysis (9). At high ATP levels the enzyme is inhibited, resulting in decreased glycolytic flux and decreased production of ATP. Low levels of ATP result in high levels of AMP and ADP via the adenylate equilibrium reaction (10), which stimulates PFK activity. This results in an increased glycolytic rate and production of ATP.

There is some evidence that PFK activity is also regulated by covalent phosphorylation and dephosphorylation. The liver and muscle enzymes have been reported to contain phosphate covalently bound to a serine residue (11),(12),(13),(14). The activity of the liver enzyme appears to be stimulated by phosphorylation, but no change in the activity of the muscle enzyme due to phosphorylation has yet been demonstrated. As yet the nature of the Kinase and phosphatase enzymes involved in the phosphorylation of PFK, and the regulation of these enzymes has not been elucidated.

PFK activity is also affected by hormones. Infusion of glucagon or epinephrine in rats causes a rapid reduction of PFK activity (15),(16), while infusion of insulin increases activity (15). The biochemical mechanism responsible for these hormone dependant changes in PFK activity is not yet clear. One possibility

is that the effects of these hormones are linked to phosphorylation and dephosphorylation of PFK via cAMP, a Kinase and a phosphatase enzyme, in a system comparable to that involved in glycogen metabolism (17).

Sheep heart PFK is a multimeric enzyme. It appears to consist of protomers of molecular weight 80,000-90,000. Brennan et al (19) determined a protomer molecular weight of 80,000-90,000 by SDS gel electrophoresis. However the protein was further dissociated to subunits of molecular weight 40,000 when carboxymethylated PFK was maleylated in 7.5M urea. The number of trypic peptides determined by electrophoretic mapping was found to be consistent with a protomer of molecular weight 85,000 and which consisted of two identical subunits. Similar results were obtained with rabbit muscle PFK (20) which were later found to be incorrect because of limitations of the peptide mapping system used. Walker et al (21) performed peptide mapping under different conditions. They demonstrated that the rabbit muscle PFK subunits appeared to have a molecular weight of 80,000, consisted of a single unique sequence and that the subunits are similar if not identical.

The sheep heart and rabbit muscle enzymes appear to be very similar. They have similar amino acid compositions (23) and some sequence homology (21),(24). The kinetic and allosteric properties of the two enzymes are similar (22). In addition the same isozyme has been reported for the rabbit skeletal muscle enzyme and the rabbit heart muscle enzyme (25). Hence the molecular weight of the sheep heart PFK subunits may also be 80,000 and the subunits similar or identical.

The smallest active form of the sheep heart enzyme is a tetramer of molecular weight 320,000 (26). The tetramer can be reversibly dissociated to a less active dimer of molecular weight 180,000 (18), and reversibly associated to several higher polymeric forms at high PFK concentrations. PFK polymers of molecular weight up to 2×10^6 have been reported (22).

The amino acid sequence of a protein is primarily responsible for the conformation that the protein adopts, and is therefore also responsible for the biological activity of the protein.

Therefore a knowledge of the primary structure of an enzyme is necessary for a full understanding of how that enzyme functions. This project is part of an ongoing investigation of the amino acid sequence of sheep heart PFK being performed in this laboratory. It is useful when sequencing proteins to determine the sequence of the amino and carboxyl terminals at an early stage. This gives information about the number of polypeptide chains in a multichain protein in the absence of more complete sequence data. It also provides points of reference when reconstructing the entire sequence of the protein by ordering cleavage products. The amino terminal sequence of PFK has already been determined (60), and it was the aim of this project to elucidate the carboxyl terminal sequence.

There are three general methods for investigating the carboxyl terminal sequence of proteins. The first is by exclusively labelling the carboxyl terminal residue, hydrolysing the protein and then identifying the labelled amino acid in the mixture of amino acids. The second method is by sequentially removing amino acids from the carboxyl terminus and identifying them. This can be performed either chemically or enzymically. The third method is by isolation of the carboxyl terminal peptide from the mixture of peptides generated by enzymic or chemical cleavage of a protein. It is then sequenced from the "back door" via its amino terminal using conventional sequencing techniques.

The two most widely used methods in the first group are the Akabori hydrazinolysis method, and the tritium labelling method of Matsuo. In the former method (27) the protein is exhaustively hydrazinolysed. This results in the release of all the constituent amino acids as amino acid hydrazides except for the carboxyl terminal residue which is released as a free amino acid. Consequently the carboxyl terminal amino acid can be separated from the bulk hydrazides by taking advantage of the charge differences between them.

In the Matsuo tritium labelling method (29),(30) the protein is dissolved in a solvent system consisting of acetic anhydride and pyridine in tritiated water. In the presence of acetic

anhyride a cyclic oxazolinone is formed at the carboxyl terminal of proteins. The carbon at position 4 of the oxazolinone has a hydrogen atom which is capable of being exchanged with water in a base catalysed reaction. In the presence of pyridine and tritiated water the carboxyl terminal residue is quantitatively labelled with tritium. It can therefore be identified if the protein is hydrolysed after the tritiation reaction (See Figure 1).

Recently a new method for determination of the carboxyl terminal amino acid has been reported (31). The protein is reacted with dimethyl-biguanide. This results in the formation of a carboxyl terminal triazine structure. The modified protein is then digested by a protease from Streptomyces griseus which releases the triazine and other amino acids. The triazine amino acid can then be identified (See Figure 2).

The carboxyl terminal amino acid can also be identified by reducing it to the corresponding amino alcohol. This can be identified after the protein has been hydrolysed. Various hydride reducing reagents have been used in this reaction. For example - $LiAlH_4$ (32), $LiBH_4$ (33), $NaBH_4$ (34) and sodium dihydrobis(2-methoxyethoxy)aluminate (35).

There are two general methods by which proteins can be sequentially degraded from their carboxyl termini. They are by digestion with a carboxypeptidase enzyme and by chemical sequential degradation. The former method has been used extensively in sequencing work. However the use of carboxypeptidase enzymes has been limited by their inability to release certain residues. For example carboxypeptidase A does not release carboxyl terminal proline, arginine or lysine. Carboxypeptidase B only releases arginine and lysine residues. Recently a number of new carboxy-peptidases with considerably broader specificity than carboxy-peptidases A and B have been isolated. For example carboxypeptidase C from citrus fruit (36), carboxypeptidase Y from bakers' yeast (37),(39) and penicillocarboxypeptidases S-1 and S-2 from the mould Penicillium janthinellum (38),(40). These enzymes release most amino acids including proline from the carboxyl termini of proteins.

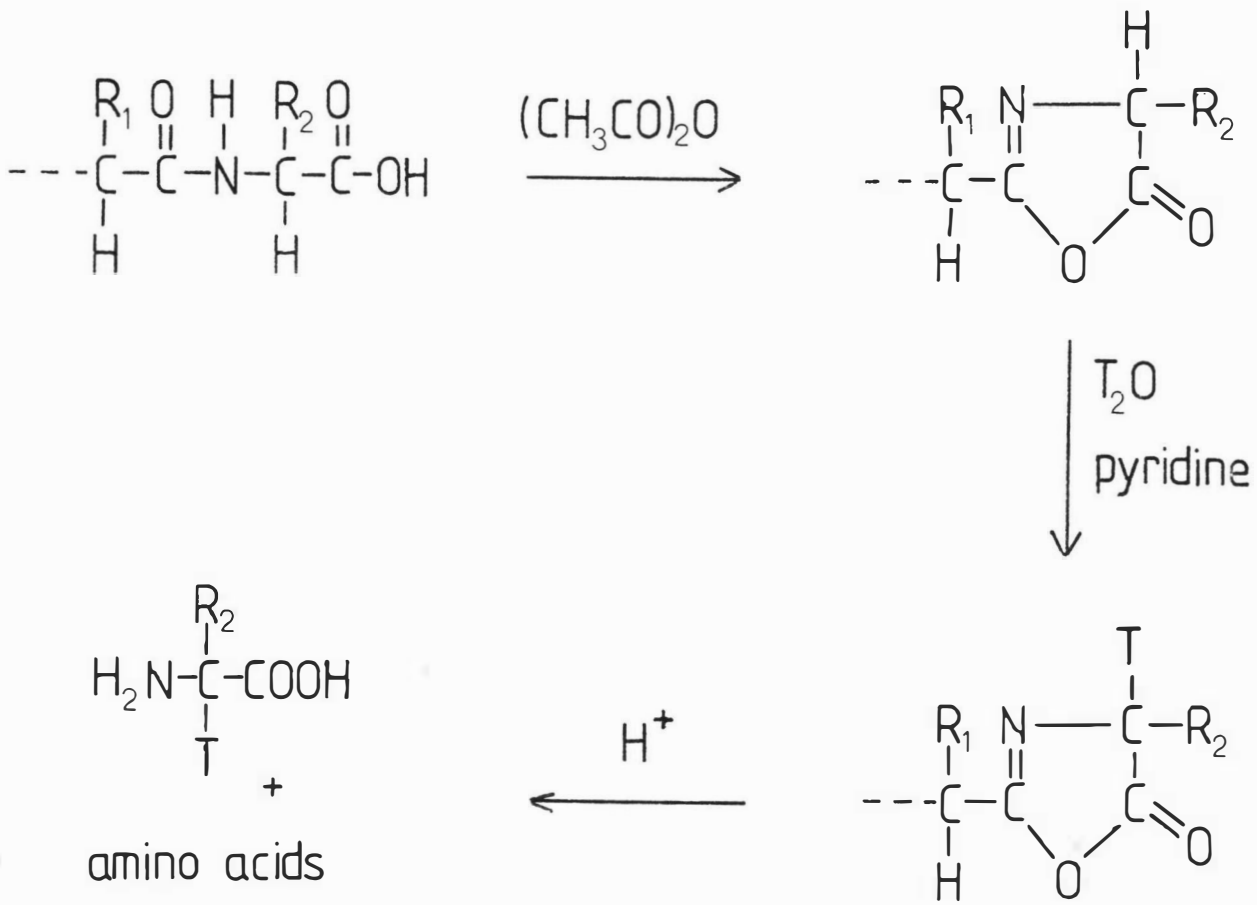


Figure 1 The tritium labelling reaction

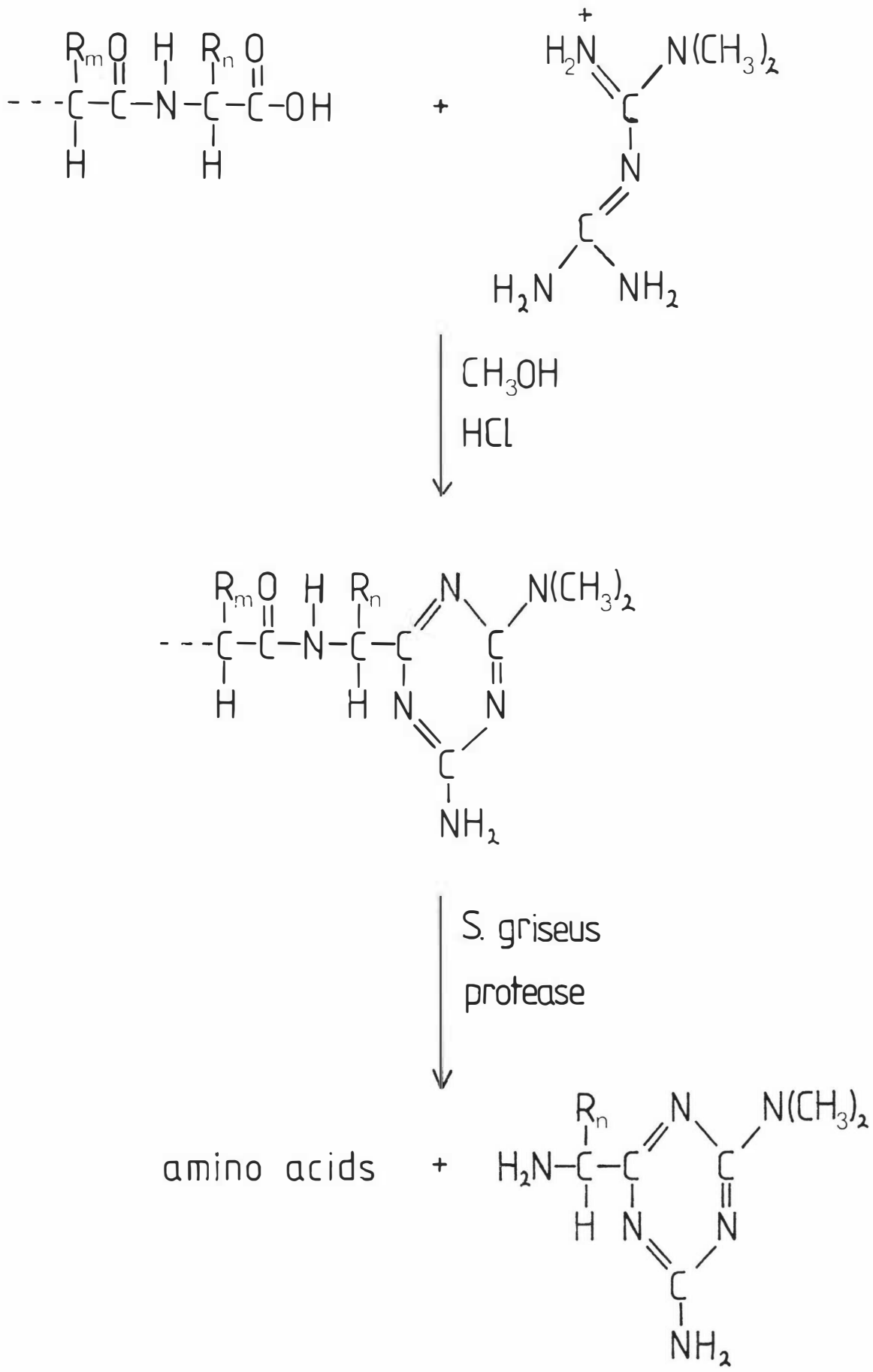


Figure 2 The triazine reaction

Several carboxyl terminal chemical degradative strategies have been devised. However all of them have limitations and are not as well developed as amino terminal sequencing methods. Bailey (42) extended the amino alcohol end group determination strategy to a sequential method. In this method the carboxyl terminal amino acid was reduced to an amino alcohol with $LiBH_4$, and then cyclised to an imidate ester with $POCl_3$. The imidate ester opened in acid to give an open chain ester. This could be reduced with $LiBH_4$ to give a free amino alcohol and a shortened polypeptide on which the reaction could be repeated (see Figure 3). The use of this method was limited by side reactions which resulted in reductive cleavage of peptide bonds.

A method originally developed by Schlack and Kumpf (44) has recently been revitalised by Stark (45) and others (46). The polypeptide was reacted with ammonium thiocyanate and acetic anhydride resulting in the formation of a carboxyl terminal thiohydantoin. This could be cleaved under mild conditions to give a free thiohydantoin characteristic of the carboxyl terminal residue. The shortened polypeptide could then be reacted with ammonium thiocyanate again (see Figure 4). Lengthy sequence determinations were precluded in this method because of the failure of carboxyl terminal proline and aspartic acid to be released, and because of incomplete hydrolysis of the acylthiohydantoin.

A strategy similar to the Stark degradation scheme is currently being developed by Tarr (47). This method involves the reaction of the polypeptide with an alkylisothiourea. This results in the formation of a carboxyl terminal cyanamide which can then be cyclised and hydrolysed as an iminohydantoin characteristic of the carboxyl terminal residue (see Figure 5). As in the Stark method carboxyl terminal proline and aspartic acid are resistant to cleavage.

