STRUCTURE AND ACTIVITY OF BACTERIAL PENICILLINASES

The primary structure of penicillinase from

Bacillus licheniformis.

by

Richard John Meadway.

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Department of Molecular Biology
University of Edinburgh.

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PREFACE

This thesis consists of work done in the Department of Molecular Biology, University of Edinburgh, under the supervision of Dr. R.P. Ambler, between 1965 and 1969. The techniques of protein chemistry used are very largely those used by Dr. Ambler, and I am deeply grateful to him for his encouragement and support throughout this work. I am also very grateful to Professor M.R. Pollock, F.R.S., for his interest and encouragement.

The growth of the large quantities of bacterial culture used for this work could not have been done without the help of several members of the Department, and I would particularly like to thank Dr. J.F. Collins and Messrs. S.G. Hughes, C.A. Shah and P. Thompson. Mrs. Margaret Thompson gave valuable help with the purification of the largest batches of enzyme.

I am grateful to Miss Joan Fleming for easing my first acquaintance with penicillinase, and for determining the data in Table 9.4.1., and to Mrs. A.P. Ambler for performing the computer studies.

I am grateful to the Medical Research Council for a Scholarship which I held while this work was done.

The Council's extension of this Scholarship for a fourth year enabled me to complete the amino acid sequence discussed here.

Except as noted above, the experimental work in this thesis is my own. Part of the results were published as a joint paper with Dr. R.P. Ambler (Appendix II). The experiments recorded in another joint paper (Appendix I) are peripheral to the work in this thesis.

SUMMARY

The amino acid sequence of penicillinase from Bacillus licheniformis 749/C was determined and proved to be one chain of 265 residues:

Lys-Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys-Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys-Leu-Gly-Ile-Phe-Ala-Leu-Asp-Thr-Gly-Thr-Asn-Arg-Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg-Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys-Ala-Leu-Thr-Val-Gly-Val-Leu-Leu-Gln-Gln-Lys-Ser-Ile-Glu-Asp-Leu-Asn-Gln-Arg-Ile-Thr-Tyr-Thr-Arg-Asp-Asp-Leu-Val-Asn-Tyr-Asn-Pro-Ile-Thr-Glu-Lys-His-Val-Asp-Thr-Gly-Met-Thr-Leu-Lys-Glu-Leu-Ala-Asp-Ala-Ser-Leu-Arg-Tyr-Ser-Asp-Asn-Ala-Ala-Gln-Asn-Leu-Ile-Leu-Lys-Gln-Ile-Gly-Gly-Pro-Glu-Ser-Leu-Lys-Lys-Glu-Leu-Arg-Lys-Ile-Gly-Asp-Glu-Val-Thr-Asn-Pro-Glu-Arg-Phe-Glu-Pro-Glu-Leu-Asn-Glu-Val-Asn-Pro-Gly-Glu-Thr-Gln-Asp-Thr-Ser-Thr-Ala-Arg-Ala-Leu-Val-Thr-Ser-Leu-Arg-Ala-Phe-Ala-Leu-Glu-Asp-Lys-Leu-Pro-Ser-Glu-Lys-Arg-Glu-Leu-Leu-Ile-Asp-Trp-Met-Lys-Arg-Asn-Thr-Thr-Gly-Asp-Ala-Leu-Ile-Arg-Ala-Gly-Val-Pro-Asp-Gly-Trp-Glu-Val-Ala-Asp-Lys-Thr-Gly-Ala-Ala-Ser-Tyr-Gly-Thr-Arg-Asn-Asp-Ile-Ala-Ile-Ile-Trp-Pro-Pro-Lys-Gly-Asp-Pro-Val-Val-Leu-Ala-Val-Leu-Ser-Ser-Arg-Asp-Lys-Lys-Asp-Ala-Lys-Tyr-Asp-Asp-Lys-Leu-Ile-Ala-Glu-Ala-Thr-Lys-Val-Val-Met-Lys-Ala-Leu-Asn-Met-Asn-Gly-Lys.

The material responsible for the different bands found on starch gel electrophoresis was separated by ion-exchange chromatography on DEAE-cellulose/

DEAE-cellulose and the products formed from each by cyanogen bromide degradation were examined. The results showed that there were two causes for the multiple bands found with extra-cellular enzyme. Some of the molecules lacked the first two residues (Lys-Thr-) and some had aspartic acid as the antepenultimate residue through deamidation. Combination of these phenomena, each giving a single charge difference, was responsible for the three bands. Material released from cells with trypsin lacked the first residue entirely, and the two electrophoretic bands were due to deamidation at the same place.

Residue tyrosine-77 was exceptionally reactive with tetranitromethane, a specific reagent for tyrosine.

Experiments were performed with penicillinase after reaction with maleic anhydride and with 2-hydroxy-5-nitrobenzyl bromide.

The results are discussed in relation to penicillinases from other bacteria, particularly Staphylococcus aureus.

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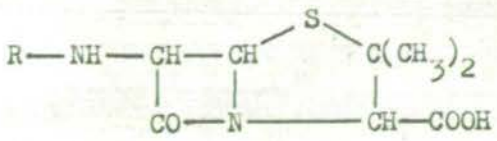
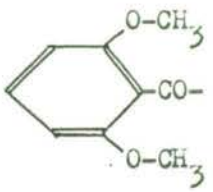
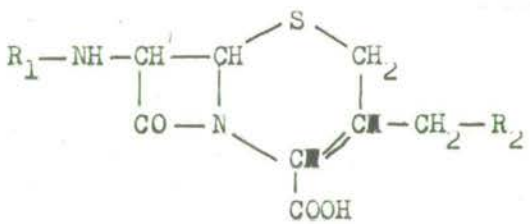
Table 1.1. Penicillins and cephalosporins.

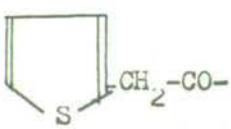
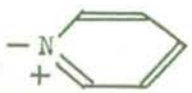
Penicillins.



Name	R
6-aminopenicillanic acid.	H-
Benzylpenicillin.	$C_6H_5-CH_2-CO-$
Phenoxymethylpenicillin.	$C_6H_5-O-CH_2-CO-$
Ampicillin.	$C_6H_5-CH(NH_2)-CO-$
Methicillin.	

Cephalosporins.



Name	R_1	R_2
Cephalosporin C.	$NH_2-CH(COOH)-(CH_2)_3-CO-$	$-O-CO-CH_3$
Benzylcephalosporin.	$C_6H_5-CH_2-CO-$	$-O-CO-CH_3$
Cephaloridine.		

CHAPTER I

INTRODUCTION

Penicillins and cephalosporins have related nuclei in which a four membered (β-lactam) ring is fused to a five or six membered ring containing sulphur. The structures of some of these compounds are shown in Table 1.1. These compounds are antibiotics because they interfere with the synthesis of cell wall peptidoglycan (Strominger et al., 1967). The final synthetic reaction is cross-linking a pentaglycine side chain and a D-alanyl-D-alanine side chain. The reaction is catalysed by peptidoglycan transpeptidase and proceeds with the elimination of D-alanine. This reaction is prevented by penicillin, possibly because the CO-N bond in the β-lactam ring is structurally analogous to the peptide bond in D-alanyl-D-alanine.

The β-lactamases (penicillinases) destroy the antibiotic activity of these compounds by opening the β-lactam ring (Figure 1.1.). Penicilloic acid is stable, but further reaction follows with cephalosporins. These enzymes have recently been reviewed

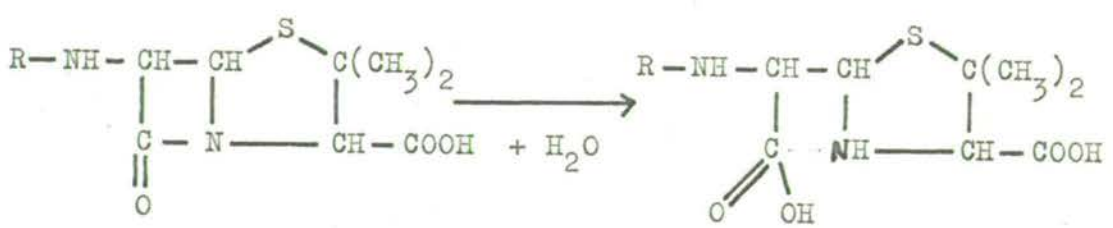


Figure 1.1.

(Citri & Pollock, 1966). Work quoted without references in this introduction will be found in this review.

These enzymes are not the only ones for which penicillins are substrates. Another group is the penicillin amidases, which remove the side chain (R) leaving 6-aminopenicillanic acid. The systematic and trivial names of these enzymes are in Table 1.2.

Table 1.2.

Trivial Name	Penicillinase	Penicillin amidase
Systematic name (I.U.B., 1965)	Penicillin amido-β-lactam-hydrolase	Penicillin amidohydrolase
E.C. number	3.5.2.6.	3.5.1.11.
Former systematic name (I.U.B., 1961)	Penicillin amidohydrolase	Benzylpenicillin amidohydrolase
Other names	β-lactamase Cephalosporinase	Penicillin acylase

I.U.B.: International Union of Biochemistry

E.C.: Enzyme Commission

The unit of enzyme activity and the determination of penicillinase activity are the subject of Chapter 4.

Reports (Saz & Lowery, 1964) of the hydrolysis of oligopeptides by purified penicillinase have not been confirmed (Pollock, 1967, 1968). At present it seems justifiable to regard the specificity of penicillinases as directed almost entirely against the β-lactam ring.

β-lactamase/

β-lactamase is apparently entirely restricted to bacteria. There are many bacterial species and genera from which it has consistently been reported absent. In addition to the species from which it has been purified (Bacillus cereus, B. licheniformis, Staphylococcus aureus, Escherichia coli & Enterobacter cloacae), recent reports on S. albus, B. anthracis, Herellea sp., Klebsiella spp., Proteus spp. and Pseudomonas spp. show that they certainly possess β-lactamases. Many other bacteria probably have β-lactamases, but the possibility of penicillin amidase has not always been adequately excluded.

Emphasis in what follows is on penicillinases which have been purified. Exo-enzymes have been prepared from culture supernatants, while preparations from Gram-negative organisms were started by ultrasonic disintegration of cell suspensions. A selective adsorption step has often been used early, with cellulosic ion-exchangers or powdered glass. Later stages have included ammonium sulphate precipitation and precipitation with organic solvents, while gel-filtration has been used in recent preparations. Many preparations were checked for homogeneity in the ultra-centrifuge; constant specific activity on refractionation has also been used as a criterion. Selected amino acid analyses are given in Table 1.3., while Table 1.4. summarises relative activity data against various substrates - "substrate profiles".

Esch. coli genes for penicillinase are often carried on extra-chromosomal/

chromosomal R-factors which are potentially transferable to other enterobacteria. The enzyme from strain TEM has been purified (Datta & Richmond, 1966) and has comparable activities against penicillins and cephalosporins. The enzyme is entirely intra-cellular.

Another intra-cellular Gram-negative β-lactamase has been purified from Enterobacter cloacae (Hennessey & Richmond, 1968). The gene for this is chromosomal, and the enzyme is predominantly a cephalosporinase.

a: Method of Goodwin & Morton (1964).

b: Including asparagine and glutamine.

c: From sequence (Ambler & Meadway, 1969: Appendix II).

d: From sequence (this work).

e: J. Imsande (personal communication).

f: Hennessey & Richmond (1968).

g: M.H. Richmond (personal communication).

Table 1.3.

Amino acid analyses of β-lactamases

Organism	S. aureus	B. licheniformis	B. cereus	Enterobacter cloacae.	Esch. coli
Strain	PC1	749/C	569/H	214	TEM
Molecular weight	28,800	29,400	31,000	14,000	16,700
Lysine	43	24	21	9	6.6
Histidine	2	1	4	2	1
Arginine	4	15	11	5	3.8
Aspartic acid[b]	39	37	34	10	12.3
Threonine	13	21	24	8	8.2
Serine	19	11	13	7	5.2
Glutamic acid[b]	18	27	24	12	12.2
Proline	9	11	9	9	6.1
Glycine	12	15	20	10	6.2
Alanine	18	26	33	15	9.4
Valine	16	15	18	10	5.9
Methionine	3	5	4	5	2.1
Isoleucine	19	14	17	6	3.9
Leucine	22	27	20	10	9.4
Tyrosine	13	6	11	5	2.4
Phenylalanine	7	7	8	3	2.9
Cyst(e)ine	0	0	0	0?	0
Tryptophan	0	3	5[a]	?	?
Total	257	265	276	126	98
Source	c	d	e	f	g

a:/

Table 1.4.