In 1955 Boissonas (48) proposed an electrochemical procedure in which oxidation of the carboxyl terminal residue lead to fragmentation with loss of CO_2 and formation of a carboxyl terminal methylolamine. It was claimed that the methylolamine could then be selectively hydrolysed (see Figure 6). However Boissonas was

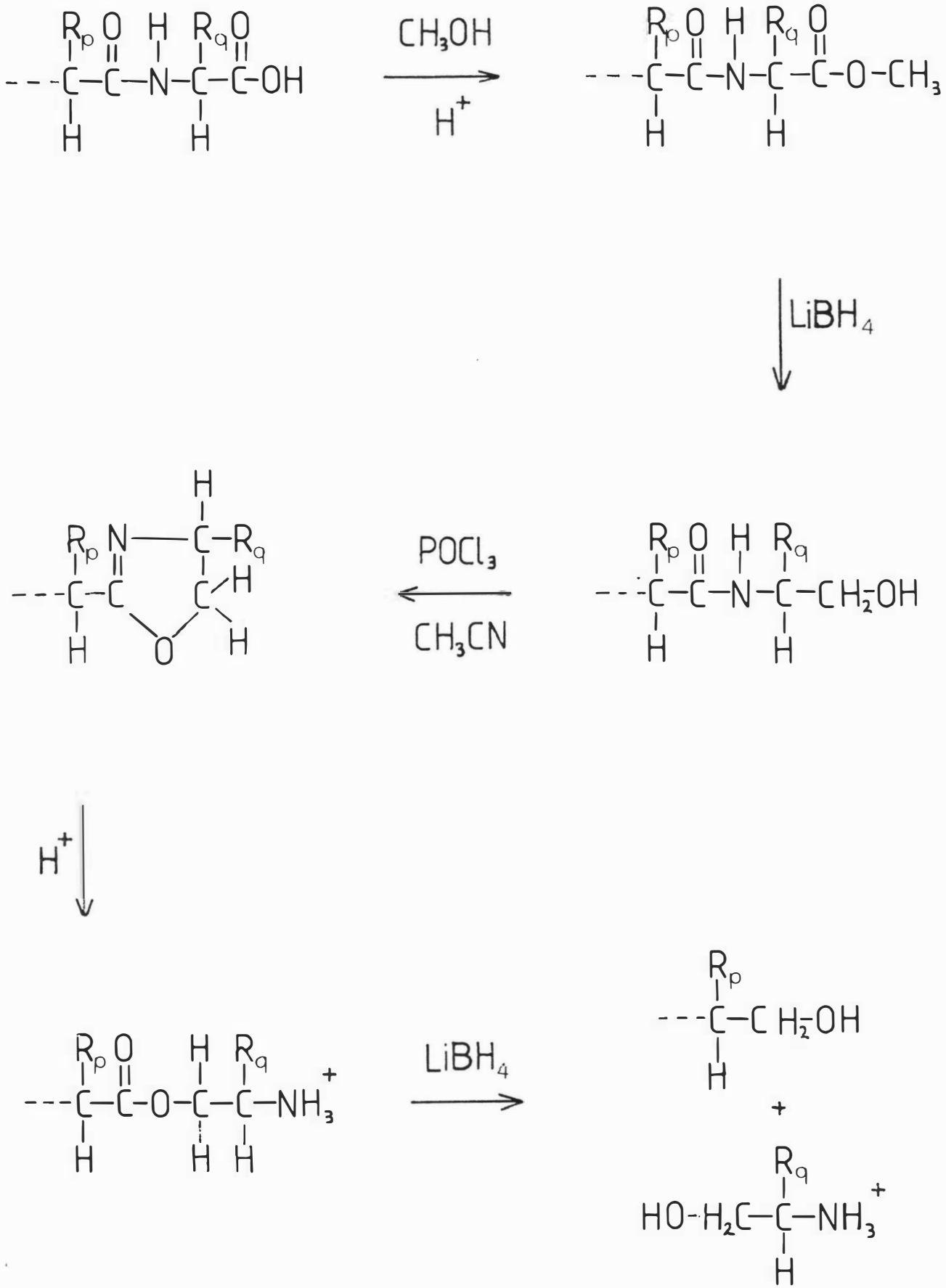


Figure 3 The sequential method of Bailey

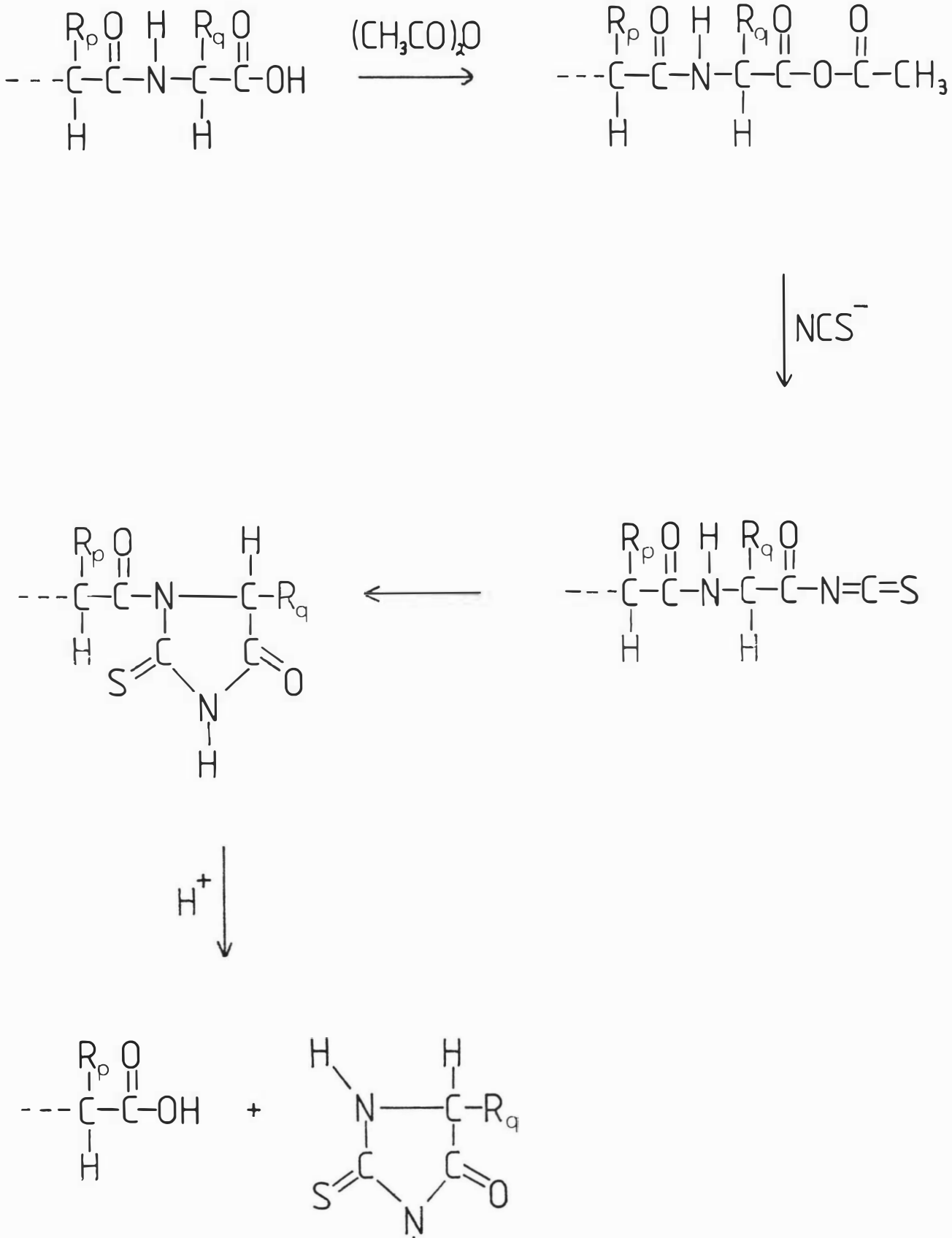


Figure 4 The sequential method of Stark

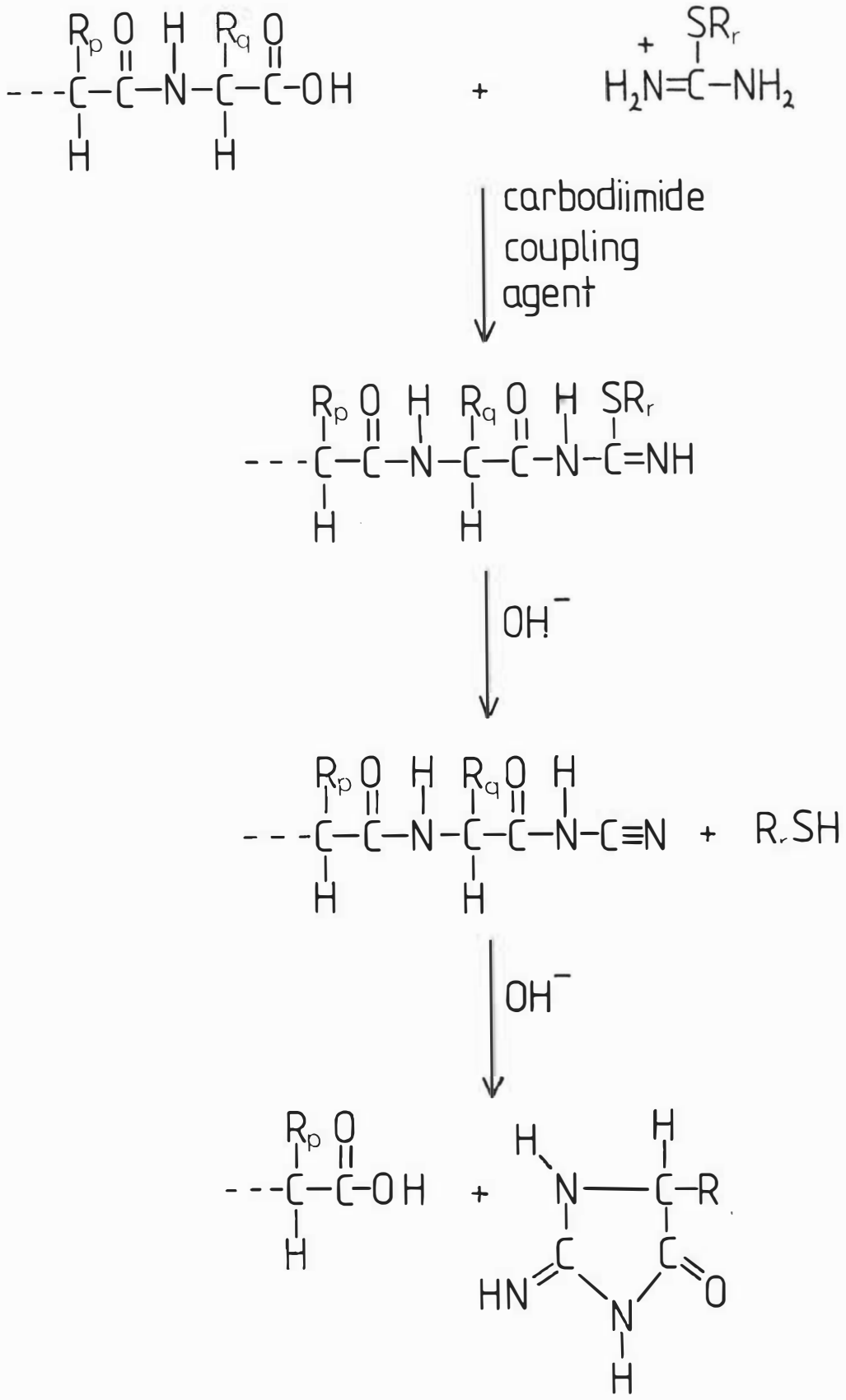


Figure 5 The sequential method of Tarr

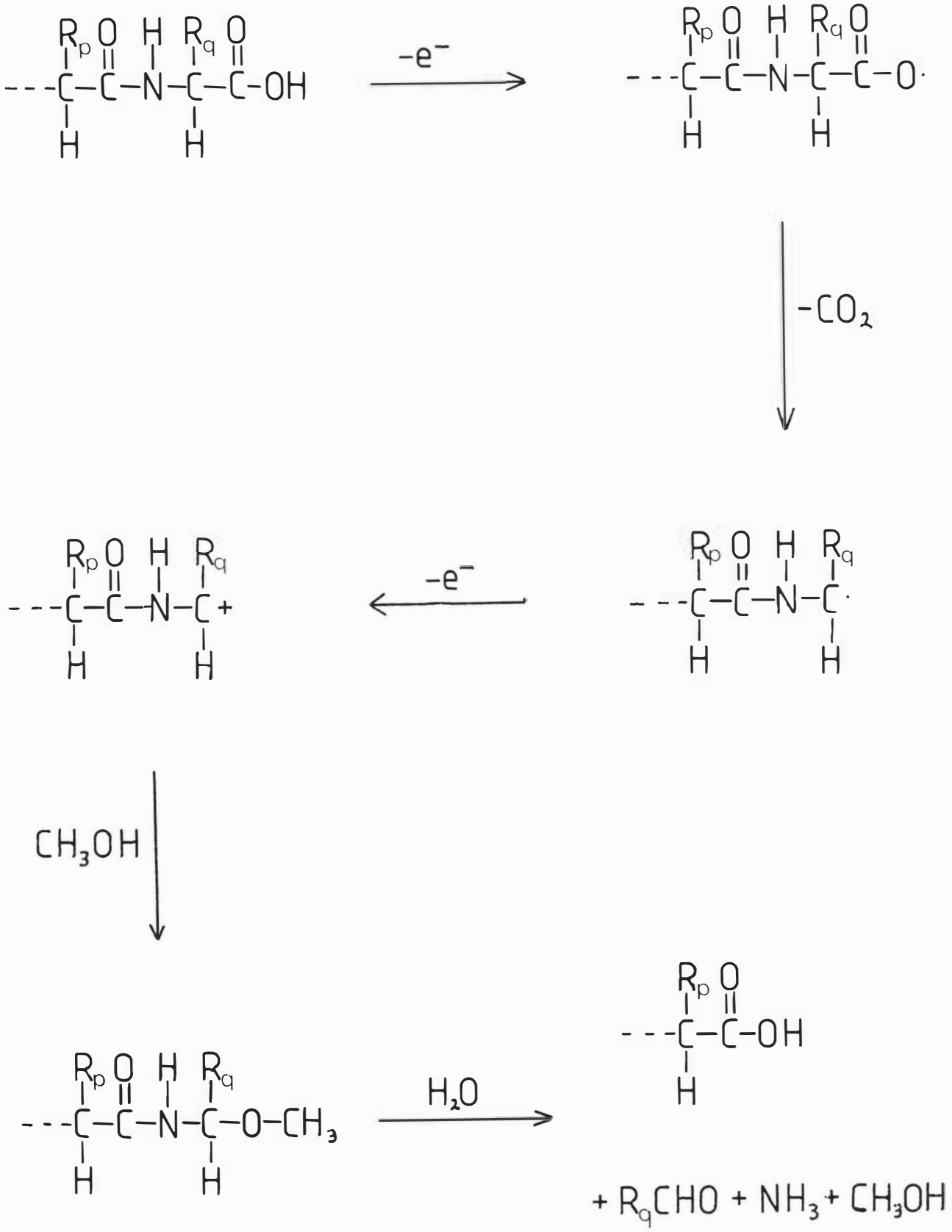


Figure 6 The Boissonas electrochemical reaction

unable to proceed more than two or three residues into a polypeptide chain. It is likely that the methylolamine was being only partially hydrolysed and partially converted to an unreactive amide (41).

Very recently Parham and Loudon (49), (50) reported upon the development of a solid phase sequence degradation strategy. The peptide was attached to porous glass beads and an azide synthesized at its carboxyl terminus. Degradation of this azide results in the release of an aldehyde characteristic of the carboxyl terminal residue, and formation of a shortened peptide amide. Reaction of the peptide amide with 1,1-bis (trifluoroacetoxy) iodobenzene caused its conversion to an isocyanate. This could then be hydrolysed giving a new peptide amide which could be degraded by repetition of the hydrolysis reaction (see figure 7).

There are a number of strategies by which the carboxyl terminal peptide can be separated from, or identified in a mixture of peptides generated by enzymic or chemical cleavage of a protein. Hargrave and Wold (51) developed a method involving the blockage of protein carboxyl groups with glycinamide. After fragmentation of the modified protein only the carboxyl terminal peptide is totally devoid of free carboxyl groups. It can consequently be separated from the other peptides on the basis of charge differences.

The same general idea was proposed by Duggleby and Kaplan (52) who preferred the use of ethanolamine as the blocking group. Fong and Hargrave (53) proposed a method in which the protein is maleylated and digested with trypsin. The trypsin peptides are then remaleylated to block their α-amino groups. The carboxyl terminal peptide will then have no positively charged group at low pH. This fact can be employed to separate it from all other peptides, each of which will contain a positively charged arginine residue.

The carboxyl terminal peptide can be identified by a combination of electrophoretic peptide mapping and carboxypeptidase digestion (56),(57). One sample of the protein is digested by a carboxypeptidase enzyme and is then selectively cleaved and subjected to electrophoretic peptide mapping. A sample of the protein which has not been digested by the carboxypeptidase

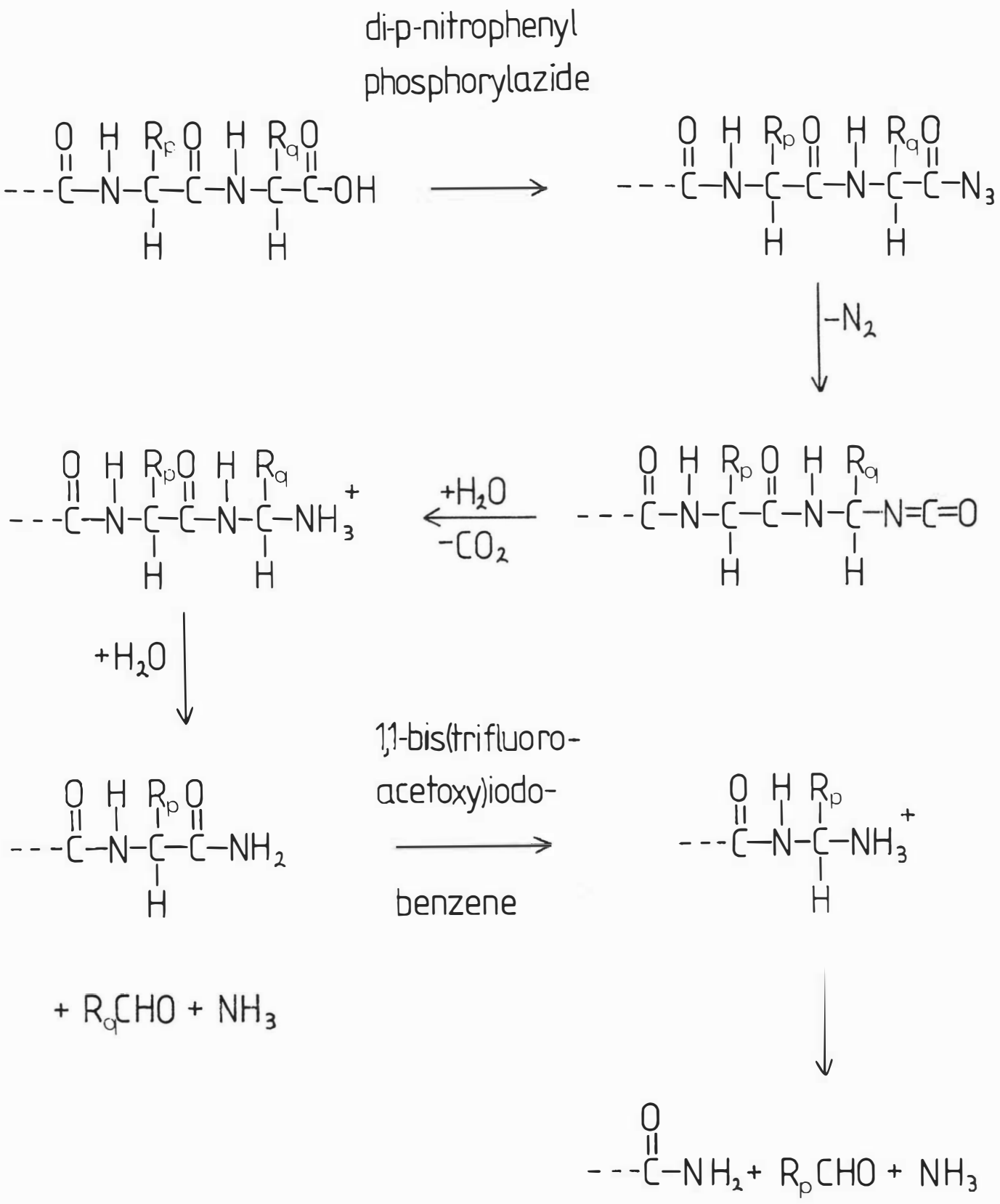


Figure 7 The sequential method of Parham and Loudon

is selectively cleaved in the same way is electrophoresed in parallel to the first sample. By comparison of the two peptide maps the carboxyl terminal peptide can be identified. It will be the only peptide to migrate to a different location on the two maps because of its reduced size in the sample subjected to carboxypeptidase digestion (56).

Proteins can be labelled to facilitate identification of the carboxyl terminal peptide. If the carboxyl terminal residue is labelled by the Matsuo tritium labelling reaction prior to selective cleavage, then the carboxyl terminal peptide will be radioactively labelled (54).

Horn (55) has proposed a method in which all peptides except the carboxyl terminal peptide are radioactively labelled. The protein is selectively cleaved by cyanogen bromide which specifically cleaves on the carboxyl side of methionine residues. This results in a mixture of peptides all with carboxyl terminal homoserine or homoserine lactone residues, except for the carboxyl terminal peptide. All the former peptides can then be aminated with ^{14}C ethylene diamine.

In this project the carboxyl terminal sequence of sheep heart phosphofructokinase was investigated by three methods. By the tritium labelling method of Matsuo, by carboxypeptidase Y digestion and by the peptide isolation strategy of Hargrave and Wold.

2. METHODS

2.1 Materials

General laboratory chemicals were supplied by British Drug Houses, May and Baker, and the Sigma Chemical Company. Sheep hearts were obtained from the Co-operative Wholesale Society. Rabbit muscle aldolase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, bovine pancreas ribonuclease A, DCC treated trypsin and bovine serum albumin were all purchased from the Sigma Chemical Company. Carboxypeptidase Y was donated by Drs C.H. Moore and J.C. McIntosh. Sepharose 6B was obtained from Pharmacia Fine Chemicals. Dowex 1-X4 and 50W-X2 (200-400 mesh) ion exchange resins and Bio-Gel P2 were from Bio-Rad Laboratories. DEAE-cellulose was from Whatman, and Amberlite MB-3 resin from British Drug Houses. Tritiated water (1.0Ci per 0.2 ml) was purchased from the Radiochemical Centre.

Before use maleic anhydride was recrystallised from chloroform, iodoacetic acid was recrystallised from carbon tetrachloride, and solutions of urea were deionised by passing through Amberlite MB-3 resin. Py ridine was redistilled after refluxing over ninhydrin, then redistilled after refluxing over NaOH. Acetic anhydride was redistilled after refluxing over calcium carbide. Hydrochloric acid was distilled in an all glass distillation apparatus.

2.2 Purification of phosphofructokinase

Fat and connective tissue was removed from fresh sheep hearts. The heart muscle was then chopped into small pieces and stored frozen. The purification was started by mincing and blending 1.8kg of frozen heart in cold wash buffer (10mM tris-HCl pH 8.6, 2mM EDTA). The enzyme was then dissolved by gently blending it in cold extraction buffer (10mM tris-HCl pH 8.6, 50mM $MgSO_4$, 5mM 2-mercaptoethanol, 0.68mM ATP, 0.1mM EDTA).

The extract (fraction 1)was adjusted to a pH of 8.0 with saturated tris solution. It was then heated rapidly to 57^{o}C, held at this temperature for three minutes and then cooled rapidly to

between 0^{o} and $4^{o}C$ in a methanol bath. Insoluble material was removed by centrifugation while the soluble enzyme remained in the red supernatant (fraction 2). PFK was precipitated with ammonium sulphate between 38% and 55% saturation (the supernatant after 38% saturation is fraction 3). The enzyme was extracted from the precipitate by dialysis overnight against phosphate buffer (20mM phosphate pH 8.0, 10mM 2-mercaptoethanol). The redissolved enzyme (fraction 4) was precipitated by adjusting the pH of the solution to 6.1 with saturated KH_2PO_4 solution.

At the final step in the purification of PFK one of three different fractionation procedures was used. In method A the enzyme in the pH 6.1 precipitate was redissolved by extraction in tris buffer (10mM tris-HCl pH 8.6, 5mM 2-mercaptoethanol, 0.1mM ATP, 10μM F-1,6-bisP). The soluble enzyme (fraction 5A) was then precipitated by dialysis against a buffer containing a high magnesium ion concentration (20mM imidazole-HCl pH 7.0, 50mM $MgCl_2$, 5mM 2-mercaptoethanol, 0.1mM ATP). The final insoluble PFK fraction is fraction 6A.

In method B the pH 6.1 precipitate was dissolved in a minimum volume of tris buffer (10mM tris-HCl pH 8.0, 5mM 2-mercaptoethanol, 1.0mM $MgSO_4$, 0.1mM ATP, 10μM F-1,6-bisP). The enzyme (fraction 5B) was loaded onto a 3.0cm x 17.0cm column of DEAE-cellulose which had been equilibrated in the above solution. It was then eluted with a linear gradient consisting of 350ml of equilibration buffer and 350ml of equilibration buffer containing 0.8M KCl. The column was eluted at a flow rate of 24.0ml per hour and 8.0ml fractions were collected. Fractions containing high levels of PFK activity were pooled and concentrated by ultrafiltration (fraction 6B).

Method C is an adaptation of the method of Hussey et al (58). The pH 6.1 precipitate was dissolved in a minimum volume of high salt buffer (50mM tris-phosphate buffer pH 8.0, 5mM 2-mercaptoethanol 1mM EDTA, 1M ammonium sulphate). The enzyme (fraction 5C) was then chromatographed on a 2.6cm x 80.0cm column of Sepharose 6B equilibrated and eluted with high salt buffer. The column was eluted at a flow rate of 20ml per hour and 10ml fractions were collected. Fractions containing high PFK activity were pooled and precipitated by 75% ammonium sulphate saturation. The precipitate

was dissolved in a minimum volume of low salt buffer (50mM tris-phosphate pH 8.0, 5mM 2-mercaptoethanol, 1mM EDTA, 0.2mM F-1,6-bisP). The enzyme (fraction 6C) was then chromatographed on a 2.6cm x 70.0cm column of Sepharose 6B equilibrated and eluted in low salt buffer. The column was eluted at a flow rate of 20ml per hour and 10ml fractions were collected. Those containing high PFK activity were precipitated by 75% ammonium sulphate saturation and then redissolved in a small volume of distilled water or low salt buffer (fraction 7C).
The methods used to prepare PFK are summarised in Figure 8.

2.3 Characterisation of phosphofructokinase

Protein was estimated by the Coomassie blue method (59) with bovine serum albumin as the standard protein. Absorbances were determined at 595nm in a Hitachi 101 spectrophotometer.

PFK activity was measured by coupling the PFK reaction to the oxidation of NADH to NAD^+. This was done by the addition of the enzymes aldolase, α-glycerophosphate dehydrogenase, and triose phosphate isomerase to the assay solution. (see Figure 9).

The rate of NADH oxidation was measured by the rate of decrease of absorbance at 340nm. Activities were measured in a 0.5ml microcuvette using a Unicam SP800 recording spectrophotometer. The assay solution consisted of 0.35ml tris buffer (70mM tris-HCl pH 8.0, 1.5mM $MgCl_2$, 5mM 2-mercaptoethanol, 3mM F-6-P, 1.5mM ATP, 0.4mM NADH, 0.015% BSA, 0.34 units per ml aldolase, 3.8 units per ml α-glycerophosphate dehydrogenase and triose phosphate isomerase). To this was added 0.05ml of appropriately diluted PFK solution to initiate the reaction. (Composition of the diluent was 10mM tris-HCl pH 8.0, 7mM 2-mercaptoethanol, 0.1% BSA).

The homogeneity of the purified enzyme was determined by SDS polyacrylamide gel electrophoresis (61). The gels were poured in 0.6cm by 10cm glass tubes. They consisted of 1.0cm of 5% stacking gel (5% acrylamide, 0.2% N,N methylene bisacrylamide, 0.1M tris-HCl pH8.6, 0.04% TEMED, 0.04% ammonium persulphate, 0.1% SDS). This was layered upon 8.0cm of 15% running gel (15% acrylamide, 0.1% N,N methylene bisacrylamide, 0.1M tris-glycine pH 8.9, 0.05% TEMED, 0.05% ammonium persulphate, 0.1% SDS).