Relative activities of different β-lactamases (benzylpenicillin = 100)

Enzyme	6-Amino penicillanic acid	Methicillin	Ampicillin	Phenoxymethylpenicillin	Cephalosporin C	Benzyl Cephalosporin	Cephaloridine
TEM	85	1	110	50	0.8	16	65
Enterobacter cloacae	-	-	13	86	7180	1830	8170
S. aureus	10	ca.1	150	-	ca.1	-	-
B. cereus 5/B	9	3	-	96	4	-	-
B. cereus 569/H	40	3.5	120	153	less than 0.1		-
B. cereus 569/H β-lactamase II	-	89	64	-	80	-	41
B. licheniformis 749/C	5	0.5	-	-	0.5	1.2	-
B. licheniformis 6346/C	13	1	-	-	7.5	21	-

The control and structure of penicillinase from S. aureus has been studied extensively. Although there are strains in which the gene is chromosomal (Asheshov, 1966), in most strains the gene is located on an extra-chromosomal plasmid. In most isolates, the enzyme is inducible, but constitutive mutants may readily be prepared. About 30-60% of the activity is generally extracellular, although plasmids are known of whose product only 5-10% is liberated (see Pollock, 1968). There is little, if any, crypticity.

The enzyme can be subdivided into types A, B & C. These differ in specific activity and in immunological properties. These are generally very closely related in structure, although slight differences have been observed in peptide maps. All the proteins which have been analysed have very similar amino acid compositions. Differences in relative activities are also very slight.

Ambler has determined the complete amino acid sequence of the penicillinase from strain PC1. (Ambler & Meadway, 1969; Appendix II). The protein consists of a single chain of 257 amino acids, without tryptophan or cyst(e)ine. It has not been possible to locate the differences between the different types in the sequence (R.P. Ambler, personal communication).

Bacillus/

Bacillus cereus produces an exo-enzyme. The enzymes from strains 5/B and 569/H are produced constitutively and differ in immunological and electrophoretic properties. Pollock (1968) showed that anti-serum prepared against B. licheniformis 749/C penicillinase reacts slightly with 569/H enzyme and more with that from another strain, 820/B. The enzyme from 569/H runs as three bands on electrophoresis in polyacrylamide gels (J. Imsande, personal communication). There are two other forms of penicillinase, δ-penicillinase and β-lactamase II (Kuwabara & Abraham, 1967), whose structural relationship with exo-enzyme are not clear.

The organism on which the work described in this thesis has been done is Bacillus licheniformis. The differences between this species and B. subtilis are not very great (Smith, Gordon & Clark, 1952) and the strains whose penicillinases have been purified (NCTC6346 & 749) were previously classified as B. subtilis (e.g. Kushner & Pollock, 1961). They were reclassified chiefly because of their production of gas on anaerobic reduction of nitrate (Pollock, 1965). It was then found that no strain, classified as B. subtilis, produced more than traces of β-lactamase, either inducibly or constitutively. The enzymological and immunological properties of penicillinase in natural isolates of B. licheniformis fall into two groups, of which 6346 and 749 are typical (Pollock, 1965, 1967).

The physiology of penicillinase has been studied with these strains and constitutive mutants (6346/C & 749/C) isolated (Pollock, 1963) from/

from them. The strain used in this work, 749/C, was made with ethylmethanesulphonate. In all strains, logarithmic cultures have about half the enzyme in the medium and the other half fixed to the cells. The cell-bound enzyme is bound to the outside of the cell membrane, since it is not released on the formation of protoplasts, but is liberated by treatment with trypsin, which does not penetrate the membrane. There is evidence that the cell-bound enzyme is an intermediate in the natural formation of exo-enzyme, and newly formed enzyme in induced cells is cell-bound. All this enzyme is fully accessible to substrate.

Collins (1964) examined the amounts of the enzyme in single cells of strains 749 & 749/C. The results showed the activities of cells of 749 were clustered around 1.6×10^{-8} units and small integral multiples of this, and in 749/C round 8.3×10^{-7} units and small integral multiples. The results suggest that penicillinase molecules may be produced in groups of about 750 & 37500 respectively (using the specific activity figure determined in Section 8.6.).

Trypsin releases the activity from its cell-bound form into a soluble form. When purified the properties of this trypsin-released material are very similar to those of exo-enzyme. Partial purification (Lampen, 1967a) of penicillinase bound to membrane fragments gave a product with enzymological and immunological properties substantially identical to those of exo-enzyme.

The/

The nature of the release process has been extensively investigated. The liberation of the enzyme may be separated from its net formation. The liberation is temperature dependent and does not occur below pH 6.0, and is insensitive to several protease inhibitors. Disintegration of membrane structure inhibits the release activity almost completely (Lampen, 1967b). It is not at present possible to tell whether cell-bound enzyme is covalently attached to the membrane or not (Lampen, 1967a; Sargent, Ghosh & Lampen, 1969).

Ghosh, Sargent & Lampen (1968) described electron microscopic observations of characteristic organelles, which consisted of tubules and vesicles and were formed in response to induction with cephalosporin C. However the possibility of artefacts of specimen preparation does not appear to have been fully excluded, and Sargent, Ghosh & Lampen (1969) considered that such structures were not necessary for secretion of penicillinase. Highton (1969) considered that such tubules and vesicles were formed from a single membrane during sample preparation, and could not find any difference between penicillinase producing and other cells.

Yudkin (1968) has shown that 749/C continues to synthesise penicillinase much longer after the addition of actinomycin D than other constitutive strains. It spontaneously reverts to full inducibility and normal behaviour with actinomycin, suggesting very strongly that it is a point mutant. This result has not been explained.

Pollock/

found by starch gel electrophoresis

Pollock (1965) described multiple bands from pure penicillinase preparations from strains 6346/C & 749/C. The patterns differed between exo-enzyme and trypsin-released enzyme.

The control of biosynthesis of penicillinase in B. licheniformis is currently under investigation. In strain 749 the penicillinase gene is very near the end of an apparently linear chromosome (D.J. Sherratt & J.F. Collins, personal communication).

The data show that there are distinct differences between penicillinases from Gram-negative and Gram-positive organisms. Thus Gram-negative penicillinases are usually little inducible, intracellular and inaccessible to substrate. The two molecular weights which have been determined are around 15,000. On the other hand, Gram-positive penicillinases are usually inducible, partly extracellular and fully accessible to substrate. Their molecular weights are around 30,000.

The differences in substrate profile tell us little about the relation of these enzymes. The differences between strains 6346/C & 749/C are apparently due to very few amino acid residues (Chapter 18). Pollock (1967) has shown that very large alterations in relative activity against penicillins and cephalosporins can be caused by single-step mutations.

A rough measure of the difference between proteins where only amino acid analyses are available is a "% difference" determined by summing the difference in residues per cent for each amino acid without/

without regard to sign, and dividing the answer by 2. This measure, calculated from the data in Table 1.3. is shown in Table 1.5. together with the values for some other pairs of proteins (Ambler, 1968).

Table 1.5.

Differences in composition and sequence of certain proteins

Proteins	% difference in composition	% difference in sequence
S. aureus - *B. licheniformis* penicillinases	18.2	
S. aureus - *B. cereus* penicillinases	18.0	
B. cereus - *B. licheniformis* penicillinases	10.1	
Esch. coli TEM - *Enterobacter cloacae* β-lactamases	11.8	
Azurins, between genera	9 - 13	32 - 39.
Penicillinase - Flagellin	14	Unrelated

It is obvious that unrelated proteins could have identical amino acid compositions. It is not clear whether the example quoted is typical of unrelated proteins. However, we can say that the staphylococcal and bacilliary penicillinases are as different as unrelated proteins. It is possible that the *B. cereus* & *B. licheniformis* enzymes, and also the *Esch. coli* TEM & *Enterobacter cloacae*, enzymes have more similar compositions than unrelated proteins of the same size.

Citri/

Citri & Pollock (1966) summarised the questions which arose from their study of penicillinases in the form of two questions:

1) To what extent have the β-lactamases from different bacterial species a shared evolutionary history?

2) To what extent do the different groups of the enzyme share properties beyond those essential for manifestation of the specific catalytic function on which they have been identified and selected?

In view of the very different size of the Gram-negative penicillinases the chief possibility of fundamental relation with the larger penicillinases is that the latter originated through duplication of the Gram-negative chain (Pollock, 1967). Such a phenomenon has been described in the amino acid sequence of clostridial ferredoxin (Tanaka et al., 1966). Thus it might not be fortuitous that the molecular weights of the Gram-positive penicillinases are about twice those of the Gram-negatives. This is a question which is susceptible to amino-acid sequence analysis.

The serological cross reaction and the compositional similarities between the enzymes from B. licheniformis & B. cereus suggest that some degree of structural relation exists (Pollock, 1968). It would seem, however, that were the enzymes from S. aureus and a Bacillus species shown to be related, the two types of bacilliary enzyme would also be related, probably more closely.

Penicillinase/

Penicillinase certainly existed before the days of penicillin chemotherapy. Sneath (1962) revived penicillinase forming strains of B. licheniformis from spores which had remained untouched in the British Museum since 1689. The natural habitat of bacilli is soil, where penicillin forming moulds are also found. Staphylococci have been found in soil (e.g. Holding, Franklin & Watling, 1965) and also in lesions on the skin of hedgehogs also inhabited by penicillin producing fungi (Smith & Marples, 1964).

It may be asked whether penicillinase might have evolved from some other bacterial enzyme. There might well be even now a structural similarity between whatever enzyme penicillin inhibits and penicillinase. Structural analogies have been postulated between various components and precursors of cell-wall mucopeptide (the synthesis of which is very sensitive to penicillin) and penicillin. Thus the various enzymes responsible for cell-wall synthesis are among possible ancestors of penicillinase (Pollock, 1967). Comparison of amino acid sequences is relevant to this point.

Over the last fifteen years techniques have been developed which enable us to determine the complete structure of a protein molecule, locating the position of every atom in a molecule with a molecular weight of tens of thousands. This advance has come from two techniques fundamental to molecular biology; the chemical determination of/

of the covalent structure of proteins and the X-ray crystallographic determination of three dimensional structure.

The technique which led to the determination of the covalent structure of proteins was chromatography. Until this method made quantitative analysis of amino acid mixtures on a small scale possible, no worthwhile progress was made on the sequence of amino acids in proteins. Following the determination of the structure of insulin (Ryle et al., 1955) and ribonuclease (Hirs, Moore & Stein, 1960), the primary structures of many proteins of steadily increasing complexity have been determined; the latest compilation (Atlas of Protein Sequence and Structure, 1968) lists about fifty substantial sequences, not counting comparatively minor variants.

Parallel with this chemical work have been the X-ray crystallographic studies of Kendrew, Perutz and others (reviewed by Perutz, 1969). This has given us a detailed picture of the tertiary structure of some enzymes. Combined with structural studies on various pathological haemoglobins (Perutz & Lehmann, 1968) we are beginning to obtain some idea of the effect of replacements of individual amino acids on the overall tertiary structure of molecules. Comparison of proteins (e.g. cytochrome c) from a wide variety of organisms enables us to see that there are very few sites in a protein where one particular amino acid is necessary; some where only the general type of side chain (e.g. hydrophobic, charged) need be maintained; and many where most amino acids are permitted.

These/

These studies enable us to form some vague idea of tertiary structure in molecules where we have primary structures. It appears that the primary structure determines the tertiary structure, and using the results of the studies on myoglobin and haemoglobin, it was possible to predict approximately which parts of the known primary sequence of lysozyme would be α-helical (Periti, Quagliarotti & Liquori, 1967). The prospect of determining the tertiary structure by the application of rules to the primary structure alone appears very distant.

The methods and problems of protein sequence determination have been discussed by Canfield and Anfinsen (1963) and Schroeder (1968), while Volume 11 of Methods in Enzymology (1967) is a very useful compilation of techniques.

Despite increasingly sensitive methods, the quantity of material required is still a barrier to structural studies on enzymes. For a protein of 30,000 molecular weight, a gram of purified material would be unlikely to be sufficient for a complete primary sequence determination even in experienced hands. The complexity of the problem increases greatly with slightly larger proteins; thus although about a dozen complete sequences in the 200-350 residues range have been determined, the only larger protein of which most of the sequence has been determined is catalase (Schroeder et al., 1969). Although many proteins of immense biological interest are still totally inaccessible to sequence work, it is certainly possible to determine/

determine the structure of large proteins which are not commercially available.

It is also necessary that the starting material be pure according to the normal criteria for proteins. The most sensitive step is overall amino acid analysis, where quite a small proportion of another protein can give misleading results. However, slightly impure material may be quite satisfactory for the preparation of peptides, since peptides from minor impurities will usually be lost in the course of fractionation of the mixture.

X-ray crystallography cannot determine the complete primary structure of a protein incidentally to the determination of tertiary structure. There are side chains which are indistinguishable by crystallography (e.g. asparagine and leucine) and the fitting of side chains to the electron density map is greatly facilitated if extensive primary structure data is available. So if enough material is available for crystallography, it should be possible to obtain primary structure information. It may very well be, however, that X-ray crystallography can assist or make unnecessary the final determination of the most difficult overlaps, similarly to the way the existence of a related sequence does. For example, the primary structure investigation of papain (Light et al., 1964) has been extended and corrected by X-ray crystallography (Drenth et al., 1968).

There are fundamentally two ways to determine the amino acid sequence of a protein. The first, considered impracticable until recently/

recently, is to degrade the protein sequentially from one end, identifying either the residue removed or the new terminal exposed at each step. A machine has now been constructed (a "sequenator"; Edman & Begg, 1967) capable of performing sequential degradation by a chemical method and delivering consecutively derivatives of the amino acids ready for characterisation. Before this method can be considered universally applicable, it has to be shown that it can cope with various difficult residues, such as glutamine and tryptophan and also that the change in solubility properties of the protein as the C-terminus approaches does not cause difficulties.