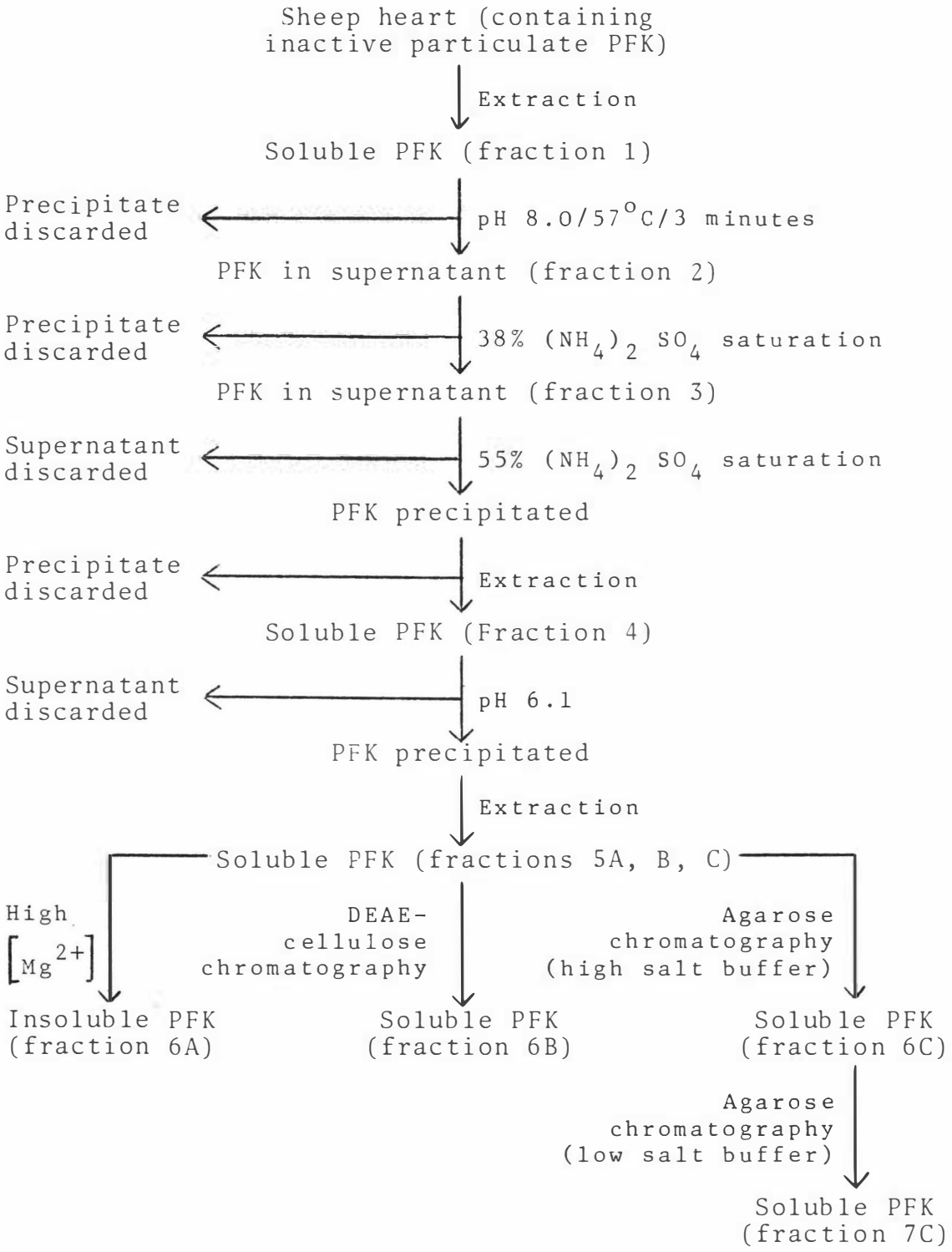


Figure 8. Summary of methods used to prepare phosphofructokinase.

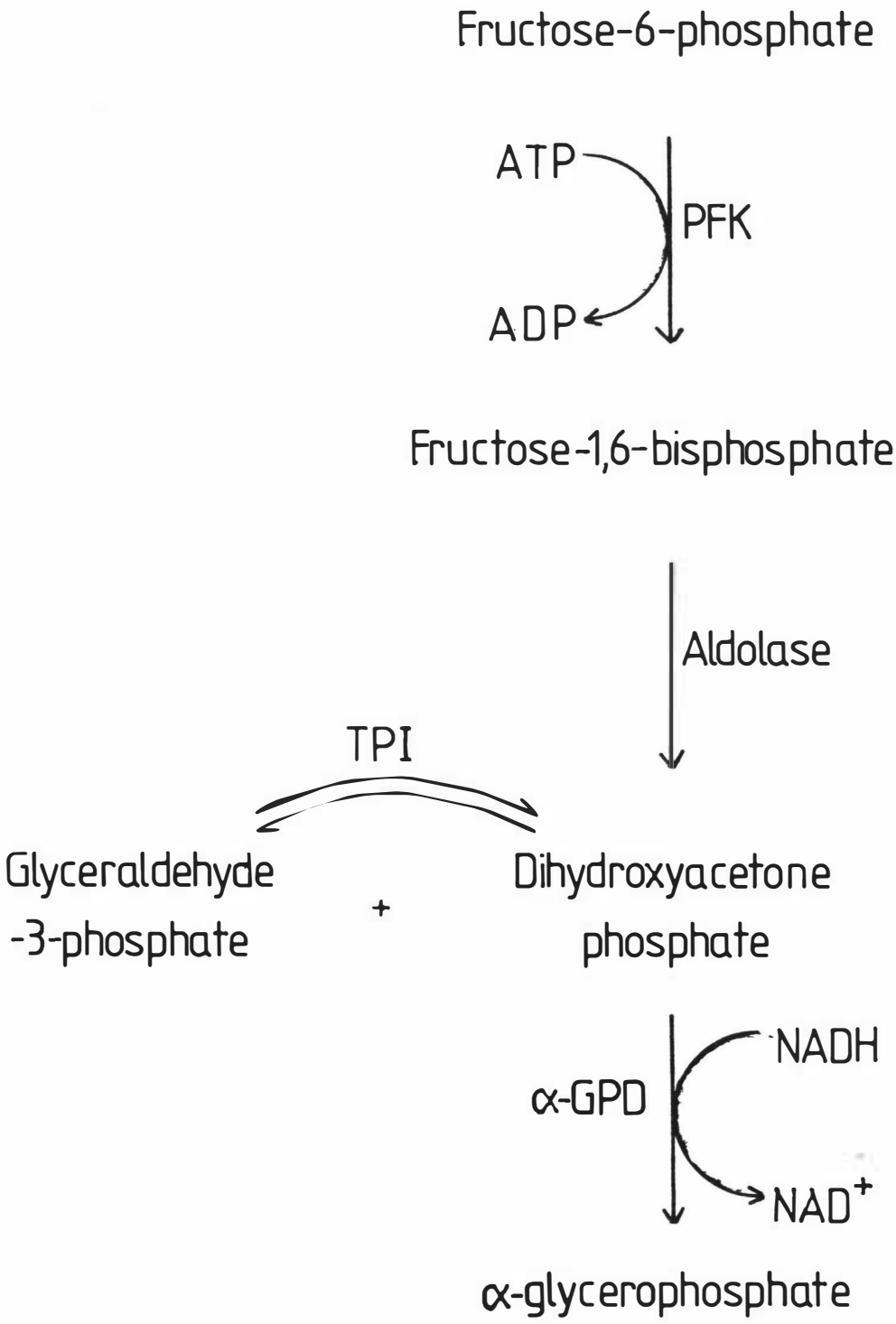


Figure 9 The phosphofructokinase assay pathway

TPI = Triose phosphate isomerase

α-GPD = α-glycerophosphate dehydrogenase

The electrode buffer consisted of 0.1M tris-glycine buffer pH 8.9, 0.1% SDS. The protein to be electrophoresed was dissolved in a small volume of tris buffer (0.01M tris-glycine pH 8.9, 1.0% SDS, 0.15M 2-mercaptoethanol). It was heated for two minutes in a boiling water bath, after which three drops of glycerol and bromophenol blue indicator were added. Up to 100 µg of protein was applied to each gel, which were then electrophoresed at 5mA per gel tube until the marker dye reached the bottom of the gels. Gels were stained by immersion in 0.1% amido black in 10% acetic acid for two hours, and decoloured by repeated washes in destain solution (glacial acetic acid : ethanol : water, 1:7:7).

2.4 Carboxymethylation (63)

The protein to be carboxymethylated was dissolved in 0.1M tris-HCl buffer pH 8.0, 8M urea at a concentration of 10-20 mg per ml. It was reduced and dissociated by adding an amount of dithiothreitol equal to the molar cysteine content of the protein, and stirring overnight under nitrogen at room temperature. After this period a volume of partially neutralised iodoacetic acid solution was added to give a 2.5-fold molar excess over the thiol content of the solution. Carboxymethylation was allowed to proceed under nitrogen and in the dark for 45 minutes. It was then terminated by the addition of 2-mercaptoethanol in a 10-fold molar excess over the iodoacetic acid. The carboxymethylated protein was dialysed against several changes of cold distilled water to remove excess reagents and then lyophilised.

2.5 Maleylation (64)

The PFK solution was dialysed against cold 0.1M borate buffer pH 9.0 (which also contained 5mM 2-mercaptoethanol, 1mM EDTA, 0.2mM F-1,6-bisP and 0.5M K_2SO_4). This was to remove tris and ammonium sulphate. Solid maleic anhydride was added slowly over a period of two hours, the pH of the solution being maintained at 9.0 by the addition of small amounts of 5M NaOH. Enough maleic anhydride was added to give a 200-fold molar excess over the amino and thiol content of the solution. After the maleylation reaction excess reagents were removed by dialysis against either distilled water or 0.1 M pyridine - acetic acid buffer pH6.0. If

necessary the maleylated protein was lyophilised after dialysis.

Polyacrylamide gel electrophoresis in nondenaturing conditions (62) was used to determine whether the PFK had been successfully maleylated or not. The gels were poured in 0.6cm by 10cm glass tubes. They consisted of 1.0cm of 2.5% stacking gel (2.5% acrylamide, 0.6% N,N methylene bisacrylamide, 0.1M tris-HCl pH 6.7, 0.06% TEMED, 20% sucrose, 0.005% riboflavin). This was layered on 6.0cm of 7% running gel (7% acrylamide, 0.2% N,N methylene bisacrylamide, 0.4M tris-HCl pH 8.9, 0.03% TEMED, 0.09% ammonium persulphate).

The electrode buffer was 0.1M tris-glycine buffer pH 8.9. The protein to be electrophoresed was dialysed against electrode buffer and three drops of glycerol and bromophenol blue were added. Electrophoresis, staining and destaining were performed as described for SDS polyacrylamide gel electrophoresis.

2.6 Carboxyl terminal tritiation

The tritiation procedure of Matsuo was used. The experiment was first performed with reduced and carboxymethylated bovine pancreas ribonuclease A to work out experimental procedures, and then with maleylated PFK. The tritiation procedure for both proteins was the same. Fifty nanomoles (0.69mg) of reduced and carboxymethylated ribonuclease or 200nmole (15mg) of maleylated PFK was weighed into a Quickfit tube and dissolved in a minimum volume of distilled water. To this was added 100mCi of tritiated water and the tube was cooled in ice for five minutes. A 0.3ml volume of pyridine and 0.1ml acetic anhydride was added. The tube was stoppered, cooled in ice for 30 minutes and then left overnight at room temperature.

After the tritiation reaction pyridine, acetic anhydride and tritiated water were removed by lyophilisation. The Quickfit tube was connected to another Quickfit tube via a glass U tube as shown in Figure 10. The reaction mixture was frozen in liquid air and the apparatus was evacuated through a sidearm in the empty Quickfit tube. By warming the tube containing the reaction mixture and cooling the empty tube in liquid air the highly

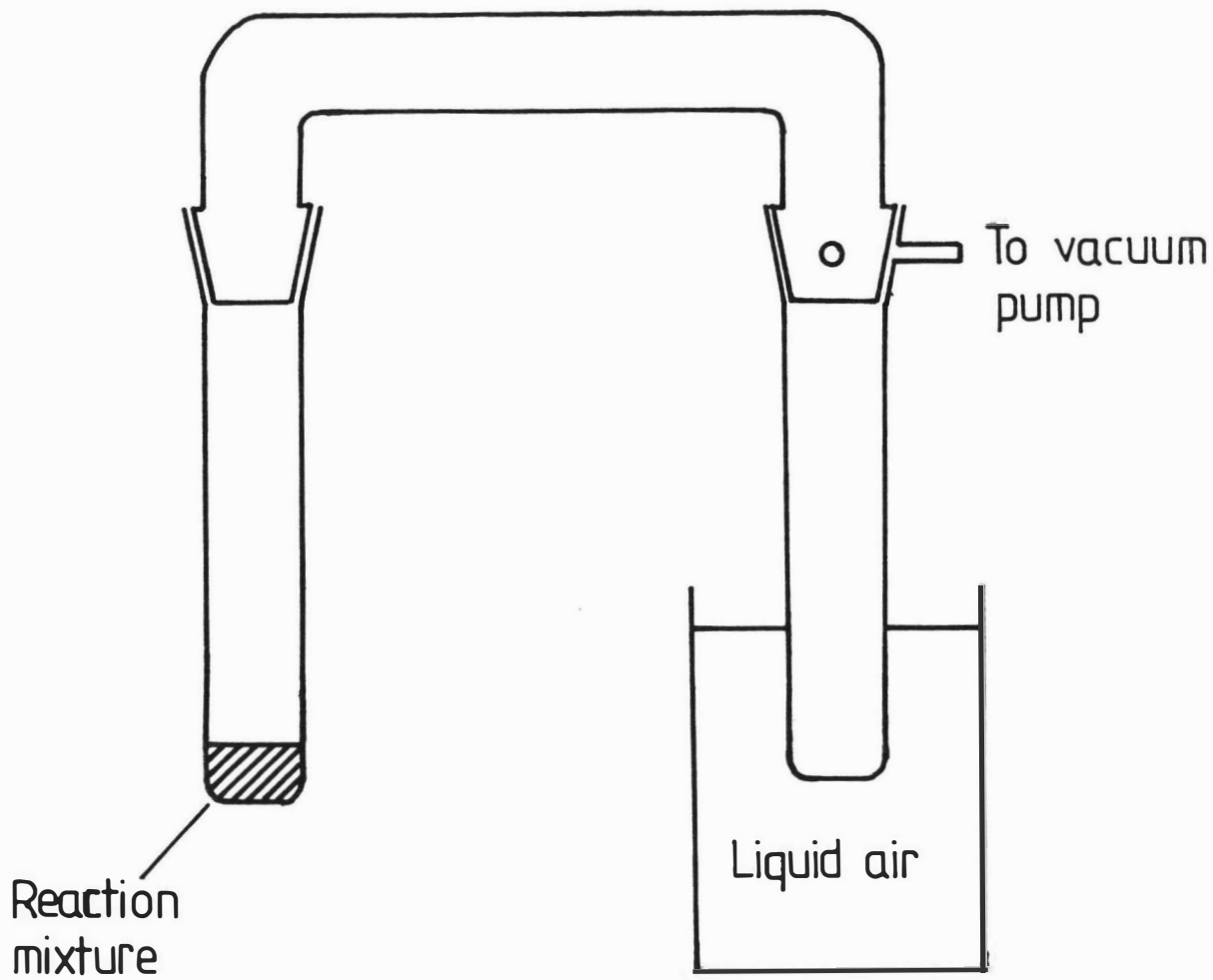


Figure 10 Apparatus used in tritiation reaction

radioactive solvent could be sublimed off and trapped in the empty tube. The protein residue was redissolved in 0.5ml distilled water and the lyophilisation procedure repeated. This was done five times to remove reversibly exchangeable tritium in carboxyl and amide groups of the protein.

The lyophilised protein was then hydrolysed. It was dissolved in 0.5ml of constant boiling point hydrochloric acid (5.9M) and transferred to a 0.7cm by 8cm heavy walled, acid washed pyrex test tube. The tube was sealed under vacuum (0.05mmHg) and heated at 110^{o}C for 24 hours. After this period the tube was opened and the contents dried rapidly over NaOH pellets in an evacuated dessicator.

Tritiated amino acids in the ribonuclease hydrolysate were identified by analytical high voltage paper electrophoresis (65). Ion exchange chromatography was used to determine labelled amino acids in the PFK hydrolysate. In the former method the protein hydrolysate was dissolved in a small volume of 20mM ammonia solution. It was spotted onto a sheet of Whatman No. 1 chromatography paper in a 3cm streak. Marker amino acids were spotted on both sides of the streak and the sheet was electrophoresed in pH 2.1 buffer (formic acid-acetic acid-water, 1:4:45) at 3KV for 45 minutes. After electrophoresis the sheet was dried and the strips containing the marker amino acids were cut off and stained in cadmium-ninhydrin stain (prepared by mixing 1.7% cadmium acetate in 33% acetic acid with 1% ninhydrin in acetone in the ratio 3:17).

Amino acids in the PFK hydrolysate were characterised by ion exchange chromatography on the ion exchange resin of a Locarte single column amino acid analyser. The hydrolysate was dissolved in 0.5ml distilled water and 0.125ml of this was applied to the column which was eluted normally. Fractions of 1.0ml were collected at the bottom of the column before the eluate was reacted with ninhydrin in the analyser.

Tritiated amino acids were detected by liquid scintillation counting in either a Packard Tri-Carb Liquid Scintillation Spectrometer or a Beckman LS8000 Series Liquid Scintillation Spectrometer. Electrophoretograms were cut into 0.5cm by 1.0cm rectangles and counted with 5.0ml of scintillation solvent, while

0.05ml of ion exchange fractions were counted with 9.0ml of scintillation solvent. All samples were counted for ten minutes. The scintillation solvent in both cases consisted of 0.1g POPOP and 4.0g PPO dissolved in 333ml of Triton X-100 detergent and 667ml of toluene.

2.7 Digestion by carboxypeptidase Y

2.7.1 Digestion of ribonuclease

The experiment was first performed with a protein of known sequence to work out experimental procedures, and to determine whether the carboxypeptidase Y preparation was contaminated with endopeptidase activity. Five milligrams (350 nmole) of reduced and carboxymethylated bovine pancreas ribonuclease A was dissolved in 0.1M pyridine-acetic acid buffer pH 5.5, 5M urea. An equimolar amount (350 nmole) of internal standard norleucine was added and the solution was incubated at 30^{o}C. The carboxypeptidase Y enzyme, which was stored as an ammonium sulphate suspension, was prepared by dialysing for an hour against cold 10mM phosphate buffer pH 7.0. After this period a volume containing 2.5 µg carboxypeptidase Y was added to the ribonuclease solution, giving a 0.05% enzyme/substrate ratio (w/w). At time intervals of 0,10,20,40,60,90 and 120 minutes after the addition of carboxypeptidase Y, aliquots containing 50nmole of ribonuclease were removed from the digestion mixture. The reaction was terminated by the addition of trichloroacetic acid. Control digestions containing carboxypeptidase Y only and ribonuclease only were performed in parallel to the above reaction.

Following digestion precipitated protein was removed by centrifugation. The aliquots were then desalted by ion exchange chromatography on 0.5cm by 4cm columns of Dowex 50W-X2, prepared in the pyridinium form and equilibrated in 0.2M pyridine-acetic acid buffer pH 3.1. Aliquots were applied to the columns and washed with 1.0M acetic acid to remove urea, and eluted with a small volume of distilled water and then 1.0M ammonia solution. The distilled water and ammonia washes were collected separately to avoid formation of ammonium acetate which would have interfered in subsequent amino acid analysis. The eluted amino acids were

lyophilised and analysed on a Locarte single column amino acid analyser, which had an automatic loading accessory. Peaks on the chromatograms were integrated manually by the half height by width method. The analyser was calibrated regularly with a Beckman standard mixture containing 50 nmole of each amino acid.

2.7.2 Digestion of phosphofructokinase

The digestion of PFK by carboxypeptidase Y was performed differently to the ribonuclease digestion. Twenty seven milligrams (300 nmole) of PFK, freshly prepared by method C was maleylated. It was then dialysed against 0.1M pyridine-acetic acid buffer pH 6.0 until the pH of the solution was 6.0. An equimolar amount (300 nmole) of norleucine standard was added and the solution was equilibrated at 30^{o}C. Carboxypeptidase Y was added to give a 0.05% enzyme/ substrate ratio (w/w). At time intervals of 0,10,20,30,45 and 60 minutes after the addition of the enzyme, aliquots containing 50 nmole of PFK were removed and placed in a boiling water bath for five minutes to terminate the reaction. Carboxypeptidase Y and PFK control experiments were performed in parallel to the above digestion. Precipitated protein in the aliquots was removed by centrifugation and the supernatants were lyophilised and analysed on the amino acid analyser.

2.8 Carboxyl terminal peptide isolation

The method of Hargrave and Wold (51) was used to isolate the carboxyl terminal peptide of PFK. Thirty milligrams of PFK was dialysed to remove salts and then lyophilised. Protein carboxyl groups were blocked with alanineamide. The PFK was dissolved in a small volume of 8M urea and solid L-alanineamide HCl was added. The solution was titrated to a pH of 4.75 with 0.1M HCl, and the reaction was initiated by the addition of the condensing agent 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide in a minimum volume of water. The concentrations of alanineamide and the carbodiimide in the solution were 1.0M and 0.1M respectively. The solution was stirred for several hours at room temperature, the pH being maintained at 4.75 by the periodic addition of dilute HCl. The reaction was then terminated by dilution with distilled water

and reagents were removed by dialysis against 1mM HCl.

The modified protein was recovered by lyophilisation and redissolved in 1mM HCl. The pH of the solution was adjusted to 8.3-8.5 with a few drops of N-ethyl morpholine. An appropriate volume of 1 mg per ml DCC treated trypsin solution was added to give a 1% enzyme/substrate ratio (w/w) and the solution was incubated at 37°C for 12 hours. After this period 1 mg of pig pancreas carboxypeptidase B was added to the solution, which was incubated for a further 12 hours at 37°C and then lyophilised.

The trypsin-carboxypeptidase B digest was subjected to ion exchange chromatography in alkaline urea on a strong base anion exchange resin. The digest was dissolved in a small volume of 8M urea and the pH was adjusted to 11.0 with 4M NaOH. It was applied to a 1.0cm by 8.0cm column of Dowex 1-X4 which was prepared in the hydroxide form, and equilibrated with 8M urea pH 11.0-11.2. The column was eluted with alkaline 8M urea at a flow rate of 1.0ml per minute and 3ml fractions were collected and neutralised with a drop of formic acid. Ion exchange fractions were desalted by gel chromatography on a 2cm by 50cm column of Bio-Gel P2 equilibrated and eluted with 20mM ammonia solution. The column was eluted at a flow rate of 0.5ml per minute and 2ml fractions were collected. Eluted peptides were detected by measurement of the conductivity and absorbance at 215nm of the fractions.

Fractions believed to contain peptides were lyophilised and examined by amino acid analysis and by preparative high voltage paper electrophoresis at pH 2.1. The latter procedure was performed similarly to analytical paper electrophoresis which has been described already, except that Whatman 3MM chromatography paper was used instead of Whatman No. 1 chromatography paper. After electrophoresis strips containing the marker amino acids and the edges of the unknown material were cut off and developed in cadmium ninhydrin stain to locate the position of any peptides. These were eluted from the chromatgraphy paper with 50mM acetic acid, lyophilised and hydrolysed. They were then analysed on the amino acid analyser.

3. RESULTS

3.1 Purification and characterisation of phosphofructokinase

Results of a typical purification of PFK are shown in Table I. No activity data is given for the final fraction prepared by method A because of the insoluble nature of the enzyme at that stage. No activities are given for the fractions prepared by method B. This is because this method was performed only once, during which no activity assays were made, before being found to be unsuitable for the preparation of PFK.

Frozen sheep heart retains PFK activity for several months. In frozen extracts the enzyme exists in an inactive particulate form and must be solubilised by incubation with ATP and $MgSO_4$ (22). The presence of adenine nucleotides or hexose phosphates is required throughout the purification (66). This is to stabilise the enzyme which is otherwise markedly labile, particularly at a mildly acidic pH (18). The greatest decrease in PFK activity during the purification usually occurred in the heat fractionation step, however it also resulted in the removal of a large amount of unwanted protein.

During some preparations a slightly higher activity was obtained in fraction 3 than in the preceding fraction 2. This was probably due to activation of the enzyme in fraction 3 by ammonium ions from ammonium sulphate fractionation (67). On one occasion the 55% ammonium sulphate PFK precipitate was extracted in phosphate buffer supplemented with 0.1mM ATP. The activity of the redissolved enzyme (fraction 4) was found to be greater than when no ATP was present. In the absence of ATP the activity of this fraction was usually about 19,000 units, while in the presence of ATP the activity was 34,000 units. However in the presence of ATP the enzyme could not be precipitated in the subsequent acidification step. In this situation the PFK had to be redialysed against phosphate buffer to remove ATP and then acidified again to precipitate the enzyme. This behaviour was probably due to the greater stability of PFK in the presence of adenine nucleotides.

Table I. Purification of phosphofructokinase.

Fraction	Volume (ml)	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1. Heart extract	5940	40.6	42,170	0.2	100
2. Heat step supernatant	3800	5.0	28,120	1.5	67
3. 38% $(NH_4)_2SO_4$ supernatant	3950	4.5	25,280	1.4	60
4. 55% $(NH_4)_2SO_4$ precipitate redissolved	111	29.2	19,800	6.1	47
5A. pH 6.1 precipitate redissolved	100	2.1	12,390	59.0	29
6A. High $[Mg^{2+}]$ precipitate	15	4.0			
5C. pH 6.1 precipitate redissolved	11.5	15.2	12,236	69.2	29
6C. Agarose eluate (high salt buffer)	9.0	11.4	11,102	110.7	26
7C. Agarose eluate (low salt buffer)	6.0	10.9	10,369	158.5	24

Table 1. (Contd).

Note 1. The letters A and C in the preparation table refer to the different fractionation methods used.

2. A unit of activity is defined as the amount of enzyme required to catalyse the formation of 1 μmole F-1, 6-bis P per minute at room temperature.

Usually two extractions of the acid precipitate with pH 8.0 or 8.6 buffer were required to dissolve the enzyme. Most of the material dissolved during the second extraction. Two extractions may have been necessary to raise the pH of the precipitate sufficiently for it to dissolve. If magnesium ions were present in the extraction buffer at a concentration of 10mM the precipitate usually failed to redissolve completely even after five or six extractions.

SDS polyacrylamide gel electrophoresis of PFK prepared by method A gave a single band indicating the preparation to be homogeneous. Although a reasonable yield of pure PFK was obtained by method A, it was very difficult to subsequently redissolve the enzyme on a large scale. It failed to dissolve in 1.0M ammonium sulphate and in mild denaturing solutions such as 0.01-1.0% Brij 35 and Triton X-100 detergents.

Removal of magnesium ions from the preparation by dialysis against 0.2M EDTA solution or 0.2M EDTA in 1.0M ammonium sulphate solution also failed to solubilise the enzyme. The precipitate could only be redissolved by constant stirring in harshly denaturing solutions such as 1.0% SDS or 8.0M urea. However polyacrylamide gel electrophoresis of PFK dissolved in these solutions indicated that some fragmentation of the protein had occurred during dissolution. Therefore it was undesirable to use these methods. The insoluble nature of PFK in the presence of magnesium ions cannot be explained.

Because of these solubility problems it became necessary to replace the magnesium ion precipitation step with a fractionation method which yielded soluble enzyme. That a further purification step was necessary after the pH 6.1 precipitation step was shown by SDS polyacrylamide gel electrophoresis of the pH 6.1 precipitate. This indicated it to be too impure to be used for structural work. Fractionation by DEAE-cellulose chromatography and agarose chromatography was investigated.

The protein elution profile obtained when the redissolved pH 6.1 precipitate was subjected to DEAE-cellulose chromatography (method B) is shown in Figure 11. Protein estimation and SDS polyacrylamide gel electrophoresis of the enzyme prepared by this

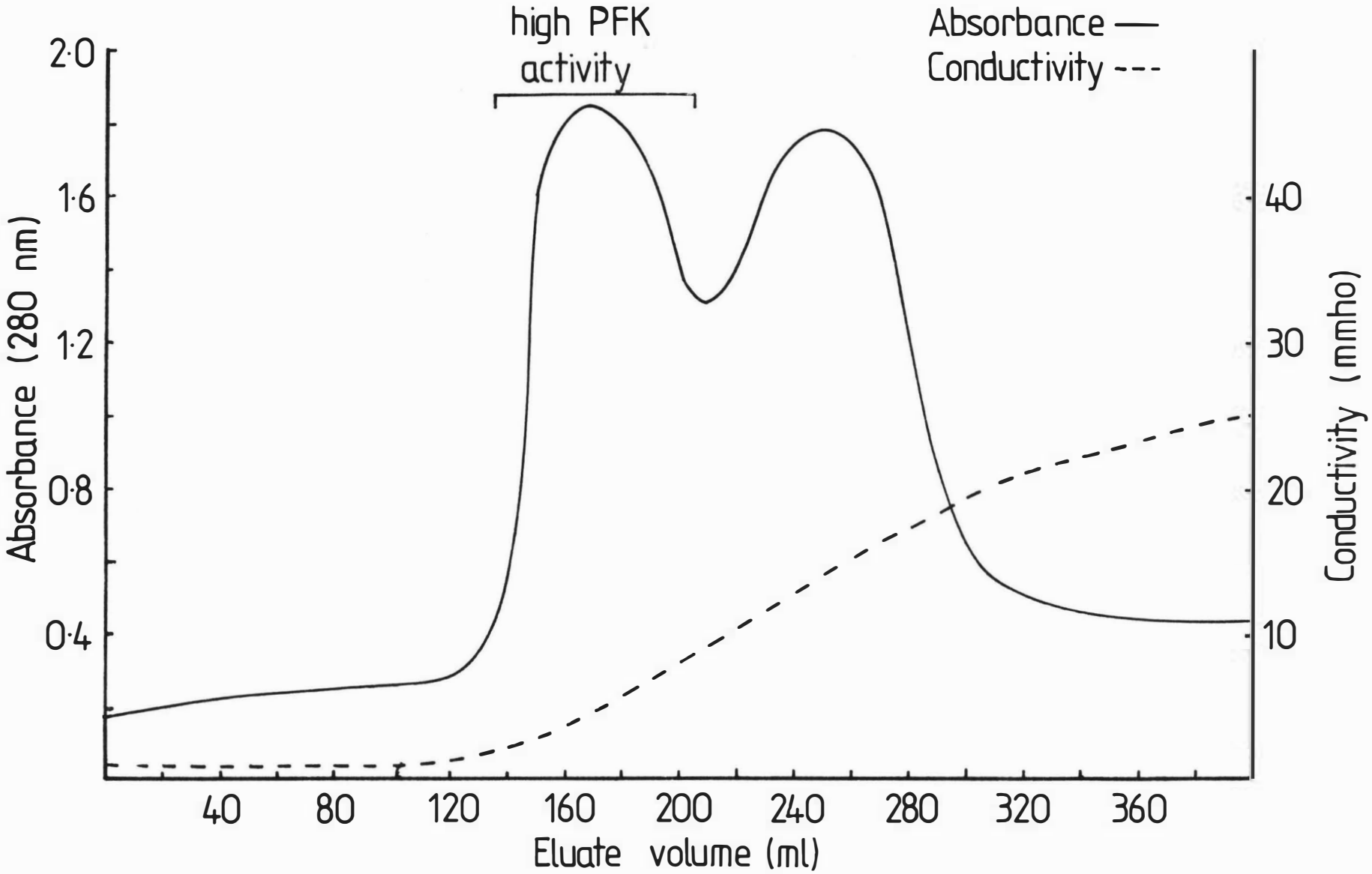


Figure 11 DEAE cellulose chromatography of phosphofructokinase

method indicated that a low yield (about 30mg protein) of impure PFK was obtained. Consequently this method was not pursued further.

Preparation method C takes advantage of the association-dissociation properties of PFK. In high salt buffer (which contained 1.0M ammonium sulphate) PFK exists predominantly as a 13S particle corresponding to a tetramer of molecular weight 320,000. Thus under these conditions the PFK was included by the Sepharose 6B gel which has molecular weight exclusion limits of 10^4—$4x10^6$. In the low salt buffer PFK exists mainly as a mixture of 18S and 30S particles with some 13S material also present. Consequently under these conditions it is largely excluded from the agarose gel. Since all other high molecular weight material has been removed in the first column step, PFK is eluted from the second column at the void volume in a pure state (58).