The second method, used for all complete protein sequences so far, is to degrade the protein with various proteases and reagents which split the chain specifically at a few amino acids only, separate and purify the peptides formed and determine the sequence of each peptide. The only proteases with specifities sufficiently narrow for digests prepared on large proteins to be useful are trypsin, chymotrypsin and pepsin, and the last two will generate some identical peptides. With smaller proteins, less specific proteases may also be useful. Clearly, under sufficiently favourable circumstances, the complete characterisation of the products of two digests will suffice to determine the sequence of the protein. In one case (Wilker, et al., 1967) the chymotryptic and tryptic peptides from a large protein were sufficient to permit the determination of its sequence. More usually, however, other enzymic or chemical digests have had also to be examined. The principal other enzymes used for primary/

primary digests are pepsin and thermolysin (Matsubara, et al., 1966) although primary digests have also been performed with less specific enzymes. Methods are available for modifying the action of trypsin on the protein; aminoethylation of cysteine (Raftery & Cole, 1966) provides an extra basic residue at which trypsin may split, whereas various lysine-blocking agents (Section 14.1.) leave only arginine as a basic residue. The only chemical reagent which has been much used is cyanogen bromide, splitting at methionine residues (Gross & Witkop, 1962).

There is a great variety of experimental approaches to the problem of peptide separation and purification. Investigators in the "ribonuclease school" have considered gradient elution from columns of ion-exchange resin as an invariable first step in separation of enzymic digests. This is an exceedingly powerful method of high capacity, but some peptides are very little recovered from columns. Workers in the "insulin school" have preferred high voltage paper electrophoresis at pH 6.5 as a first step, and this is quite comparable in resolving power to ion-exchangers for non-neutral peptides. Some peptides, however, are largely lost in the course of paper purification. The limited capacity of paper methods can be circumvented by a preliminary separation of peptides on a size basis by gel-filtration, which also introduces a completely different basis of separation. Paper chromatography or further high-voltage paper electrophoresis is almost invariably necessary before a peptide will give/

give a stoichiometric amino acid analysis, whatever initial separation was used.

Thus paper and column methods are both very powerful, and if sequence investigations are to encompass larger proteins, both will have to be used to the limit of their resolving power. The difficulties of paper methods have frequently been overestimated; the "severe limitations" (Schroeder, 1968) of low capacity and poor recovery have not prevented workers using them solving the sequences of proteins as large as those solved by column methods on about the same amounts of material (e.g. Hartley, 1964; Milstein, Clegg & Jarvis, 1968; Ambler & Meadway, 1969).

The peptides thus purified are ready for sequence determination. The principal techniques recently used involve degradation by the method of Edman (1950). The amino acid phenylthiohydantoins released may be characterised directly. Alternatively the residual peptide at each step may be analysed ("subtractive Edman") although this is a method which consumes a lot of material. In the DNS-PTC ("dansyl"-Edman) method the N-terminus of the residual peptide is determined after each cycle of sequential degradation, and this is probably the most sensitive technique. None of these methods is infallible, and evidence obtained by degradation with exo-peptidases is also often necessary. Partial acid hydrolysis is still sometimes used, although it is not as generally powerful as methods based on the Edman reaction.

Often/

Often the peptides found after primary digestion are too large for convenient degradation by these methods. Digestion with another protease (particularly trypsin for peptides produced by other methods) often provides valuable data on overlaps as well as reducing the peptide to a suitable size for direct sequence determination.

In considering the techniques and results of other sequence investigations I have limited myself to proteins over 200 residues, firstly to keep the number of papers to be considered within reasonable bounds and secondly because investigations of large proteins are most closely comparable with the present work. Table 1.6. is a list of proteins over 200 residues whose complete sequences have been published. References have been selected somewhat arbitarily and many more will be found in the Atlas of Protein Sequence and Structure (1968).

Table 1.6.

Protein sequences longer than 200 residues

Protein	Reference	Remarks
Esch. coli tryptophan synthetase A protein	Guest *et al.*, (1967)	Full details in papers cited therein.
Subtilisin BPN'	Markland & Smith (1967)	Full details in papers cited therein.
Subtilisin Carlsberg	Smith *et al.*, (1968)	Full details in papers cited therein.
Trypsinogen	Walsh & Neurath (1964)	
Chymotrypsinogen	Hartley (1964)	
L-type Bence-Jones protein	Milstein, Clegg & Jarvis (1968)	Full details. Several other complete Bence-Jones protein sequences have been published but without full experimental details (e.g. Wilker *et al.*, (1967).
S. aureus penicillinase	Ambler & Meadway (1969)	
Lobster glyceraldehyde 3-phosphate dehydrogenase	Davidson *et al.*, (1967)	Pig; Harris & Perham (1968).

In all the proteins listed tryptic and chymotryptic digests have been examined. Some form of Edman degradation has been used almost invariably on the peptides; the subtractive Edman method was used in the subtilisins and tryptophan synthetase, and the DNS-PTC method with S. aureus penicillinase and Bence-Jones protein. Peptic and cyanogen bromide digests have also been used extensively.

It is interesting to observe that similar proteins may require very different digests. Thus the sequence of subtilisin Carlsberg was determined using the data from tryptic and chymotryptic digests, while subtilisin BPN' which differs in less than 30% of its amino acids, required peptic and cyanogen bromide digests also.

A great variety of techniques has been used to separate cyanogen bromide peptides. Drapeau & Yanofsky (1967b) had to use preparative polycrylamide gel electrophoresis in 8 M-urea to separate some of the peptides found. The fragments formed from subtilisin BPN' were not separated by this or by ion-exchange chromatography. The unresolved material was digested with trypsin and one of the peptides found gave the required information. Ambler used CM-cellulose and 8 M-urea solutions to separate the fragments formed from S. aureus penicillinase (Ambler & Meadway, 1969), while gel-filtration was adequate to separate the fragments formed from a Bence-Jones protein (Milstein, Clegg & Jarvis, 1968).

There are frequently regions of the molecule which are not detected in some digests. Tryptic digests in particular are subject

to/

to this difficulty. The material is often called "core" and is usually characterised by a high proportion of hydrophobic residues. Thus Drapeau & Yanofsky (1967a) report a tryptic peptide which could only be obtained in reasonable yield from a tryptic digest of cyanogen bromide treated protein, which contained it in two parts. Markland & Smith (1967) considered parts of subtilisin BrN' to be missing in both tryptic and chymotryptic digests. Milstein, Clegg & Jarvis (1968) also had a region which could not be recovered in tryptic digests. There do not appear to be reports of "core" peptides in peptic digests.

A different approach was used for the studies on glyceraldehyde 3-phosphate dehydrogenases. Lysine-blocking agents were used to restrict tryptic splitting to arginine residues - trifluoroacetyl groups by Davidson et al., (1967) and maleyl groups by Harris & Perham (1968). This did not much diminish the total number of different digests which required to be examined.

Even after all this accumulation of data there are usually a few overlaps which are ill-demonstrated. The weight of evidence from the rest of the molecule is usually convincing, but this often leaves a few peptide bonds which have not been located in fact in any digest. Thus Markland & Smith (1967) have a peptide, isolated from both peptic and cyanogen bromide digests from a region which is not found in any other digest. The peptide is placed between two other cyanogen bromide peptides essentially on the ground that there is nowhere/

nowhere else to put it. Smith et al., (1968) have two regions of sequence which end in lysines in tryptic peptides and two which start with lysine in chymotryptic peptides. The sequence is written so as not to exclude a large part of the molecule altogether. Similarly Carlton, Guest & Yanofsky (1967) argue that, were two peptides linked, a portion of protein would be excluded from the molecule. These arguments are all perfectly sound, and the sequences undoubtedly correct, but the degree of formal proof which has been attained with unique sets of overlapping peptides from smaller proteins is no longer possible.

Before we can consider the similarities found between different proteins we must define the term "homology". I shall follow Margoliash (Nolan & Margoliash, 1968; Margoliash, 1969) in defining homologous proteins as ones with a common ancestor, the sense in which the term has been used in studies of evolution of organisms. Winter, Walsh & Neurath (1968) have argued that the term should mean "having a greater degree of similarity than would be expected by chance alone" and consider that it is not possible to prove formally that proteins are homologous on Margoliash's definition. It would seem, however, that Neurath's definition fails to distinguish the two methods whereby proteins might come to be similar, namely convergence and divergence.

Similarities between proteins, in the primary sequence or in the structure of an active centre, may arise in two ways. One protein/

protein may be the ancestor of many, either through duplication and alteration in one organism, or through response to different selection pressures in different organisms. This is divergent evolution and the proteins are homologous. Alternatively, two organisms, requiring a particular function to be carried out, may evolve recognisably similar proteins from different starting material to perform the function, there being a limited number of ways in which the function can be carried out. This is convergent evolution and the proteins are analogous.

The proteases contain examples of both these processes. The group of enzymes chymotrypsin A & B, trypsin, elastase and thrombin have varying degrees of similarity in their primary structures and the same residues at their active sites (Brown, Kauffman & Hartley, 1967; Blow, Birktoft & Hartley, 1969). It is difficult to believe that they are not homologous enzymes. On the other hand subtilisins have no similarities with these in primary structure or overall molecular architecture, but also have residues of serine, histidine and aspartic acid in similar relation to each other in the active centre (Wright, Alden & Kraut, 1969). This may well be a case of convergent evolution.

Enzymes of different function may also be homologous. The function of α-lactalbumin is probably synthetic, but it has considerable primary structure similarities to lysozyme from hen's egg, of which the tertiary structure has been determined. Indeed, a model of the tertiary structure of α-lactalbumin has been postulated on the/

the basis of the tertiary structure of lysozyme (Brown et al., 1969).

Thus it appeared that the problem of evolutionary relations between penicillinases could not be solved without the determination of protein sequences. The work reported in this thesis is part of such an investigation, complementary to the determination of the S. aureus sequence. Indeed, as will appear in Chapter 16, without the S. aureus sequence it is doubtful whether a unique sequence could be drawn up for the B. licheniformis enzyme.

The protein is unusual for its size in lacking cystine and cysteine. Although this simplifies some aspects of sequence determination, and removes the problem of disulphide bridges, which has complicated other investigations, it renders ineffective various reagents which can denature proteins by reacting with cysteine without affecting tryptophan. So denaturing this protein has frequently conflicted with keeping the tryptophan residues intact, since oxidation is the only reliable chemical method of denaturation available.

The multiple starch gel bands observed with pure preparations were a little disturbing and it was desirable to find out the cause of them. This was possible early in the investigation, and the result left no reasonable doubt that only one primary sequence was present, with minor post-synthetic modifications.

The sequence of the protein has been determined principally by the/

the examination of the tryptic, chymotryptic, peptic and cyanogen bromide digests. The other studies reported are to some extent peripheral, and have not been carried to a state of completeness.

CHAPTER 2

ABBREVIATIONS, MATERIALS AND NOMENCLATURE

2.1. Abbreviations.

BAWP : Butan-1-ol : acetic acid : water : pyridine 15:3:12:10 by volume.

BIS : N,N'-methylene bis acrylamide.

CM- : Carboxymethyl-

CPA : Carboxypeptidase A.

CPB : Carboxypeptidase B.

DEAE- : Diethylaminoethyl-

DFP : Diisopropylfluorophosphate.

DNP- : 2,4-dinitrophenyl-

DNS- : 1-dimethylaminonaphthalene-5-sulphonyl-

DNS-PTC : Using PTC to degrade peptides and DNS-chloride to label the N-termini.

DPCC- : Treated with diphenylcarbamyl chloride.

HNB- : 2-hydroxy-5-nitrobenzyl-

N-t. : N-terminus.

PTC : Phenyl isothiocyanate

SBTI : Soya bean trypsin inhibitor.

SE- : Sulphoethyl-

TEMED : N,N,N',N'-tetramethylethylenediamine.

TNM : Tetranitromethane.

Tris : 2-amino-2-hydroxymethylpropane-1,3-diol.

XCFF : Xylene cyanol FF.

2.1. Abbreviations Contd.

m : Electrophoretic mobility at pH 6.5
m' : Electrophoretic mobility at pH 3.5
} Defined in Section 2.4.

R_x : R_F relative to that of XCFF on descending paper chromatography in BAWP.

% : Unqualified, means by volume for two liquids.

u : Units (of penicillinase). ku and Mu are 10^3 and 10^6 units respectively.