The protein elution profiles obtained when the redissolved pH 6.1 precipitate was subjected to agarose chromatography in high and low salt buffers are shown in Figures 12a and 12b. It was found that if the equilibration period in the preceding 38% ammonium sulphate fractionation step was increased, then the size of the first peak to be eluted from the high salt column was decreased.

PFK prepared by method C migrated in 15% SDS polyacrylamide gels with a mobility of 0.09 relative to the movement of the bromophenol blue marker band. The gels were mainly single banded with a minor amount of small molecular weight protein becoming apparent with heavy loading of the gels (see Figure 13). However the PFK was considered to be pure enough to perform sequencing work on. Polyacrylamide gels of peaks eluted after the high PFK activity peak in both columns showed they contained PFK and significant amounts of small molecular weight proteins.

The PFK prepared by method C was pure, obtained in reasonable yields and was readily soluble in mild conditions. Therefore it was suitable for sequencing work. One disadvantage of this method however was the length of time required to perform the preparation. It usually took from five to seven days depending on how fast the two agarose columns were eluted. In comparison method A required

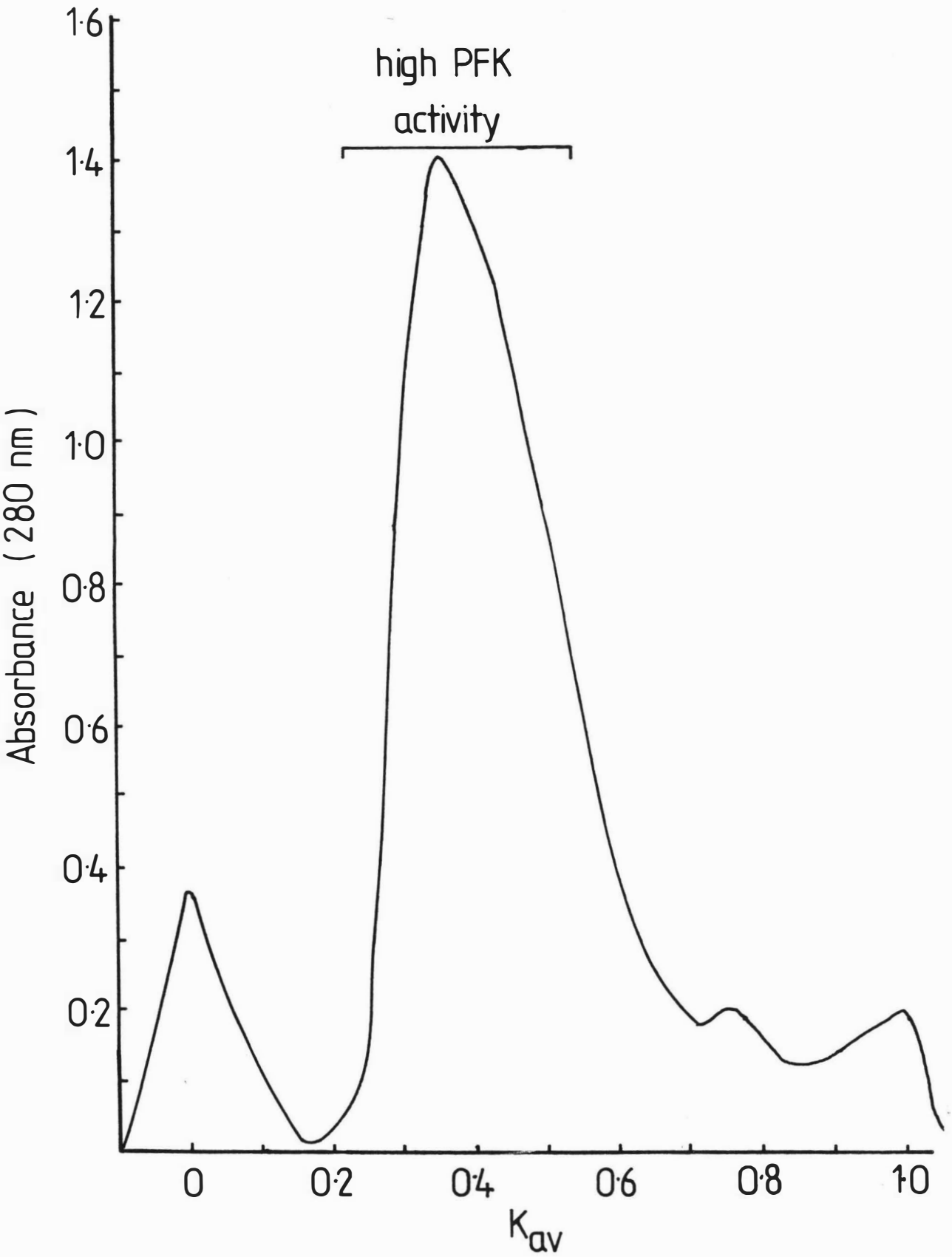


Figure 12a Agarose chromatography of PFK in high salt buffer

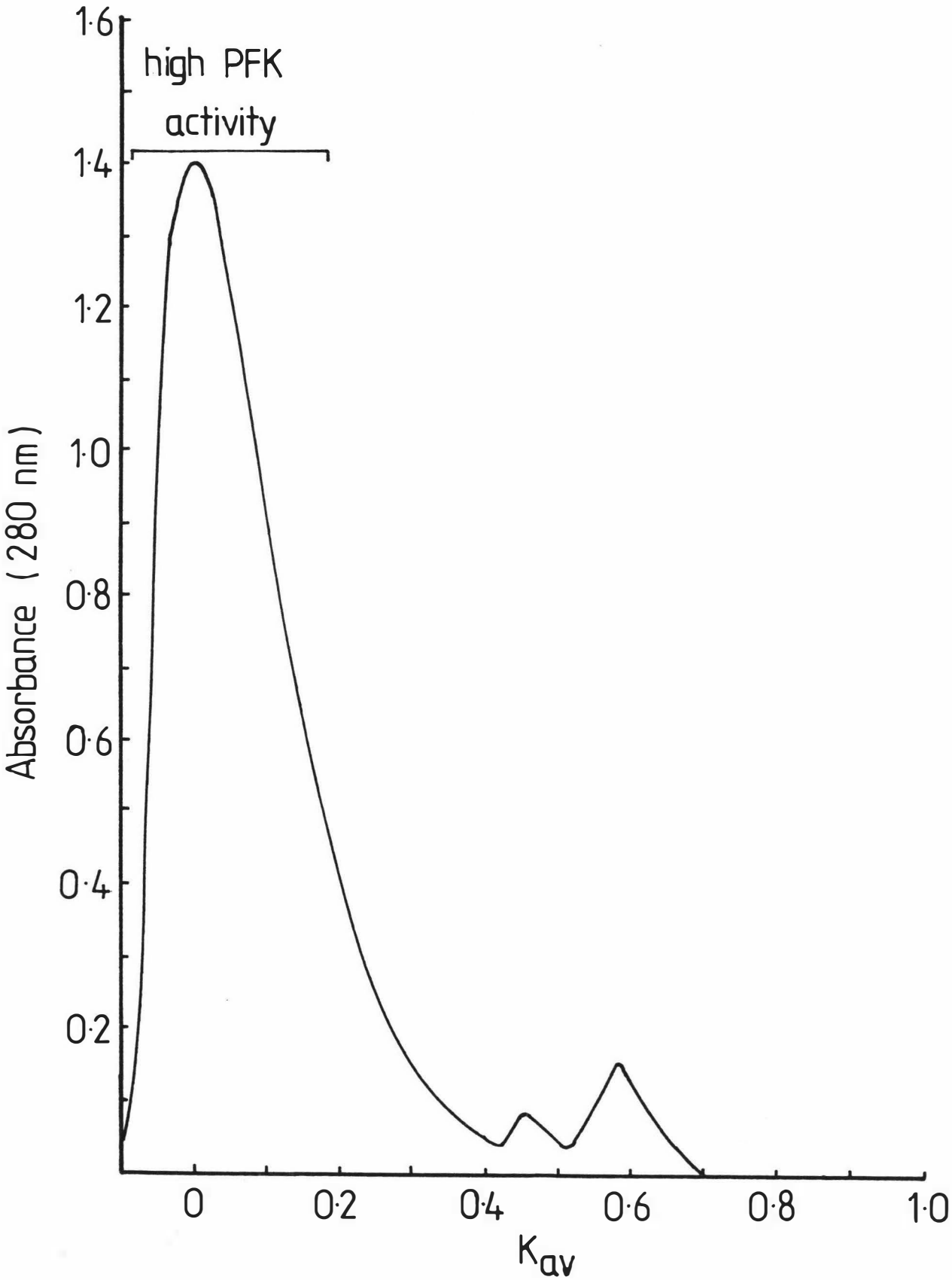


Figure 12b Agarose chromatography of PFK in low salt buffer

Figure 13 SDS polyacrylamide gel electrophoresis of PFK prepared by method C

only two and a half days.

3.2 Maleylation of phosphofructokinase

There were two prerequisites for determining the carboxyl terminal sequence of the purified enzyme. The first was that the PFK be dissociated and denatured so that the carboxyl terminal was accessible for reaction, the second was that the denatured protein be soluble. Attempts to denature the protein by the addition of solid urea to the solution (fraction 7C), resulted in the protein becoming insoluble and precipitating. This anomolous reaction of PFK toward urea, which usually facilitates the dissolution of proteins, cannot be explained. Carboxymethylation was not used to denature the protein because it had been found previously that carboxymethylated PFK becomes insoluble in low salt concentrations.

It was decided to attempt to denature the PFK by maleylation. Reaction conditions were required under which the PFK would remain soluble and which would allow the maleylation reaction to occur. Maleylation is usually performed in the presence of a denaturing agent such as urea or guanidine HCl to ensure that the protein is fully denatured and accessible for reaction. These reagents had to be avoided on this occasion since urea had been found to precipitate the enzyme.

PFK prepared by method C was obtained dissolved in tris-phosphate buffer which also contained 2-mercaptoethanol, fructose-1,6-bisphosphate, EDTA and ammonium sulphate. The maleylation reaction was performed in similar conditions except that the tris buffer and ammonium sulphate were replaced with borate buffer and potassium sulphate respectively. This was done by dialysing the PFK solution against borate buffer containing 2-mercaptoethanol, EDTA, fructose-1,6-bisphosphate and potassium sulphate. This was necessary because both tris and ammonium sulphate would interfere in the maleylation reaction. To ensure that complete maleylation of PFK occurred in the absence of a denaturing agent a larger excess of maleic anhydride was added (200-fold) than is usual (50-fold).

When native PFK was subjected to polyacrylamide gel electrophoresis it formed a band on the surface of the running gel

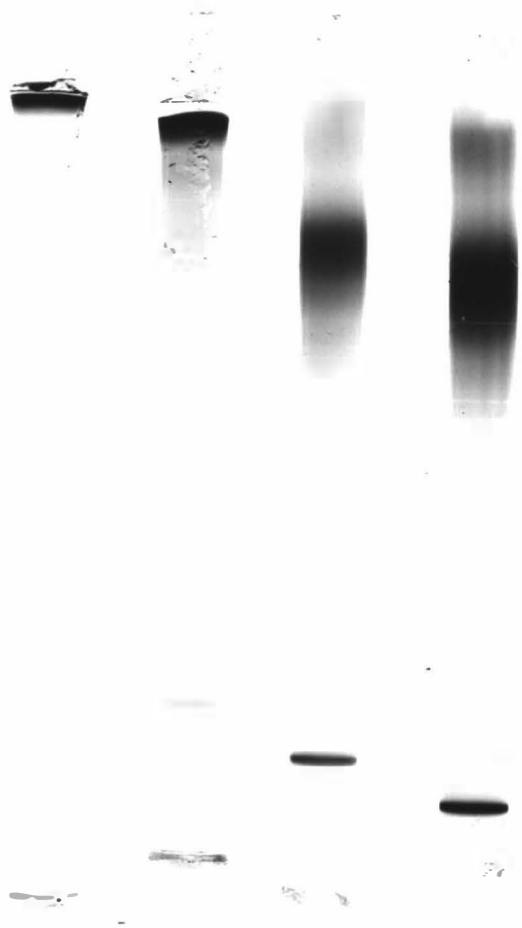
(see Figure 14a). In contrast maleylated PFK migrated into the running gel as a diffuse band with a mobility of 0.22 to 0.33 relative to the marker dye (see Figure 14b). Native sheep heart PFK is a large tetrameric enzyme which in high concentrations forms aggregates (67). Consequently it is too large to migrate into polyacrylamide gels containing more than 2.5% acrylamide. In contrast maleylated PFK can migrate into gels containing up to 12.5% acrylamide (68). The movement of the maleylated PFK into the gels in this experiment indicates that maleylation had occurred successfully despite the absence of a denaturing agent. However the broadness of the band indicates that maleylation had occurred to differing extents.

When the maleylated PFK was dialysed against distilled water or pyridine-acetic acid buffer it remained in solution in contrast to the native or carboxymethylated enzyme which become insoluble in low salt concentrations. Maleylated PFK is probably soluble while the urea denatured protein is insoluble because of the large excess of negative charges that maleylated proteins have. This results in electrostatic repulsion of the protein chains which has a dissociating and solubilising effect. Carboxymethylation also introduces negative charges onto the polypeptide chain, but carboxymethylated PFK was insoluble at low salt concentrations. This is probably because of the fewer number of carboxymethylation sites than maleylation sites in PFK. Under normal reaction conditions only cysteine residues are carboxymethylated, while both lysine and cysteine residues are alkylated by maleic anhydride. The latter forms stable S-(2-succinic acid) cysteine (64). There are 13.6 moles of cysteine and 43.5 moles of lysine per mole of PFK (23).

3.3 Carboxyl terminal tritiation

3.3.1 Tritiation of ribonuclease

Bovine pancreas ribonuclease was reduced and carboxymethylated prior to tritium labelling to make the carboxyl terminal accessible for reaction. After tritiation, labelled amino acids were characterised by high voltage paper electrophoresis at pH 2.1. The electrophoresed ribonuclease hydrolysate was not stained with



14a 14b

Fig. 14a Polyacrylamide gel electrophoresis of native PFK

Fig. 14b Polyacrylamide gel electrophoresis of maleylated PFK

ninhydrin to avoid quenching of the tritium label, and because tritiated amino acids are partially decomposed by the ninhydrin reaction (28). Tritiated amino acids in the hydrolysate were identified by comparison with the ninhydrin stained marker amino acid strips.

The results of this experiment are represented in Figure 15. There was a peak of radioactivity associated with the valine spot. This indicates that the tritiation reaction had occurred successfully since valine is the carboxyl terminal residue of ribonuclease (69). The serine, isoleucine and leucine marker spots slightly overlapped the peak of radioactivity, probably because of their poor separation from valine. There was a broad radioactive peak associated with the glycine and alanine spots. This may have been due to residual tritiated pyridine derivatives.

3.3.2 Tritiation of phosphofructokinase

After tritiation of maleylated PFK labelled amino acids in the PFK hydrolysate were characterised by ion exchange chromatography on the ion exchange resin of a Locarte single column amino acid analyser, rather than by paper electrophoresis. This was done to increase the resolution of amino acids and the sensitivity of tritium label detection. Fractions were collected at the bottom of the column before being reacted with ninhydrin in the analyser to avoid quenching of any tritiated amino acids.

The elution of radioactivity was correlated with amino acid elution by analysing 0.2ml samples of fractions with 5.0µl of Beckman standard amino acid mixture (containing 5 nmole of each amino acid), on the Locarte analyser. Amino acids could be readily identified because they superimposed on the appropriate 5 nmole calibration peak. It was found for example that glutamic acid eluted in 51.0ml and isoleucine at 94.0ml. In this way the position of amino acids in the chromatogram could be correlated with their elution volume, and hence with the radioactivity elution profile.

The results of this experiment are represented in Figure 16. One large and one small peak of radioactivity were detected at the beginning of the chromatogram. However these were eluted before the first amino acid (aspartic acid) was eluted from the

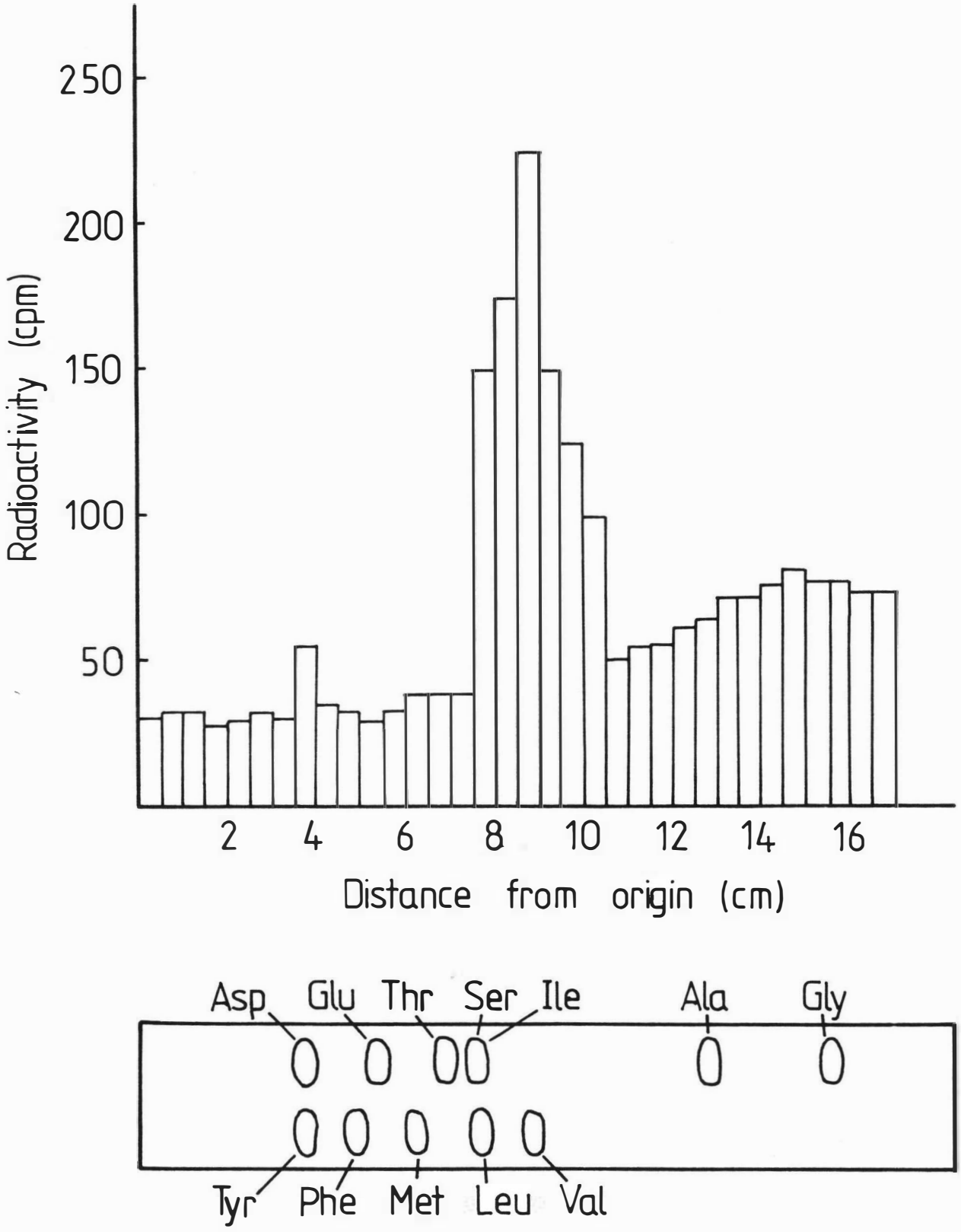


Figure 15 Electrophoresis of tritiated ribonuclease hydrolysate at pH 2·1

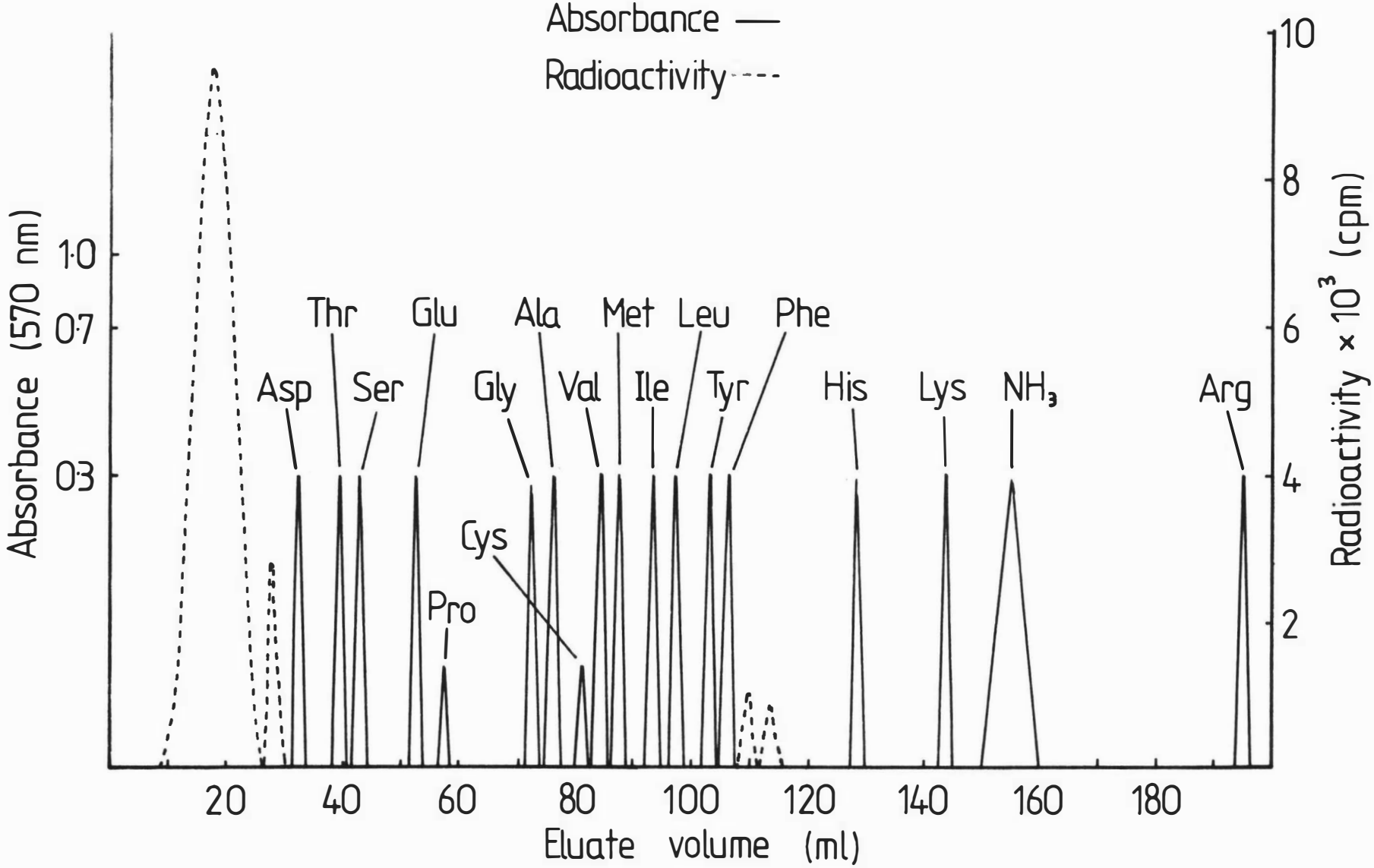


Figure 16 Ion exchange chromatography of tritiated PFK hydrolysate

column. These peaks may have been due to residual tritiated water in the hydrolysate. Two smaller peaks of radioactivity occurred at 109.0ml and 113.0ml. However these peaks eluted after phenylalanine and tyrosine in a part of the chromatogram where no amino acids are present. Analysis of these radioactive fractions confirmed the absence of amino acids in them. One explanation for these peaks is that they were residual tritiated pyridine derivatives. No amino acids in the PFK hydrolysate were labelled with tritium.

The tritiation of maleylated PFK was performed twice, and on both occasions no amino acids were labelled. However ribonuclease was successfully tritiated under similar reaction conditions. Therefore the failure of PFK to be labelled cannot have been due to faulty experimental technique, but rather to some intrinsic property of PFK. In the conditions used in this experiment, all carboxyl terminal amino acids were capable of being tritiated except the imino acid proline (28). This is because it is incapable of forming the carboxyl terminal oxazolinone structure which is necessary for tritiation to occur (see Introduction). Therefore the possibility exists that the carboxyl terminal residue of PFK is proline. The significance of the failure of PFK to be tritiated will be discussed later.

3.4 Digestion by carboxypeptidase Y

3.4.1 Digestion of ribonuclease

Bovine pancreas ribonuclease was reduced and carboxymethylated prior to digestion to make the carboxyl terminal accessible for reaction. Native proteins are generally resistant to carboxypeptidase digestion (28). Although ribonuclease is readily soluble the digestion was performed in the presence of the denaturing agent urea. This was because it was anticipated that the subsequent digestion of PFK would have to be performed in urea because of the insoluble nature of that enzyme. The carboxypeptidase Y enzyme retains 80% of its activity after being incubated in 6M urea at 25^{o}C for one hour (70).

The non-protein amino acid norleucine was added to the ribonuclease solution on a mole per mole basis as an internal

standard. After digestion losses of amino acids and inaccuracies introduced during sampling can be accounted for by comparing the amount of norleucine in each aliquot. Evaluation is based on the ratio of the amount of amino acid released and the amount of norleucine present in the aliquots, as shown in the equation below (71).

$$\left[\text{A.A.released}\right]_{\text{theor.}} = \frac{\left[\text{norleucine}\right]_{\text{theor.}}}{\left[\text{norleucine}\right]_{\text{found}}} \times \left[\text{A.A.released}\right]_{\text{found}}$$

A non-protein amino acid must be used as the internal standard so that after digestion the only internal standard present is that which was added originally. Norleucine was chosen because it is chromatographically well separated from other amino acids during analysis (eluting between isoleucine and tyrosine). In addition it is commercially available and very stable (71).

After digestion the aliquots were subjected to ion exchange chromatography to remove urea which otherwise would have interferred in amino acid analysis. The recovery of some amino acids from ion exchange chromatography was not complete, these amino acids probably being eluted with the urea. These low recoveries were accounted for by desalting a sample containing 50µl Beckman standard amino acid mixture (containing 50 nmole of each amino acid). From the recoveries of these amino acids correction factors could be calculated for each amino acid (see Table II).

Amino acids in the digestion aliquots were quantitated by amino acid analysis, and corrected by the norleucine and ion exchange chromatography correction factors. Any amino acid yields in the zero time samples were then subtracted from these values. No amino acids were released in the parallel carboxypeptidase Y control digestion indicating that autodigestion of carboxypeptidase Y had not occurred.

The results of this experiment are shown in Figure 17. The carboxyl terminal sequence of ribonuclease is -Pro-Val-His-Phe-Asp-Ala-Ser-Val-COOH (69). From my data the correct sequence of the first six residues can be deduced. The increased rate at which valine is released towards the end of the digestion probably

Table II. Recoveries of amino acids from ion exchange chromatography.

Amino acid	Amount recovered (nmole)	Calculated correction factor
Aspartic acid	33.2	1.47
Threonine	29.3	1.66
Serine	34.4	1.42
Glutamic acid	34.2	1.42
Proline	39.2	1.24
Glycine	40.3	1.21
Alanine	41.3	1.18
Valine	45.5	1.07
Methionine	39.2	1.24
Isoleucine	46.8	1.04
Leucine	46.1	1.06
Norleucine	46.1	1.06
Tyrosine	48.0	1.02
Phenylalanine	48.8	1.0
Histidine	48.0	1.02
Lysine	48.1	1.01
Arginine	46.5	1.05

Note 1. Fifty nanomoles of each amino acid was applied to the column.

2. Correction factors were calculated relative to phenylalanine which was recovered in the greatest amount.

3. Amino acids are listed in the order in which they are eluted during amino acid analysis.

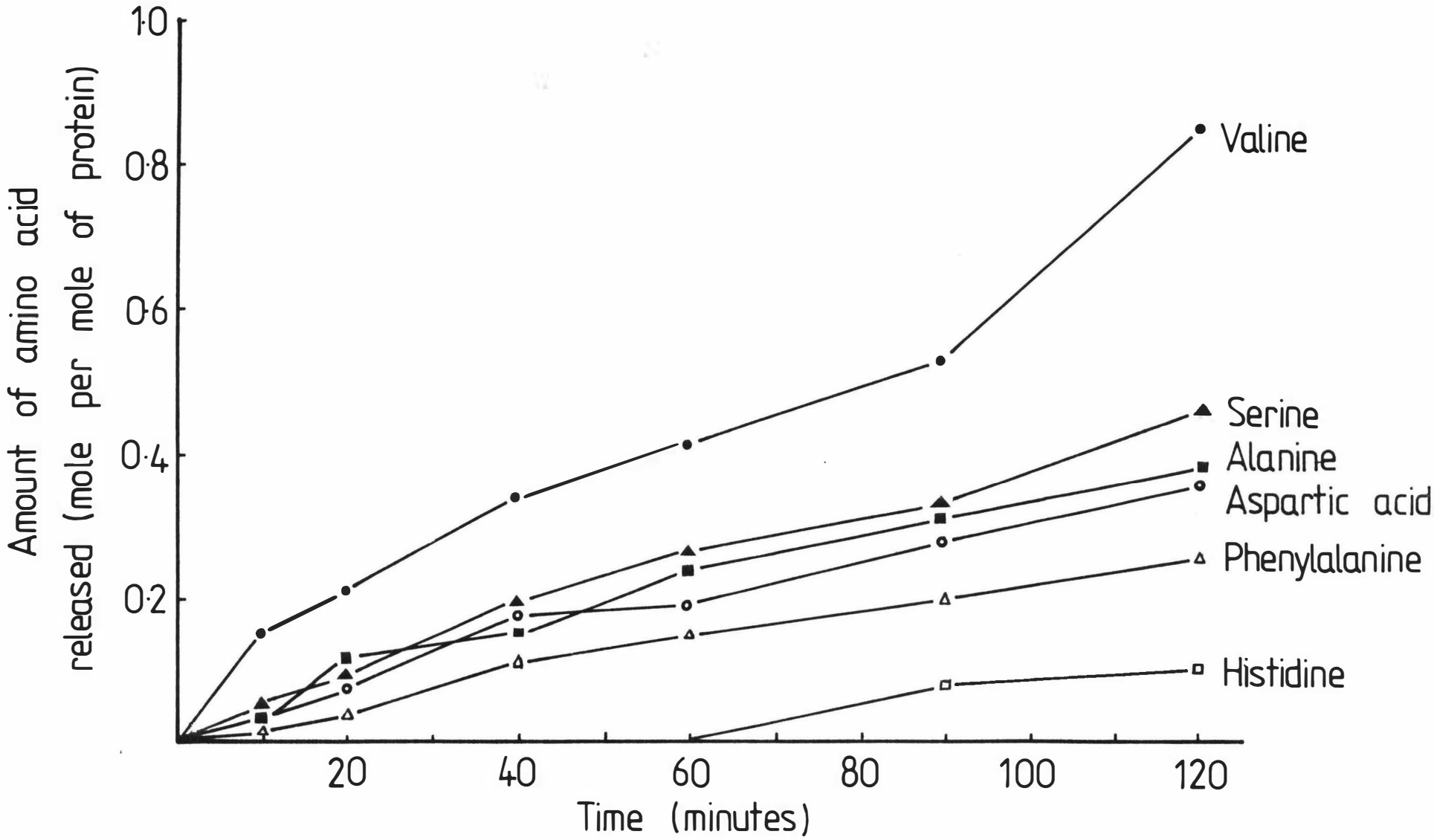


Figure 17 Carboxypeptidase Y digestion of ribonuclease

represents the release of the second valine residue in the sequence.