Two types of abbreviation have been used for amino acids. Generally, three letter abbreviations (Commission on Biochemical Nomenclature, 1967) have been used, but single letter abbreviations (Commission on Biochemical Nomenclature, 1969) are used in Chapter 16 for concise comparison of sequence. The abbreviations are

Amino acid.	Three letter.	One letter.	Amino acid.	Three letter	One lett
Lysine	Lys	k	Glutamic acid	Glu	e
Histidine	His	h	Glutamine	Gln	q
Arginine	Arg	r	One of these two	Glx	
Aspartic acid	Asp	d	Proline	Pro	p
Asparagine	Asn	n	Glycine	Gly	g
One of these two	Asx		Alanine	Ala	a
Methionine	Met	m	Valine	Val	v
Methionine Sulphone	Mes		Isoleucine	Ile	i
Homoserine	Hsr		Leucine	Leu	l

Homoserine/

2.1. Abbreviations Contd.

Amino acid.	Three letter.	One letter.	Amino acid.	Three letter.	One lett
Homoserine lactone	Hsl		Tyrosine	Tyr	y
Threonine	Thr	t	Phenylalanine	Phe	f
Serine	Ser	s	Tryptophan	Trp	w
Pyrrolidone Carboxylic acid	Glp				

Residues joined by hyphens are in that sequence. Residues in brackets are in unknown order.

2.2. Materials.

Spores of B. licheniformis 749/C (Pollock, 1963) and rabbit anti-penicillinase antiserum were kindly supplied by Professor M.R. Pollock, as were various penicillins and cephalosporins (see Pollock (1965) for manufacturers). Benzylpenicillin was a gift from Glaxo Laboratories Ltd.

Where available Analytical Reagent grade chemicals were used. The sources of less common chemicals, the batches of proteases used and the addresses of suppliers are listed in Appendix III.

Acrylamide and BIS were recrystallised from chloroform and acetone respectively. N-ethyl morpholine was redistilled, and maleic/

maleic anydride was distilled under reduced pressure. Urea solutions were de-ionised by passage through a column of mixed-bed ion-exchange resin (Biodeminrolit, Permutit).

The molarity of buffers refers to the anion. Buffers were made by titrating a more concentrated solution of the anion with acid or base to the required pH, diluting to the correct volume and checking the pH. If further adjustment was required a very concentrated acid or base was used. Ammonium acetate buffers at pH 8.5 were made starting with ammonia. The ionic strengths are about 25% less than buffers made starting from acetic acid. Molarities of this buffer refer to ammonia.

2.3. Peptide nomenclature.

The first letter shows the primary digestion by which the peptide was made: X, Cyanogen bromide; T, Trypsin; C, Chymotrypsin; P, Pepsin.

The second letter shows the mobility of the peptide at pH 6.5: B, Basic; N, neutral; A, acidic.

The peptides in each series are then distinguished by numbers. Thus PA25 is an acidic peptic peptide. The series of numbers are not complete.

Peptides produced by PTC degradation are distinguished by the letter F followed by a number indicating the number of cycles. Thus CN7 gives CN7F3 after 3 cycles of PTC degradation and its N-terminus is the fourth residue of CN7.

Peptides produced by further digestion with another protease are/

are distinguished by the same system. At this stage other enzymes have also been used: H, Thermolysin; S, Subtilisin B; M, papain.

Thus peptide TA8 was degraded with chymotrypsin and one of the peptides produced is neutral at pH 6.5, and is called TA8CN1. This was degraded with thermolysin and two neutral peptides were produced, TA8CN1HN1 & TA8CN1HN2. After two cycles of PTC degradation the latter gives TA8CN1HN2F2.

This system was not used for peptides produced in the experiments described in Chapters 14 and 15. Soluble cyanogen bromide peptides are labelled X1, etc. Different numbers distinguish the regions of sequence giving rise to the peptides. Prefixed E & T show whether the peptide originated from exo-enzyme or trypsin-released enzyme. X1a & X1b, etc. come from the same region but differ in some respect. XC was an insoluble peptide which was characterised.

2.4. Peptide mobilities.

The electrophoretic mobility of a substance is the distance it moved relative to the movement of a standard substance.

Mobilities at pH 6.5 (m) have been measured from the mono-aminomonocarboxylic acids to correct for endosmosis. Lysine has been given a mobility of +1, and aspartic acid -1. XCFF has a mobility/

mobility of -0.40 and was used as a reference where necessary. Mobilities at pH 6.5 can indicate whether side chain carboxyl groups are free or amidated. Offord (1966) has compiled data from which the charge on a peptide of known mobility and molecular weight can be deduced. In that paper basic peptides were also measured relative to aspartic acid, on which system lysine is + 0.92, but the scatter of the data is so great that the difference is not significant.

Mobilities at pH 3.5 (m') are measured from taurine using alanyl-glycine as +1. A similar system was used by Milstein, Clegg & Jarvis (1968).

Movement in descending chromatography in BAWP has been expressed relative to XCFF (R_x). The R_F of XCFF is about 0.69.

CHAPTER 3

METHODS

This chapter summarises techniques which have been used frequently. Other techniques will be found in

Chapter 4	:	Penicillinase assay.
Chapter 7	:	Penicillinase purification.
Section 8.2	:	Polyacrylamide disc electrophoresis.
Section 8.3	:	Isoelectric focusing.
Section 9.3	:	Fractionation of penicillinase by chromatography on DEAE-cellulose.
Section 9.4	:	Antiserum titration of penicillinase.
Section 10.1	:	Removal of large peptides by precipitation with trichloracetic acid.
Section 11.4	:	Separation of peptides on Sephadex G25.
Section 11.4	:	Desalting of peptides.
Section 12.3	:	Separation of peptides with SE-Sephadex.

3.1. Protein determination.

Protein has been determined by the method of Lowry *et al* (1951). The sample was made up to 0.5 ml. and 3 ml. of reagent A was added and mixed.

Reagent A/

Reagent A. Make fresh daily.

To 100 ml. 2% (w/v) Na_2CO_3 in 0.1 M-NaOH add 1 ml. 2% (w/v) aq. NaK tartrate and 1 ml. 1% (w/v) aq. $CuSO_4.5H_2O$.

Reagent B. Stable for several months at 4°.

Dilute Folin-Ciocalteau's reagent with an equal volume of water.

After 10 min., 0.3 ml. of Reagent B was added and mixed. The extinction at 750 nm. was read after 0.5 to 3 hr. Standards containing up to 0.2 mg. serum albumin were determined concurrently and a standard curve was drawn. Protein samples contained 0.05 - 0.2 mg. protein.

3.2. Starch gel electrophoresis.

This is the method of Smithies (1959). 19.25 g. starch (Connaught) was added to 175 ml. 0.03 M sodium borate buffer, pH 8.5, in a 1 litre spherical flask. The mixture was heated over a Bunsen burner with constant thorough swirling until the mixture gelled and began to bubble. The gel was then de-gassed with a water pump vacuum, and then poured into a mould, consisting of a Perspex tray, 14.8 x 9.0 x 1.5 cm. internally, with a plate 0.6 cm. thick fitted in the bottom. After the gel had set it was covered with polythene to prevent evaporation and allowed to set thoroughly at 4°.

To insert samples in the gel, a slit was cut with a razor blade 3 cm. from one end. The samples were loaded on pieces of 3MM paper and inserted in the slit. A gel could easily accommodate six. One piece of paper was loaded with XCFF, 0.25 mg./ml., as an external marker.

The/

The electrode vessels contained 0.3 M sodium borate, pH 8.5, and wicks of eight thicknesses of 3MM paper were used. The wicks were pressed firmly on the ends of the gel. The apparatus was enclosed in a box (in a 4° room) which cut off the power when opened.

40 ma. was allowed to flow through the gel (250 to 350 v). The run was terminated when XCFF had moved about 7 cm. (3 hr). After the gel had cooled to about 4°, the gel and false bottom were pushed up and two 0.1 cm. thick spacer plates were inserted. The gel was then replaced in the tray and sliced with a wire across the top of the tray.

To detect protein, a slice was dipped in 0.5% (w/v) Amido (Naphthalene) Black in methanol:water:acetic acid (5:5:1 by vol.) for ten minutes. The excess stain was removed by repeated washing with the same solvent.

Penicillinase was detected by spraying a slice with 1.2 g. sodium benzylpenicillin in 50 ml. 0.1 M sodium phosphate buffer, pH7.0, containing 2.5 ml. 0.32 M-I_2 in M-KI (Pollock, 1965). Consumption of iodine by penicilloic acid produced white bands on a blue ground. The bands spread quickly through diffusion of penicilloic acid and were recorded as rapidly as possible.

To compare positions of protein and penicillinase bands correction (about 10%) was made for shrinkage in the gel stained for protein.

3.3. Ion-exchange chromatography.

Cellulose phosphate (Whatman P11) was prepared as described by

Pollock/

Pollock (1965). It was de-fined by settling from a large volume of water. It was washed with 0.2 M-HCl until the washings were ammonia-free when made alkaline, and then with water. In most experiments it was then washed with 0.1 M-NaOH until the washings were alkaline to bromothymol blue and then with water followed by 0.2 M-HCl. A final water wash was monitored with small scale experiments to ensure adequate penicillinase-adsorbing capacity.

CM-cellulose (Whatman CM11) was de-fined by suspending 100 g. in 3 litres of water and discarding the supernatant after about 20 min. It was then treated with 1.5 litres 0.5 M-NaOH for 1 hr., and then washed with water to pH 8. It was next treated with 0.5 M-HCl for 30 min. and washed to near neutrality before equilibration with the buffer required. After use in penicillinase preparation (Section 7.2) it was washed with 0.5 M-acetic acid for 1 hr., then washed and re-equilibrated.

DEAE-cellulose (Whatman DE11) was treated as CM11 but with the treatments in the reverse order. After acid treatment it was washed to pH 4 with water. After use in penicillinase preparation (Section 7.6) it was washed with M-ammonium acetate, pH 4.8 and re-equilibrated.

Whatman CM52 and DE52 were supplied pre-swollen. They were de-fined and equilibrated before use.

SE-Sephadex A-25 was swollen overnight in water. It was washed with 0.5 M-HCl until the effluent was sodium free (flame test). After washing with water to pH 3.5 it was suspended in M ammonium acetate, pH 4.0. Before use it was equilibrated with the buffer required.

Gradients/

Gradients were generated with a linear device. Two vessels of the same cross sectional area were interconnected at the bottom. The initial buffer in the first was connected to the column and mixed magnetically. The final buffer was placed in the second vessel to the same level.

The extinction of effluent from columns at 254 or 280 nm. was recorded automatically with a Uvicord I or II (LKB Instruments Ltd.).

3.4. Gel filtration.

Gel filtration on columns of Sephadex was introduced by Porath & Flodin (1959). Sephadex (Pharmacia Ltd.) is dextran, cross-linked to different degrees and made into beads. It separates compounds chiefly on a size basis, G25, for example, up to molecular weight 5,000 and G100 from 15,000 to 150,000. "Fine" grades have been used throughout.

The material was first swollen in water (LH20 in equal volumes of ethanol and 5% formic acid). In a stirred suspension, overnight was long enough for G25, but G100 required 48 hr. Equilibration was accomplished by stirring in a suitable volume of concentrated buffer and changing the buffer once or twice.

Columns of grades up to G25 and LH20 were prepared simply by pouring a slurry into a column and replacing buffer as it drains with more slurry until the column was tall enough. The column was then connected to a pump and buffer passed at an appropriate rate to ensure equilibration.

With higher grades such as G100, the slurry was first degassed on a water pump. The column was then filled with slurry and immediately

connected/

connected to a pump delivering buffer at the required flow rate. From time to time the buffer in the top of the column was replaced with slurry. This procedure was necessary to prevent over-packing of the compressible beads at the start.

The sample was applied by allowing the top of the column to run just dry and layering the sample on top with a Pasteur pipette. Great care was needed to avoid damage to the top of columns of G100. The extinction of the effluent was recorded as noted in the last section.

3.5. Oxidation with performic acid.

The reagent was made (Hirs, 1956) by adding 0.5 ml. 30% (100 vol.) hydrogen peroxide to 9.5 ml. conc. formic acid. After 2 hr. it was ready for use.

An equal volume of this reagent was added to penicillinase dissolved in conc. formic acid at 40 mg./ml. (ten-fold excess over methionine). After 2 hr. at 0°, the mixture was diluted with 25 volumes of water and freeze-dried.

In early experiments, 0.05 ml. of this reagent per methionine per micromole was added to peptides to be oxidised in a tube. After 2 hr. at 0° the reagent was removed under vacuum over H_2SO_4 and NaOH. However, it was thought that this method might lead to halogenation of tyrosine (Sanger & Thompson, 1963; Hirs, 1967). Instead, the peptide, on paper prior to elution, was exposed to the vapour of performic acid for 2 hr. in a partly evacuated dessicator (Brown & Hartley, 1966).

After/

After 2 hr. the reagent was removed over NaOH in another desiccator. The peptides were then eluted in the normal way.

3.6. Cyanogen bromide degradation.

Cyanogen bromide cleaves polypeptide chains at methionine residues leaving a residue of homoserine, which is in equilibrium with its lactone (Gross & Witkop, 1962). The use of formic acid as a solvent prevents solubility problems (Ambler, 1965).

The penicillinase was dissolved in 98% formic acid at 10-40 mg./ml. Cyanogen bromide was dissolved in formic acid at the same concentration, and an equal volume was added. After 24 hr. at 25° the mixture was diluted with 25 volumes of water and freeze-dried.

These quantities represent a 45-fold excess of cyanogen bromide over methionine, and amino acid analysis generally showed less than 5% methionine remaining.

3.7. High-voltage paper electrophoresis.

Two types of apparatus have been used. In the Michl (1951) apparatus, the paper hangs in a tank of organic liquid cooled with water passing through a coil at the top. The top of the paper is held in a trough connected to one electrode, and the bottom dips in buffer at the bottom of the tank connected to the other electrode. In the Gross (1961) apparatus, the paper, insulated between sheets of polythene is pressed between two cooled metal plates. It is connected to buffer vessels

through/

through paper wicks. The buffers and coolants used at different pH's are shown in Table 3.7.1.

Whatman 3MM paper was used except for amino acids and small amounts of peptides, which were separated on Whatman No. 1 paper. In the Michl tanks papers 57 cm. long were used. At pH 2.0 & 3.5 the sample was generally placed 10 cm. from the bottom, positive, end of the paper; at pH 6.5 & 9.5 near the middle. At pH 4.38 pre-cut papers 19 x 79 cm. were used, and the samples were placed 30 cm. from the negative electrode.

Table 3.7.1. Buffers and Coolants for high-voltage paper electrophoresis

pH	System	Coolant	parts by vol.					Reference
			Pyridine	Acetic Acid	Formic Acid	TEC[a]	Water	
2.0	Michl	White spirit	-	4	1	-	45	Ambler (1963b)
3.5	Michl	White spirit	1	10	-	-	89	Ryle et al. (1955)
4.38	Gross	-	3	6	-	-	500	Gray (1967a)
6.5	Michl	Toluene[b]	25	1	-	-	225	Ryle et al. (1955)
9.5	Michl	White spirit[c]	-	-	-	5	95	Sanger & Brownlee (1967)

[a]: Triethylamine carbonate

[b]: Containing 8% pyridine

[c]: Containing 10% triethylamine

The se buffers were also used to wet the paper, except at pH 3.5, where it was diluted first with an equal volume of water.

Amino/

Amino acids and peptides were dissolved and applied in 0.1 M-ammonia, and DNS-amino acids in 50% pyridine. Amino acid mixtures and analytical samples of peptides were applied to 1 cm. in 0.01 ml. Up to 16 samples and standards were applied to a sheet for identification of DNS-amino acids at pH 4.38. For preparative purposes peptides were dissolved in about 0.03 ml./cm. of 3MM paper (less for No. 1) and loaded on a band at the origin. Two 1 cm. markers were used, separated from the band by 1 cm. On 3MM loads were about 0.2 micromoles/cm. If this gave less than 5 cm., No. 1 paper was used at 0.05 - 0.1 micromoles/cm. Care was taken to load the markers at the same level as the main band. For bands wider than 20 cm. a narrow strip was cut from the centre of the band and developed with the markers.

Amino acids were divided into two mixtures, which were applied as standards. One also contained ε-DNP-lysine. Standards for DNS-amino acid identification contained all the DNS-amino acids commonly found. For all other separations a standard mixture (Milstein & Milstein, 1968) was used. In additon to 0.25 mg./ml. XCFF it contained 5 micromoles/ml. of lysine, histidine, arginine, glycine, valine, cysteic acid, aspartic acid, glutamic acid, alanyl-glycine, taurine and ε-DNP-lysine. The coloured compounds were used as external markers to observe the length of runs (Milstein & Sanger, 1961). Red "Pentel" pen (Milstein, 1966) was used, at pH 6.5 starting from the origin and at pH 3.5 starting at the far end of the paper from which it ran back to the origin, as an external marker.

The/

The paper was placed on a glass plate with the origin raised on glass rods. The paper was wetted with buffer which was allowed to creep up to the origin from each side. Excess buffer was removed by blotting, and for the flat-plate machine by expressing it with a roller.

3.8. Paper chromatography.

Peptide mixtures were separated by descending paper chromatography with butan-1-ol:acetic acid:water pyridine (15:3:12:10 by vol.). (BAWP) (Waley & Watson, 1953). Very little equilibration was needed. The peptides were loaded as described for electrophoresis at pH 3.5 and the bottom of the sheet was serrated. The chromatograms were run for about 15 hr. The bands were not as straight as those obtained by electrophoresis, and a central guide strip was used for band over 15 cm.

Certain DNS-amino acids were identified by paper chromatography. The spots were cut out from the paper after electrophoresis at pH 4.38 and sewn (Naughton & Hagopian, 1962) to another sheet 10 cm. from the top. The trough was filled with the upper phases of light petroleum (B.P. 40-60°):acetic acid:water (10:9:1 by vol.). (Boulton & Bush, 1964). The best method of equilibration was to start a blank paper in another trough in the tank when putting the experimental paper in, and start the experimental paper two hours later. The bottom of the paper was serrated since a large volume of developer flowed off the bottom.

3.9. Detection/

3.9. Detection and elution of peptides.

Peptides containing oxidised tryptophan fluoresced on u.v. (365 nm.) irradiation.

Ninhydrin. The paper was dipped in 0.2% ninhydrin in acetone (Toennies & Kolb, 1951) and heated at 105°. Transient yellow or brown colours were given by peptides with glycine, threonine and serine N-termini, which faded on further heating to the purple given by other peptides. After pH 9.5 electrophoresis, a little glacial acetic acid was added to the ninhydrin (Levy & Chung, 1953).

Arginine. (Sagakuchi reaction - Jepson & Smith, 1953). The paper was dipped in 0.1% 8-hydroxyquinoline in acetone. When dry it was sprayed with 0.2% bromine in 0.5 M-NaOH. Arginine stains red, but the reaction did not work well after ninhydrin.

Tyrosine. (Acher & Crocker, 1952, modified by Jepson & Smith, 1953). The paper was dipped in 0.1% 1-nitroso-2-naphthol in acetone. When dry, it was dipped in 10% (v/v) conc. nitric acid in acetone and heated strongly. Tyrosine gave a red colour, while tryptophan gave a grey-brown colour (Ambler, 1960). This method works well after ninhydrin.

Tryptophan. (Ehrlich reaction - Dalgliesh, 1952). The paper was dipped in 1% p-aminodimethylbenzaldehyde in acetone freshly acidified with a little conc. HCl. Tryptophan slowly gave mauve spots, and the method worked well after ninhydrin.

Histidine. (Pauly reaction - Dent, 1947). Equal volumes of 1% (w/v) sulphanilic acid in M-HCl and 5% (w/v) $NaNO_2$ were mixed and

after/

after 10 min. at 4° the mixture was sprayed on the paper. Then the paper was sprayed with 15% (w/v) Na_2CO_3. Histidine gave bright red spots and tyrosine brownish ones.

Methionine. (Toennies & Kolb, 1951). The paper was dipped in a fresh mixture of 50 ml. 2mM aq. chloroplatinic acid, 3 ml. aq. M-KI and 10 ml. M-HCl made up to one litre with acetone. When dry the spots (white on pale purple) are intensified with HCl vapour. The method did not work with methionine sulphone or after ninhydrin.

N-Terminal Proline (Acher, Fromageot & Jutisz, 1950). The paper was dipped in 0.2% (w/v) isatin in acetone with 4% (v/v) glacial acetic acid and then heated strongly. N-terminal proline gives a blue colour. This method does not work after ninhydrin but can be followed by it.

Chlorination. (Reindel & Hoppe, 1954). The paper, wetted with acetone/ethanol (equal vols.), was exposed in a closed dish to chlorine generated from a mixture of satd. aq. $KMnO_4$ and 2.4 M-HCl in a small dish with a glass grid over it. After the ninhydrin colour had faded, excess chlorine was allowed to blow off and the paper was dipped in a fresh mixture of equal volumes of 0.05 M KI and satd. o-Tolidine in 0.5 M-acetic acid. Peptides and many amino acids give blue spots. o-Tolidine is carcinogenic and was handled carefully.

Peptides were eluted with 0.1 M-ammonia, which was allowed to flow through the strip for two to three hours. Strips were occasionally examined with ninhydrin to check completeness of elution, and no evidence

of/

of incomplete elution was found. Ammonia was dried over conc. H_2SO_4. The dessicator was evacuated with a rotary pump, and care was needed to avoid bumping.

3.10. Acid hydrolysis.

Samples for quantitative analysis (0.05 - 0.20 micromoles) were dried in Pyrex tubes (10.0 x 1.2 cm.). 0.5 ml. of 6M-HCl (prepared by dilution of freshly opened conc. HCl) was added and the tube was sealed under vacuum to prevent oxidation (Moore & Stein, 1963). A neck was drawn in the tube, the acid was frozen and the tube was evacuated to 0.15 torr and sealed. Samples were hydrolysed at 105°, usually for 24 hours.

Samples of peptide and DNS-peptide for qualitative analysis were in 3.5 x 0.8 and 3.0 x 0.6 cm. tubes respectively. 0.1 & 0.05 ml. respectively of 6M-HCl were added and the tubes were sealed and hydrolysed for 12 to 18 hrs.

The tubes were opened and the acid dried in high vacuum over NaOH pellets. Amino acid analyser samples were removed from the dessicator and stored at 4° as soon as dry. This was easier than rotary evaporation and no serine-O-glutamate (Ikawa & Snell, 1961) was observed on the analyser trace. Serine- and threonine-O-sulphates (Murray & Milstein, 1967) were not observed either.

3.11. Qualitative amino acid analysis.

0.01 micromoles of peptide was hydrolysed as described in Section 3.10. and electrophoresed at pH 2.0 on No. 1 paper (Section 3.7.).

The/

The dry paper was examined under u.v. (365 nm.) light. The decomposition products of tryptophan are fluorescent, but this must be distinguished from the general fluorescence of peptides and amino acids after strong heating (Phillips, 1948). The paper was then dipped in ninhydrin in acetone (Section 3.9.) with 1% crude collidine (Appendix III) added. (Levy & Chung, 1953). Transient, brilliant colours appeared on heating the paper. The most striking were: glycine, red; serine, green; threonine, grey; proline, yellow; phenylalanine, green; tyrosine, brown; aspartic acid, bright blue; asparagine, orange-brown. The colours faded on vigorous heating or overnight at room temperature. The amino acids were identified by their positions and colours and relative intensities were recorded. Leucine and isoleucine could not be distinguished.

The isatin reagent for proline (Section 3.9.) works after ninhydrin with collidine (Milstein, 1966). On vigorous heating a red colour appears.

3.12. Quantitative amino acid analysis.

Amino acids have been determined on acid hydrolysates. Analysis for tryptophan is dealt with in the next section. The automatic method of amino acid analysis by ion-exchange chromatography was described in great detail by its originators (Spackman, Stein & Moore, 1958). The accelerated system used here has been described (Benson & Patterson, 1964), and advances in technique have been reviewed (Spackman, 1967).

Amino/

Amino acids are identified by their positions of elution during a highly reproducible programme. They are measured by calculation of the area under the peak on the chart, compared with the area given by a known quantity of the same amino acid.

Two analysers have been used in this work. An instrument from Evans Electroselenium Ltd. (EEL) used pulverised and graded resin (Beckman 50B) (Moore, Spackman & Stein, 1958), while the Beckman 120C used resin polymerised in beads (Beckman PA35 & UR30, and Locarte No. 12) (Benson & Patterson, 1965).

The approximate sensitivity of these machines (micromoles amino acid for 0.01 absorbance.peak height) is: EEL, 0.001 (Proline, 0.003); Beckman, 0.003 (Proline, 0.012).

A check was kept on the quantity of sample applied to each column by the use of internal standards (Walsh & Brown, 1962). The sample was generally dissolved in 0.35 ml. sodium citrate buffer, pH 2.2, containing 1 micromole/ml. of norleucine and 2-amino-3-guanidopropionic acid which elute separately from the protein amino acids. 0.15 ml. was generally applied to each column, leaving 0.05 ml. for use in the case of accident. By calculating the amount of unnatural amino acid it was possible to determine precisely how much sample had been applied to the column.

Homoserine was obtained from methionine with cyanogen bromide. Mixtures containing it were converted wholly to homoserine from its lactone by treatment with pH 6.5 pyridine acetate buffer for 1 hr.

at/

at 105° (Ambler, 1965). On the EEL analyser, it was separated from glutamic acid by lowering the pH of the eluting buffer from 3.28 to 3.10, but on Locarte No. 12 resin in the 120C it eluted earlier than and well separated from glutamic acid at pH 3.28.

Constants for aspartic acid and serine have been used for methionine sulphone and homoserine respectively. The stoichiometry obtained with the peptides involved suggests that this caused little error.

Peptide analyses are presented as moles amino acid/mole peptide. The amount of peptide was calculated by assuming that the sum of the amounts of amino acid (excluding impurities) is an integral multiple of the amount of peptide.

3.13. Tryptophan determination.

Spies and Chambers' (1948) tryptophan determination was carried out only on whole protein. About 3 mg. penicillinase was used, in parallel with up to 0.5 micromoles L-tryptophan. The sample, in 0.5 ml. water, was treated with 4 ml. 65% (v/v) sulphuric acid mixed with 0.5 ml. 3% (w/v) p-dimethylamino-benzaldehyde in M-H_2SO_4. After thorough mixing, and one hour in the dark, 0.05 ml. 0.04% (w/v) $NaNO_2$ was added, and mixed. The extinctions were read at 590 nm. after another 30 min. in the dark. The tryptophan content was determined from a standard curve.