Since the correct sequence can be deduced from my data, the carboxypeptidase Y preparation must have been free of contaminating endopeptidase activity. Therefore it is suitable for use in sequence studies. It is important in sequence analysis that the carboxypeptidase preparation is free of endopeptidase activity, otherwise erroneous results can be obtained. This is especially true if the carboxyl terminal residue of the substrate protein is an amino acid which is slowly released by the carboxypeptidase enzyme. Carboxypeptidase Y preparations can be contaminated by an aminopeptidase enzyme (72), and by yeast proteinase A (73). The aminopeptidase can be inactivated by the addition of EDTA, but there is no specific inhibitor for the proteinase A enzyme (74). Therefore the preparation must be free of this contaminant.

3.4.2 Digestion of phosphofructokinase

It was intended to perform the digestion of PFK in urea, similarly to the digestion of ribonuclease. However dissolution of PFK prepared by method A in urea caused some fragmentation of the protein chain, and addition of urea to PFK prepared by method C caused the enzyme to be precipitated. The experiment was performed with maleylated PFK in the absence of urea. At acidic pH maleyl blocking groups are hydrolysed from the protein chain by an intramolecularly catalysed reaction. However at 37^{o}C and pH 6.0 (the pH at which the carboxypeptidase Y digestion was performed), the half life for hydrolysis of maleyl groups is approximately 10^3 hours (64). Therefore the maleylated PFK was sufficiently stable to perform the digestion at this pH. After the digestion amino acids were quantitated by amino acid analysis and corrected by the norleucine correction factor and zero time yield. No amino acids were released in the parallel carboxypeptidase Y control digestion.

The results of this experiment are shown in Figure 18. Leucine and isoleucine were released most rapidly from PFK by carboxypeptidase Y, followed by phenylalanine. The rates at which amino acids are liberated from a protein by a carboxypeptidase enzyme are used to deduce their sequence at the carboxyl terminal.

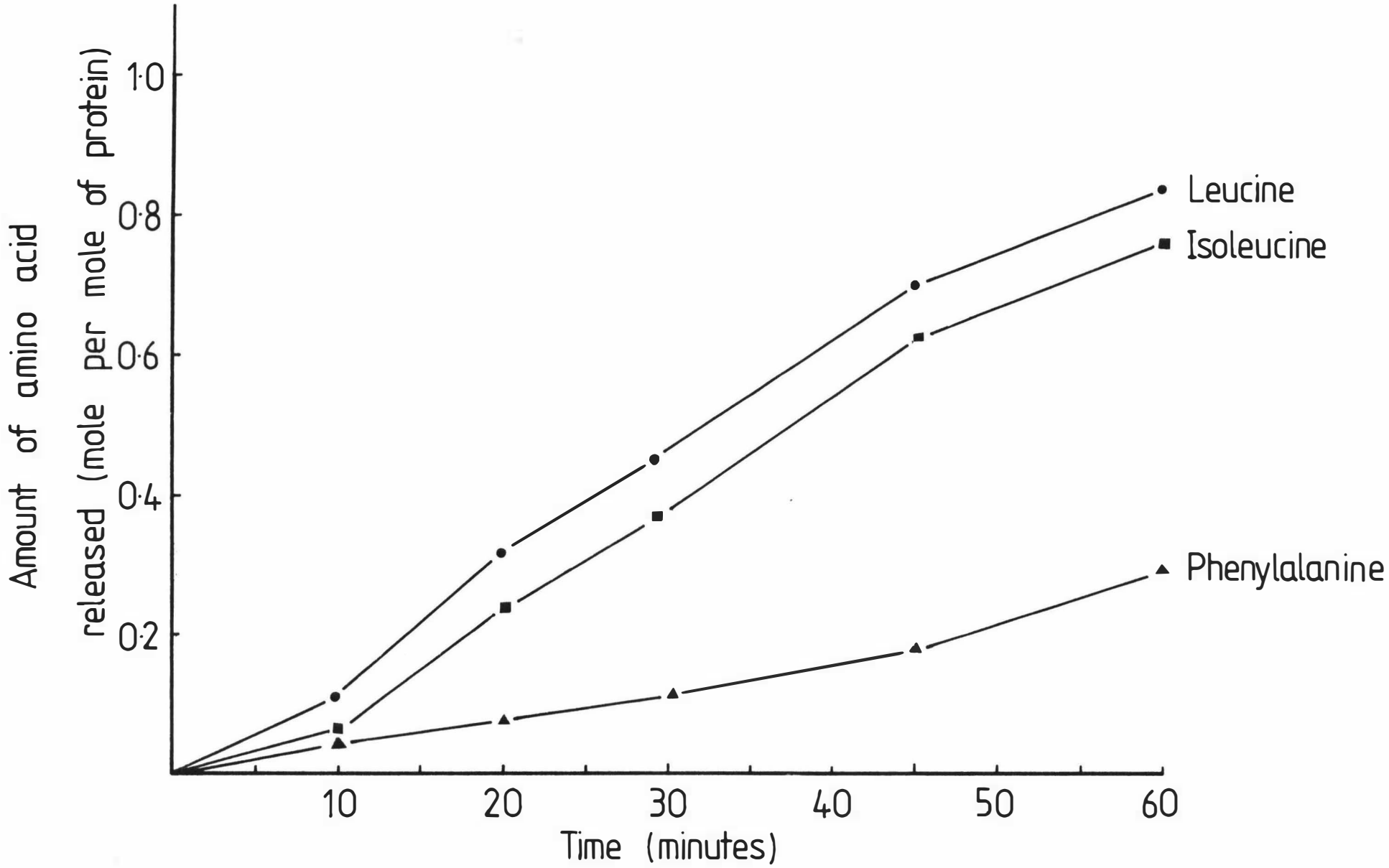


Figure 18 Carboxypeptidase Y digestion of phosphofructokinase

The closer an amino acid is to the carboxyl terminal the faster it is liberated. Amino acids are released until the penultimate or exposed residue is one which is liberated at so slow a rate that hydrolysis is effectively ended.

Isoleucine and leucine were released faster than phenylalanine and therfore must occur closer tothe carboxyl terminal than phenylalanine. But because leucine and isoleucine were released at the same rate, their relative order in the sequence cannot be determined. The carboxyl terminal sequence implied by these results is -Phe-(Leu-Ile)-COOH. Although carboxypeptidase Y does have a broad specificity for the release of carboxyl terminal residues, it does exhibit preferences for certain amino acids and amino acid sequences. In general aromatic and aliphatic amino acids are released faster than basic and acidic residues (74). This can result in the liberation of two or more amino acids at the same rate, as has occurred in this experiment.

The results of this experiment confirmed a preliminary digestion of maleylated PFK by carboxypeptidase Y. In this experiment a single aliquot of PFK was digested for 30 minutes. Analysis indicated the amino acids released in the greatest quantities were leucine and isoleucine.

The carboxyl terminal sequence of carboxypeptidase Y has recently been determined by the digestion of diisofluorophophate inactivated carboxypeptidase Y by carboxypeptidase A (75). The carboxyl terminal amino acid of carboxypeptidase Y is a leucine residue, which is the same amino acid released from PFK in the greatest yield. The possibility that the appearance of leucine was due to autodigestion of carboxypeptidase Y, rather than digestion of PFK can be excluded for two reasons. At the enzyme/substrate ratio used, the amount of carboxypeptidase Y present in each digestion aliquot was 0.037 nmole. If the enzyme had released its own carboxyl terminal residue to the maximum extent of one mole per mole of protein then 0.037 nmole of leucine would have been released. This amount is insignificant compared to the actual amount of leucine released during the digestion. In addition no amino acids were released in the parallel carboxypeptidase Y control experiment, indicating that autodigestion had not occurred.

3.5 Carboxyl terminal peptide isolation

In the method of Hargrave and Wold (51) glycineamide was used to block the carboxyl groups of proteins. Since this was unavailable L-alanineamide was used instead. After tryptic digestion of the modified PFK all of the tryptic peptides should have had a free α-carboxyl group, except the carboxyl terminal peptide which was blocked by alanineamide. Consequently during anion exchange chromatography all peptides should have been retained by the resin, except the carboxyl terminal peptide which is eluted. The digestion of the peptides by carboxypeptidase B prior to ion exchange chromatography was to remove carboxyl terminal arginine residues. (Trypsin cleaves proteins on the carboxyl side of lysine and arginine residues, hence tryptic peptides end in carboxyl terminal arginine or lysine). Digestion by carboxypeptidase B was necessary because peptides containing positively charged arginine are not retained during ion exchange chromatography.

Ion exchange fractions were desalted by gel chromatography and any peptides detected by measurement of conductivity and absorbance at 215nm. Fractions believed to contain peptides were those that absorbed at 215nm but had no conductivity.

After preparative paper electrophoresis of desalted ion exchange fractions, a spot with mobility equal to the arginine marker amino acid was detected. This material was eluted from the electrophoretogram and lyophilised. One fifth of this material was hydrolysed and then analysed. It was found to consist of arginine only. This arginine was probably released from the tryptic peptides by carboxypeptidase B. Arginine was eluted from the ion exchange resin because at pH 11-0 (the pH at which the ion exchange column was run) it has no net charge, and is not bound by the resin. In contrast free lysine has a net negative charge at pH 11.0 and is therefore retained by the column.

No peptides were eluted from the ion exchange column. The failure of this experiment may have been due to the absence of an arginine or lysine residue near the carboxyl terminal of PFK. In that case the carboxyl terminal peptide generated by tryptic cleavage would have been large and possibly insoluble.

4. DISCUSSION

Sheep heart PFK was digested by carboxypeptidase Y twice. On both occasions this resulted in the liberation of leucine, isoleucine and phenylalanine. However no tritiated amino acids were detected when PFK was subjected to the Matsuo tritium labelling reaction. An attempt to isolate the carboxyl terminal peptide of the protein by the method of Hargrave and Wold was also unsuccessful.

The carboxypeptidase Y results suggest that the carboxyl terminal sequence of PFK is -Phe-(Leu-Ile)-COOH. However despite their apparent presence at the carboxyl terminal, neither isoleucine or leucine were labelled in the tritiation reaction. This is puzzling since both of these amino acids, if occurring at the carboxyl terminal, should have readily incorporated tritium under the experimental conditions used. Under the experimental conditions used the only amino acid incapable of being tritiated was proline. However the failure of the tritiation reaction cannot have been due to the presence of proline at the carboxyl terminal since this amino acid was not released during the carboxypeptidase Y digestion of PFK. Carboxypeptidase Y is capable of liberating carboxyl terminal proline (39).

An alternative explanation for the failure of the tritiation reaction is that tritium incorporation may have been prevented by blockage of the carboxyl terminal of PFK. There is some evidence to support this view. Davis (23) investigated the carboxyl terminal sequence of sheep heart PFK with carboxypeptidase A. He found that leucine and isoleucine were released in the greatest yield, but both in recoveries of less than 0.075 moles per mole of protein. It is interesting that despite the low yields in this experiment, the two main amino acids released were those released in the greatest yield by carboxypeptidase Y in my experiment.

Paetkau et al (20) examined the carboxyl terminal sequence of rabbit muscle PFK, an enzyme which is very

similar to the sheep heart enzyme structurally and kinetically (see Introduction). The rabbit muscle enzyme was digested by carboxypeptidases A and B, and subjected to the hydrazinolysis reaction. All three methods failed to detect a carboxyl terminal residue. This was attributed at least partially to masking of the carboxyl terminal because of the great propensity of PFK to aggregate even in highly disruptive solvents.

There are two possible explanations for these results. The first is the occurrence at the carboxyl terminal of PFK of an amino acid which is not released by either carboxypeptidase A or B, and which is not detected by the hydrazinolysis method. The second possibility is that the carboxyl terminal is blocked. The former explanation seems unlikely because there are no amino acids which are not detected by both the hydrazinolysis method or by carboxypeptidase A or B digestion. With the exception of proline, all amino acids are released by carboxypeptidases A and B, although some residues are released at a slow rate (for example glycine, aspartic acid and glutamic acid). Amino acids which are difficult to identify by the hydrazinolysis method are arginine and cysteine, which are partially destroyed in the reaction (28).

The possibility that the carboxyl terminal of PFK is blocked is a more likely explanation for the failure to detect a carboxyl terminal residue. Both carboxypeptidases A and B require their substrate protein to have a free α-carboxyl group for activity (28). Similarly the hydrazinolysis method requires the carboxyl terminal residue to be unblocked in order for it to be separated from the bulk amino acid hydrazides by charge differences (see Introduction).

If the carboxyl terminal of PFK is blocked then there is some indirect evidence that it is amidated. There are a number of amino terminal blocking groups (acetyl, formyl and pyroglutamyl groups). However the only known carboxyl terminal blocking group in naturally occurring polypeptides

(for example oxytocin, secretin and gastrin) is the amide group (28)(76).

In addition carboxypeptidases A and B, which failed to release amino acids from PFK, require a free α-carboxyl group for activity. In contrast, carboxypeptidase Y, which does release amino acids from PFK, has carboxyl terminal amidase activity. In the deamidation reaction carboxypeptidase Y releases the amide group as ammonia and then the carboxyl terminal amino acid separately, rather than releasing an amino acid amide (39)(40).

If the carboxyl terminal residue of PFK is amidated then my apparently inconsistent results can be explained. Carboxypeptidase Y would have released the amide group and then the carboxyl terminal residue as a free amino acid (either leucine or isoleucine). But carboxyl terminal tritiation would have been prevented by the presence of an amide group at the carboxyl terminal (30). Apparently amidation prevents formation of the cyclic oxazolinone structure which is necessary for tritiation to occur (see Introduction). Unfortunately there is no experimental data to confirm that the carboxyl terminal of PFK is amidated. No efforts were made during the digestion of PFK by carboxypeptidase Y to quantitate the amount of ammonia released in the digestion aliquots. It was not then realised that the carboxyl terminal residue of PFK may have been amidated.

Every carboxyl terminal sequencing strategy has characteristic merits and intrinsic disadvantages depending on the chemical principle involved. Consequently it has been recommended that results should be obtained from at least two analytical methods which are governed by entirely different chemical principles, before a valid conclusion about the carboxyl terminal sequence can be made (28). In this investigation results were obtained from only one analytical method - the carboxypeptidase Y digestion of PFK. Therefore the carboxyl terminal sequence implied by that experiment cannot be said to be conclusive.

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