For spectroscopic determination of tryptophan a solution of known concentration was made 0.1 M in NaOH. Its extinction was then

determined/

determined at 280 & 294.4 nm. In the case of protein a correction was made for haze by extrapolating back from extinctions determined at 360 nm. and 320 nm.

The residues of tryptophan and tyrosine may then be calculated (Goodwin & Morton, 1946; Beaven & Holiday, 1952). E is extinction in a 1 cm. cell; c is the molar concentration of peptide or protein.

$$\text{residues Trp} = (0.263\ E_{280} - 0.170\ E_{294.4})/10^3 c.$$

$$\text{residues Tyr} = (0.592\ E_{294.4} - 0.263\ E_{280})/10^3 c.$$

U.v. absorbing impurities in peptides were removed before analysis by gel filtration on Sephadex G25 in 1% formic acid. (Ambler & Brown, 1967). The tyrosine content of two peptides was determined by this method. One containing no tyrosine, gave 0.35, and another, containing 1 tyrosine gave 1.07. Hence results for tryptophan are only approximate.

3.14. DNP method for N-termini.

The method was based on that of Fraenkel-Conrat, Harris & Levy, (1955). 15 mg. penicillinase was dissolved in 1 ml. 0.2 M N-ethyl morpholine acetate, pH 8.5, in a screw cap tube. Solid urea was added to saturation, together with 0.05 ml. 1-fluoro-2, 4-dinitrobenzene. The mixture was stirred for 3 hr. at 37°, and then excess reagent was extracted with diethyl ether. The aqueous phase was transferred to

a/

a hydrolysis tube and 6 M-HCl added until the DNP-protein precipitated. The precipitate was centrifuged and washed with M-HCl. It was then dissolved in 0.25 ml. 12 M-HCl, diluted with an equal volume of water and hydrolysed under vacuum for 12 hr. at 105° (Section 3.10).

The hydrolysate was diluted with two volumes of water and extracted thrice with 3 ml. ether. The pooled ether was washed with an equal volume of M-HCl. A portion of the aqueous phase was dried and analysed for amino acids to determine the quantity of protein. The ethereal phase, containing most of the DNP-amino acids, was dried in a steam of nitrogen and dessicated to remove the last traces of acid.

The DNP-amino acids were separated by two dimensional ascending paper chromatography. The sample was applied near the corner of a ten inch square sheet of No. 1 paper which was fitted in a metal frame. Standards were run in parallel papers. The first dimension was 2-methylbutan-2-ol:2M aq. ammonia (4:1 by vol.) and was run for 16 hr. (Ambler, 1963a). The papers were dried and then separated in the second dimension with 1.5 M sodium phosphate buffer, pH 6.0 for 7 hr. (Levy, 1954). The papers were dried and the unknown spots identified by comparison with the standards.

Spots were eluted by heating in water at 55° for 15 min. and the extinction determined at 360 nm. compared with a blank eluted from a clear piece of chromatogram. The quantity of amino acid was determined using the relevant extinction coefficient (Fraenkel-Conrat, Harris & Levy, 1955).

3.15. DNS/

3.15. DNS method for N-termini.

The DNS method (Gray, 1967a) has been used for peptides and protein. Peptide samples (0.01 micromoles) were dried in a dessicator in a 3.0 x 0.6 cm. tube. 0.01 ml. 0.1 M-$NaHCO_3$ was added and the sample dried again. 0.01 ml. water and 0.01 ml. 2.5 mg./ml. DNS-chloride in acetone were then added. The samples were kept at 37° for 2 hr., covered with parafilm to prevent loss of the acetone. The mixture was then dried and hydrolysed (0.05 ml. 6 M-HCl, 12 - 18 hr., 105°; Section 3.10), and the DNS-amino acids were separated by high-voltage paper electrophoresis at pH 4.38 (7,000 v, 130 min; Section 3.7.).

About 0.03 micromoles of protein was dissolved in 1.5 ml. 0.5 M-$NaHCO_3$. 1.5 ml. of 20 mg./ml. DNS-Cl in acetone was added. There was an immediate precipitate and the mixture was incubated at 37° for 3 hr. Excess reactants were dialysed out overnight against water. The DNS-protein was collected by centrifugation and washed with water. It was then dissolved in 0.25 ml. 12 M-HCl, diluted with an equal volume of water and hydrolysed under vacuum for 12 to 18 hr. (Section 3.10.). The DNS-amino acids were separated as described above.

The paper was heated thoroughly and examined under u.v. (365 nm.) The relative positions of the different DNS-amino acids are described by Gray (1967a). Spots considered to be DNS-Gly, DNS-Ala and DNS-Ser were cut out and sewn on to another sheet of paper and identified by electrophoresis (30 min. 110v./cm.) at pH 2.0. This separation is also shown by Gray (1967a). DNS-Thr behaves as DNS-Ser.

Spots/

Spots considered to be DNS-Thr, DNS-Pro, DNS-Phe, DNS-Leu, DNS-Ile & DNS-Val were cut out, sewn on to another sheet and identified by paper chromatography in the system of Boulton & Bush (1964) (Section 3.8.). The paper was examined with u.v. (254 nm.) light. Movement relative to DNS-Ile put equal to 1.00 were DNS-Thr, 0.00; DNS-Ala, 0.14; DNS-Phe, 0.29; DNS-Pro, 0.46; DNS-Val, 0.63; DNS-Leu, 0.82.

Where necessary, the identities of DNS-His, DNS-Arg, ε-DNS-Lys & α-DNS-Lys were checked by electrophoresis at pH 9.5.

Various dipeptides were still visible after the period of hydrolysis used. DNS-Val-Val & DNS-Ile-Ile ran more slowly than the DNS-amino acids at pH4.38. On paper chromatography the relative movement of DNS-Ile-Ile was about 0.45, and DNS-Val-Val moved slightly less. DNS-Ile-Phe behaved similarly, moving about 0.25 on paper chromatography. DNS-Ile-Arg moved similarly to DNS-His at pH 4.38, but moved in the opposite direction at pH 9.5. DNS-Ile-ε-DNS-Lys behaved like bis-DNS-Lys as remarked in Section 13.10. The structures of all these dipeptides were confirmed by DNS-PTC degradation.

Recoveries of DNS-Pro were markedly improved by reducing hydrolysis time to 4 hr. Care was needed to distinguish it from DNS-Ala, which it resembled at pH 2.0. The identification by chromatography was unequivocal.

3.16. Hydrazinolysis.

The C-terminal amino acid is the only free amino acid remaining in a hydrazinolysate (Akabori, Ohno & Narita, 1952).

About/

About 5 mg. penicillinase was weighed into a tube (10.0 x 1.2 cm.). 30 mg. Permutit ZK226 (H^+ form, 2.5% cross-linked, less than 200 mesh) was added (Braun & Schroeder, 1967) and a neck drawn in the tube. 1 ml. hydrazine (95%+) was placed in the neck and drawn in by cooling the tube, which was then sealed under vacuum (Section 3.10.). After 14 hr. at 80° the hydraxine was removed in vacuo over conc. H_2SO_4. The soluble compounds were removed from the resin by three washes with 1 ml. water, and the hydrazides were extracted from the water by two extractions with 1 ml. benzaldehyde. The aqueous phase was then extracted twice with 4 ml. ethyl acetate to remove the benzaldehyde and rotary evaporated to dryness. The amino acids were applied to the amino acid analyser in the usual way.

3.17. Carboxypeptidase digestion of protein.

The preparation of CPA & CPB is described in Section 3.19. The techniques used are those of Ambler (1967a). Performic acid oxidised penicillinase was used, dissolved with the aid of urea and passed through Sephadex G25 to equilibrate it with 0.2 M N-ethyl morpholine acetate, pH 8.5.

Preliminary experiments were performed with about 1 mg. penicillinase. After reaction with enzyme, the digestion was stopped with sulphonated polystyrene beads (Permutit ZK225, SRC13, H^+ form), added until the pH was below 3. After thorough shaking, the supernatant was discarded and the beads washed twice with a little water. The beads were eluted three times with three volumes of 2 M-ammonia and the eluted amino acids

were/

were dried and the amino acids were examined by electrophoresis at pH 2.0 (Section 3.11).

A quantitative experiment was performed with CPB. About 0.04 mg. CPB was used with 1.5 mg. portions of penicillinase. The digestions were stopped after various times at 30° by adding 0.5 ml. acetic acid to the 1 ml. of buffer. The solutions were then dried and quantitatively analysed for amino acids.

3.18. Carboxypeptidase digestion of peptides.

The enzyme solutions are described in the next section. Techniques were those of Ambler (1967b). Peptide samples were dried in tubes (3.5 x 0.8 cm.). An enzyme-free peptide control was generally used. Most peptides were examined with CPA; tryptic peptides with CPB and CPA + CPB. Enzyme blanks were invariably negative.

The peptide (0.02 - 0.05 micromoles) was dissolved in 0.2 ml. 0.2 M-ammonium acetate, pH 8.5 and the enzyme (about 0.01 mg. in 0.01 ml.) was added. After 4 hr. at 37° the solution were dried. The mixtures were electrophoresed at pH 3.5 or 6.5 on No. 1 paper. Care was taken to keep lysine and aspartic acid on the paper. The band containing the monoaminomonocarboxylic acids was located by developing standards, cut out, sewn on another sheet of paper (Naughton & Hagopian, 1962), and electrophoresed at pH 2.0 to identify the amino acids. The first electrophoresis was done at pH 6.5, unless neutral residual peptides might confuse the identification of amino acids, when it was done at pH 3.5. Amino acids and peptides were compared with

the/

the control and recorded.

3.19. Enzyme digestion.

Some proteases were pre-treated before use, and solutions were stored at -20°.

Trypsin was treated with diphenylcarbamylchloride (DPCC), an inhibitor of chymotrypsin (Erlanger & Cohen, 1963). Trypsin was dissolved at 10 mg./ml. in 0.1 M tris-chloride, pH 8.0 containing 0.1 M-$CaCl_2$. 0.005 ml./ml. of DPCC (25 micromoles/ml. in acetone) was added and after 30 min. a small precipitate was centrifuged off.

Chymotrypsin was pre-incubated with one tenth of its weight of SBTI in 0.2 M ammonium acetate, pH 8.5, for one hour at 37° (Ambler, 1963b).

CPA (DFP-treated) was solubilized immediately before use (Fraenkel-Conrat, Harris & Levy, 1955). 1.25 mg. enzyme was washed with water and centrifuged. It was then suspended in 0.1 ml. 1% (w/v) $NaHCO_3$ and cooled in ice. 0.1 M-NaOH was added with mixing drop by drop until the protein dissolved, and the pH dropped to 8.5 as rapidly as possible with 0.1 M-HCl. The mixture was diluted to 1.25 ml. with 0.2 M N-ethyl morpholine acetate pH 8.5. If the pH dropped below 8 the protein precipitated and was discarded. The solution was kept on ice and used as soon as possible.

CPB (DFP-treated) was separated from amino acids by gel filtration on Sephadex G25. The operation was performed at 4°, and the column was equilibrated with 0.01 M tris-chloride, pH 8.6. The enzyme was detected by its extinction at 280 nm., was divided into portions and rapidly

frozen/

frozen at -20°.

Large peptides were degraded with enzymes to help determine their sequences. Trypsin, chymotrypsin, subtilisin B and thermolysin were used in 0.2 M ammonium acetate, pH 8.5. Subtilisin B solution was made up freshly. 0.02 to 0.05 mg. enzyme with 0.2 to 1.0 micromole peptide were used in 0.2 to 0.5 ml. Incubation was for about 3 hr. at 37°. Trypsin was occasionally incubated overnight.

For digestion with papain, about 0.5 micromoles peptide was dissolved in 0.4 ml. pyridine acetate buffer (Table 3.7.1.), pH 6.5, with 0.04 ml. of 0.1 M-dithiothreitol. 0.04 ml. of papain suspension (17 mg./ml.) was added and the tube was flushed with nitrogen and incubated at 37° for 24 hr.

Digests were dried in a desiccator and separated by electrophoresis.

Leucine amino peptidase and partial acid hydrolysis were used on peptide TA9 (Section 11.26.).

3.20. Sequential degradation (DNS-PTC method).

The Edman (1950, 1956) method removes the N-terminal residue and leaves the next residue free at the N-terminus. The successive N-termini are determined by the DNS-method (Gray, 1967b).

The peptide was dried in a screw-cap tube, 6.0 x 1.2 cm. and dissolved in 0.2 ml. 50% aq. pyridine. 0.1 ml. of 5% PTC in pyridine was added, the tube was flushed with nitrogen (Ilse & Edman, 1963) and capped. After 1 hr. at 37° the tubes were opened and dried for 1 hr. in a heated (60°) vacuum desiccator over conc. H_2SO_4 and NaOH pellets. When the

tubes/

tubes were cool, 0.20 ml. anhydrous trifluoroacetic acid (Edman, 1964) was added and the tubes were again flushed with nitrogen and capped. After 30 min. at 37° the tubes were opened and dried *in vacuo* over NaOH pellets, the evacuated dessicator being placed in a 60° oven.

When the tubes were cool, 0.25 ml. water was added and the solution was extracted with 1.5 ml. butyl acetate to remove diphenylthiourea. The extraction was performed with vigorous agitation on a vortex mixer followed by brief centrifugation and was done three times. The final aqueous phase was dried over conc. H_2SO_4 *in vacuo*. The peptide, one residue shorter, was dissolved in a suitable volume of 50% pyridine and a sample removed for N-terminal determination, as well as for mobility determination if required. The volume was restored to 0.2 ml. with 50% pyridine and another cycle of degradation performed.

In some cases the identity of the free amino acid left at the last stage was confirmed by electrophoresis at pH 2.0 or pH 6.5.

Generally only insignificant amounts of N-termini other than the main one were observed. On occasion impurities were observed at a significant level, usually the residue before or the one after, and these have been recorded.

CHAPTER 4

PENICILLINASE ASSAY

4.1. Introduction.

The available methods have been reviewed (Citri & Pollock, 1966; Hamilton-Miller, Smith & Knox, 1963). The major methods of assay follow either the destruction of penicillin (by its formation of a coloured complex) or the formation of penicilloic acid, by its acidity or its reaction with iodine.

The liberation of carbon dioxide from a bicarbonate containing penicillin solution was followed manometrically by Henry & Housewright (1947). Other acidimetric methods have used coloured indicators to follow the release of protons in weakly buffered solutions (e.g. Imsande, 1965).

Iodometric methods were introduced by Perret (1954). His method used an excess of iodine solution, strongly buffered at pH 4.0, to stop the enzyme reaction and react with the penicilloic acid. The excess iodine was measured by titration with thiosulphate. A blank was used in which the enzyme was added after the iodine. The amount of iodine consumed may also be measured by direct spectrophotometric comparison with the blank (J.F. Collins, personal communication; A.B. Pardee, *per* Chesbro & Lampen, 1968: Sargent, 1968)./

1968).

The method of Citri (1958) measures the time for decolorisation of a buffered iodine-penicillin mixture. For a given amount of iodine the time is reciprocally related to the amount of enzyme. This method does not correct for non-specific uptake of iodine by the sample. Also it cannot be used for very iodine labile enzyme (e.g. that from S. aureus).

4.2. Unit of penicillinase.

Unit amount of penicillinase is that which hydrolyses 1 micromole of benzylpenicillin in one hour at 30° at pH 7.0 (Pollock & Torriani, 1953). This unit differs from the latest recommendations (International Union of Biochemistry, 1965) only in the use of an hour instead of a minute.

The formula used for calculation in the Perret assay (next Section) can be established as follows. Under the conditions used the degradation products of one mole of penicillin reduce 4.15 moles of iodine (I_2) (Perret, 1954). Thus one unit corresponds to the reduction of 4.15 micromoles I_2 /hr. But 1 ml. 0.0166 M thiosulphate will reduce 8.3 micromoles of I_2. Hence, for one hour, the number of units of enzyme is equal to twice the titration difference in ml. of 0.0166 M thiosulphate. The formula in the next Section follows.

The abbreviations ku and Mu have been used for 10^3 and 10^6 units/

units respectively.

4.3. Perret (1954) assay.

This assay has been used for most previous work on B. licheniformis penicillinase (Pollock, 1965).

Penicillin solution, 600 mg. sodium benzylpenicillin in 250 ml. 0.1 M sodium phosphate buffer, pH 7.0, was made daily. 5 ml. of this was placed in each of two 50 ml. Erlenmeyer flasks shaking in a 30° water bath. When the penicillin had reached the bath temperature, the enzyme was added to one flask. After a suitable time, 10 ml. of 0.08 M-I_2 in 0.06 M-KI and 2 M sodium acetate buffer, pH 4.0 was added from an automatic pipette. After one minute, the iodine solution was added to the control flask, followed by an identical enzyme sample. Five minutes after the addition of the iodine, each flask was titrated with 0.0166 M sodium thiosulphate from a burette, calibrated to 0.05 ml. The thiosulphate was made by dilution of commercial standard thiosulphate.

If the difference (control - experimental) between the titres is d ml. and the time of incubation is t minutes, then

$$\text{units of penicillinase} = 2 \cdot \frac{60}{t} \cdot d$$

(see Section 4.2. for proof). Several assays may be performed at once by suitable staggering of starting times.

Very dilute penicillinase solutions had 0.5% (w/v) gelatin added/

added to prevent absorption to glass surfaces, but this was not done with stronger solutions (Kushner, 1960).

The method was reproducible to about 5% without exceptional precautions.

4.4. Citri (1958) assay.

The penicillin solution described in the last section was used. Sufficient iodine was added to preserve a very pale yellow tint, otherwise penicilloic acid might accumulate through contamination with minute amounts of penicillinase. 2.5 ml. was placed in a tube in a 30° water bath. 0.25 ml. 0.0322 M-I_2 in 0.024 M-KI was added. After two minutes the enzyme was added and the tube immediately shaken. The time to decolorize was measured with a stopwatch. The assay was generally performed with 400 to 1100 units of enzyme, giving times of 7 to 20 sec. The time in seconds is divided into 8000 to give the number of units of penicillinase present, (see Section 4.5 for proof). Unless noted otherwise, all assays for penicillinase were performed by this method.

The iodine was kept in a brown glass bottle and the iodine concentration was checked occasionally. The titre of the iodine was 3.85 ml./ml. of 0.0166 M thiosulphate. This dilute thiosulphate was not indefinitely stable (Section 7.8.).

4.5./

4.5. Reproducibility and standardisation of Citri assay.

A solution of exo-penicillinase was assayed by the use of twelve different amounts within the range of the Citri assay. Each assay was performed three times. Each time was converted into units/ml., as was the mean of the three for each amount of enzyme. The mean assay was 1144 units/ml., standard deviation 76 units/ml. Hence the 95% confidence limits (assuming a Gaussian distribution) are ± 149 units/ml. (13%). Similar computation using the values obtained from the mean of the three determinations gave 10.5%.

In another experiment a solution was assayed seventy times giving 95% confidence limits of ± 11.2%. Citri (1958) quotes an accuracy of "well within 10%". The method has generally been performed in the past using times of the order of minutes and it is likely that the accuracy has been somewhat reduced by decreasing the time.

In view of the iodine sensitivity of B. licheniformis 749 enzyme it was necessary to check to what extent it was inactivated by the iodine used in the assay. Pollock (1965) showed that 3.8 mM-I_2 inactivated it about 30% in five minutes at 0° and pH 7.0. Solutions of exo-enzyme between 300 and 1500 units/ml. were made. Each was assayed by the Perret method (Section 4.3.) and by the Citri method using 0.0333 M iodine (Section 4.4.). Enzyme activities were calculated by dividing the time in seconds into 7200,/

7200, corresponding exactly to the stoichiometry of the Perret assay. The results are shown in Table 4.5.1.

Table 4.5.1.

Comparison of assay by Citri and Perret methods.

Solution	Activity Perret	Units/ml. Citri	Citri / Perret %
A	374	310	82.9
B	749	661	88.3
C	1085	911	84.0
D	1469	1346	91.6
E	1757	1548	88.1
Mean			87.0

Thus the Citri assay gives values about 13% low compared with the Perret method. The theoretrical figure 7200 was increased by 100/87 to correct, giving 8280. For convenience it was lowered to 8000 by decreasing the iodine concentration to 8000/8280 of its former strength, i.e. 0.0322 M.

It is evident that the Citri assay as used is likely to be unreliable/

unreliable in experiments such as chemical modification studies, where the iodine sensitivity of the enzyme might be altered.

CHAPTER 5

GROWTH OF BACILLUS LICHENIFORMIS

5.1. Apparatus.

PP1 etc. refer to the various penicillinase preparations detailed in Chapter 7.

Inocula were grown from spores in five litre flasks on a reciprocating shaker in a 37°. PP2 was grown in ten such flasks, each containing 1 litre of medium. PP3 was also grown on the reciprocating shaker, in two ten litre aspirators on their sides, each containing four litres of medium. Compressed air was blown through the culture. From time to time three litres of culture was removed and replaced with fresh medium.

The remaining experiments were performed in New Brunswick fermenters. Antifoam (PPG 2000) was added under automatic control. Some experiments were under automatic pH control; in others samples were removed, checked for pH, and additions of acid or alkali made accordingly.

Growth was followed by measurement of extinction at 675 nm. Separate curves were used to obtain mg. (dry weight)/ml. from extinction in 1.0 cm. and 0.2 cm. cells. The curves, determined on CH/S medium,/

CH/S medium, were kindly provided by Dr. J.F.Collins.

5.2. Experimental media.

The earlier experiments were done with CH/S medium (Pollock, 1965). It consisted of 1% (w/v) casamino acids, 0.02 M-potassium phosphate buffer, pH 7.2, and 1 ml./l. of salt solution, comprising, per 100 ml. very dilute HCl, 25g. $MgSO_4.7H_2O$, 100 mg. $FeSO_4.7H_2O$, 100 mg. $ZnSO_4.7H_2O$, 10 mg. $MnSO_4.4H_2O$, 1 mg. $CuSO_4.5H_2O$ and 0.2 mg. $K_2Cr_2O_7$. The medium was autoclaved at 115° for 20 minutes.

From time to time cultures were examined by plating on Andrade agar (Kogut, Pollock & Tridgell, 1956) and all colonies were shown to be constitutive penicillinase producers.

Experiments PP2 and PP3 were harvested at about 3,000 and 5,000 units /ml. respectively. In experiments PP4 and PP5 it was found that the maximum density that could be attained by continuous culture was about 1 mg./ml. corresponding to about 5,000 units/ml.

Small scale experiments showed that growth stopped because certain amino acids were rapidly exhausted. If the casamino acids were made more concentrated the high salt concentration began to inhibit growth. The amino acids most rapidly consumed were identified by amino acid analysis and a new medium incorporating them was tried. Buffer and salts were as previously, and the medium also contained per litre: 20 g. glucose, 2.9g.monosodium L-glutamate, 3.0 g.

glycine/

glycine, 1.4 g. monosodium L-aspartate, 1.2 g. DL-methionine, 2.6 g. $(NH_4)_2SO_4$. and 1 mg. thiamine.

The inoculum was prepared on CH/S medium. Experiment PP6 was performed in a continuous culture apparatus and the medium was sterilised as it was pumped in by membrane filtration. The culture tended to become acid, and the pH was controlled with 10 M-NaOH. Penicillinase assays were not performed because the methionine interfered with Citri assays. 70 litres of supernatant were prepared, but stable continuous culture could not be obtained above 1 mg. (dry weight)/ml. It appeared subsequently that $1 - 2 \times 10^8$ units penicillinase had been made.

It appeared from further small scale experiments that the glucose and other amino acids could be replaced by glutamate. Most of the penicillinase used was made with such a medium (Section 5.3.).

5.3. Glutamate medium.

The medium consisted, per litre, of 40 g. monosodium L-glutamate, 2 g. $(NH_4)_2SO_4$, 5.5. g. $KH_2(PO_4)_3$, 14.6. g. $K_2H(PO_4)_3$, 3 g. tri-sodium citrate and 0.22 g. $MgSO_4.7H_2O$, and was sterilised by autoclaving. 1 ml./l. of a separately sterilised solution of 16 g./l. $FeCl_3.6H_2O$ and 0.25 g./l. $MnSO_4.4H_2O$ in 0.01 M-HCl was added, as also was 0.1 ml./l. of 10 mg./ml. thiamine HCl sterilised

by/

by membrane filtration.

Inocula were prepared either on CH/S medium or on glutamate medium supplemented with 1% (w/v) casamino acids.

The medium has a pH of about 7.0. Growth of B. licheniformis raises the pH to 8.5, so the pH was kept around 7 by addition of HCl to neutralize the ammonia produced.

Growth on this medium reached about 5 mg. (dry weight)/ml. Penicillinase activity was about 15 - 30,000 units/ml. Growth could be maintained at these levels with a mean generation time of around 120 min. If the culture was permitted to continue into stationary phase densities of 10 mg./ml. and activities of 50,000 units/ml. were reached, but the culture became very prone to lysis.

Optimal conditions were found to be aeration reaching 14 litres/min. at 5 mg./ml. but much less in earlier stages, with mixing at 700 r.p.m. The temperature was 37°.

5.4. Harvesting.

Cultures could be stored for a few hours at 10° prior to harvesting, but cultures limited by exhaustion of medium were very prone to lysis.

The cells were separated in either a Szent-Györgyi-Blum continuous flow attachment to a Sorvall RC-2 centrifuge or in a Sharples Super Centrifuge. These developed about 25,000g and

13,000g/

13,000g respectively and operated at flow rates of up to 6 litres/hr.

Cells were stored at -20° until required. Supernatants were stored at 4° until adsorbed on ion-exchangers. (Chapter 7).

CHAPTER 6

RELEASE OF CELL-BOUND PENICILLINASE

6.1. Proteolytic release.

Lysed, nuclease-treated, cells of B. licheniformis 749/C were made from cells stored since harvesting at -20°. The cells were blended with 0.01 M sodium phosphate buffer, pH 7.0, at 25-50 mg. (wet weight)/ml. They were centrifuged at 20,000g for 20 min. and resuspended twice. The last supernatant was assayed on one occasion and showed 800 units/ml. The resuspended cells were treated with lysozyme (50µg/ml.), ribonuclease (10 µg./ml.) and deoxyribonuclease (10 µg./ml.) for 1 hr. at 37°. If not used immediately the mixture was stored at -20°.

The total activity was stable to incubation at 37° for 8 hr. with 3 mg. per million units penicillinase of trypsin, chymotrypsin, Pronase and subtilisin B. These solutions were diluted with two volumes of water and centrifuged as above, and the activity in the supernatant was compared with the total activity present. The control without protease showed 18%, SBTI-chymotrypsin released about 50% and the other proteases released all the activity.

The/

Table 6.1.2.

Release of penicillinase from cells (48,600 units/ml.) by trypsin.

Trypsin µg./ml.	Time min.	% of activity in supernatant
0	0(Control)	21
1	30	30
10	1	35
10	2	46
10	5	52
10	10	63
10	30	83
100	1	100

The time courses of release by trypsin and chymotrypsin were determined. For chymotrypsin, lysed cells were diluted with 1.5 volumes of water. 1 mg. chymotrypsin and 0.25 mg. SBTI were added to 10 ml. A control solution lacked additions. The solutions were incubated at 37°, and 1 ml. samples were removed from time to time, diluted with 4 ml. water, centrifuged as above, and the supernatants were assayed for penicillinase. The results are shown in Table 6.1.1.

Table 6.1.1.

Release of penicillinase from cells (13,500 units/ml.) by 0.1 mg./ml. chymotrypsin.

Time (hr.)	% released	
	Control	Experimental
1	29	46
2	28	53
4	26	72
10	25	105
20	25	94

These figures are plotted semi-logarithmically in Figure 6.1.1. and/

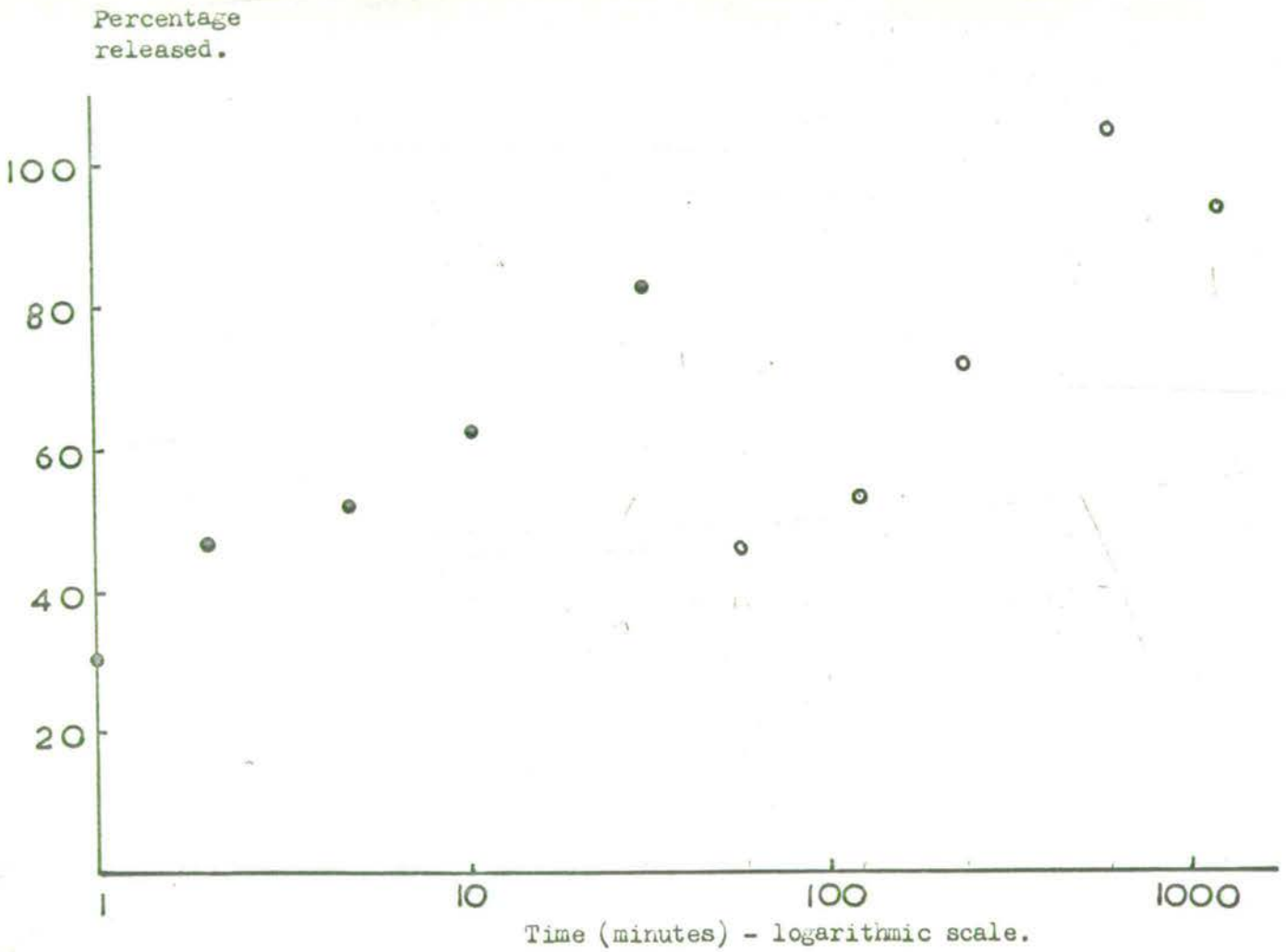


Figure 6.1.1. Release of penicillinase activity from cells (57,000 units/ml.) with trypsin (0.01 mg./ml.) (●) and chymotrypsin (0.1 mg./ml.) (o). Temperature 30°.

and the material was examined by starch gel electrophoresis (Section 3.2.). The result is shown in Figure 6.1.2.

Added phenylmethanesulphonyl fluoride (100 fold excess) made no difference to release by trypsin whether added before or after the enzyme although it is an inhibitor of the enzyme (Gold & Fahrney, 1964). Rapid freezing was therefore employed to stop the reaction. Lysed cells were buffered by addition of 0.1 vol. of M ammonium acetate, pH 8.5. A control sample was frozen with methylated spirit and solid CO_2 and stored at -20°. 10 µg./ml. DPCC-trypsin was added and 1 ml. samples were removed and frozen at intervals. All samples were thawed with 9 ml. water at 0°, centrifuged at 4° as described previously. The supernatants were stored on ice and assayed as rapidly as possible. The results are shown in Table 6.1.2. together with some obtained at other levels of trypsin, and some of the results are shown in Figure 6.1.1.

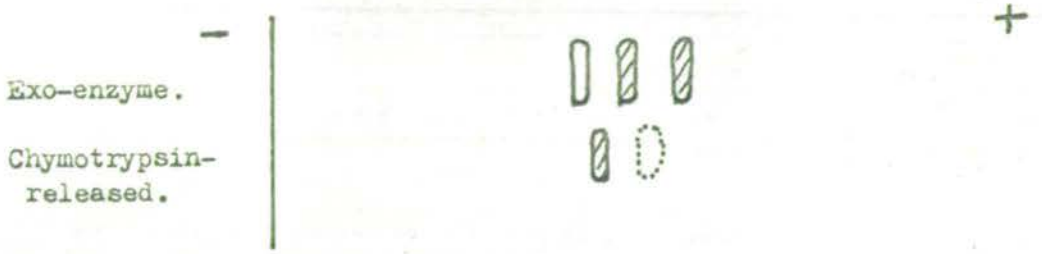


Figure 6.1.2. Starch gel electrophoresis (pH 8.5) of enzyme released from cells with chymotrypsin.

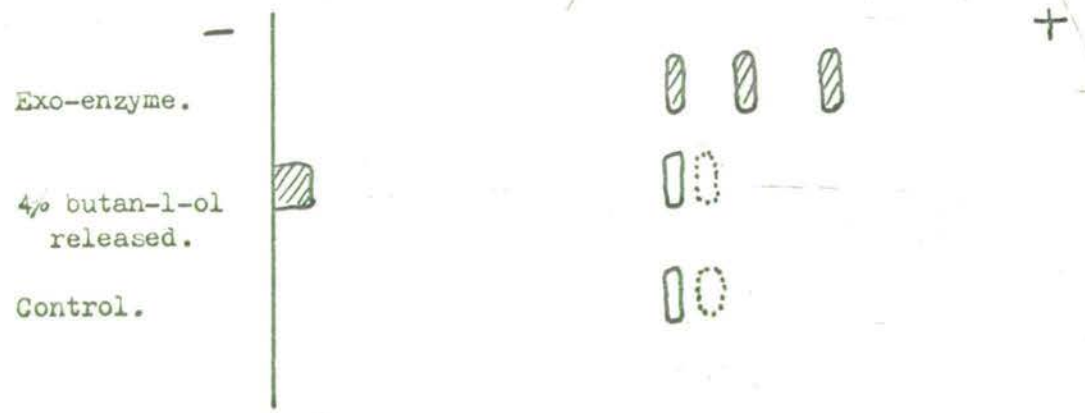


Figure 6.2.1. Starch gel electrophoresis (pH 8.5) of supernatant from cells treated overnight (22°) with 4% butan-1-ol. Control lacked butanol.

6.2. Non-proteolytic release.

The cell-bound fraction of penicillinase in B. licheniformis is attached to membrane fragments in fractionated cells, and Lampen (1967a) partially purified such a membrane fraction. He could not isolate the penicillinase from this without using protease. Various other treatments, used by investigators of lipo-protein systems (Morton, 1955; Widmer & Crane, 1958) have been tried on crude lysed cells.

The first set of treatment consisted of 4% and 6% butan-1-ol, 3% and 6% butan-2-ol, water saturated with cyclohexanol and 4% butan-1-ol saturated with benzene. None had any effect on the activity of exo-enzyme at 4° or 22° after 20 hr. At 37° they all inactivated exo-enzyme progressively. The worst was 6% butan-1-ol (86% loss of activity in 20 hr.) and the best 6% butan-2-ol (8%). The inactivation was irreversible by dialysis.

The effect of two of these treatments on a lysed cell preparation was examined at 4° and 22°. The preparation contained 160,000 units in 2 ml. After 20 hr. the samples were diluted to 10 ml. with water and centrifuged (4°, 20 min. 20,000g.). The supernatants were decanted and assayed and the acitivity expressed relative to the total activity of the sample. The result is shown in Table 6.2.1.

Table 6.2.1.

Release of enzyme from cells by organic solvents over 20 hr.

Treatment	% released	
	At 4°	At 22°
4% butan-1-ol	10	52
6% butan-2-ol	8	32
none	5	22

The material released by butan-1-ol was examined by starch gel electrophoresis. The result is shown in Figure 6.2.1. Material smeared at the origin was also described by Lampen (1967a), who ascribed it to penicillinase attached to membrane fragments too small to sediment under the conditions used.

The second set of treatments included 45% acetone, 12.5% ethanol and 50% petroleum spirit (b.p. 40-60°) all of which destroyed the enzyme activity of lysed cells. Treatment of lysed cells with 2 volumes of n-heptane or 2,2,4-trimethylpentane led to release comparable with that found with 4% butan-1-ol. On starch gel examination the extra activity was associated with smears at the origin similar to those in Figure 6.2.1.

CHAPTER 7

PENICILLINASE PURIFICATION

7.1. Adsorption of exo-enzyme on cellulose phosphate.

Exo-enzyme from cultures grown on experimental media (up to PP6) (Section 5.2.) was adsorbed on cellulose phosphate (Pollock, 1965). The preparation of the ion-exchanger has been described in Section 3.3.

The pH of culture supernatant was adjusted to about 5.5 with acetic acid. About 50 ml. (sedimented volume) cellulose phosphate per litre culture was added. If the pH differed much from 4.8 it was adjusted. The amount of penicillinase remaining in the supernatant was determined after a few minutes stirring. If necessary more cellulose phosphate was added and the pH further adjusted until only about 10% remained in the supernatant. After settling the cellulose phosphate, most of the supernatant was poured off and the cellulose phosphate was suspended in the rest and poured into a column. It was washed with a few volumes of 0.01 M sodium phosphate buffer, pH 4.8 and then eluted with 0.2 M sodium phosphate, pH 7.6. The eluate was assayed in fractions and those containing most of the activity were kept.

In/

In one experiment 86% of 115 x 10^6 units in 37 litres was adsorbed on 1 kg. cellulose phosphate. This made a column 70 cm. x 8.5 cm. diam., which flowed at 130 ml./hr. under compressed air at a pressure of 7 pounds/sq. in. 44 x 10^6 units were in the fractions kept and 5 x 10^6 units were discarded. Thus 49% of the activity adsorbed was recovered.

7.2. Adsorption of exo-enzyme on CM-cellulose.

The glutamate medium (Section 5.3.) has a much higher ionic strength than previous media, so penicillinase would no longer adsorb directly to cellulose phosphate. In view of the recoveries obtained from cellulose phosphate it was considered desirable to find out whether other ion-exchangers would work better.

Preliminary experiments showed that penicillinase could be adsorbed to CM-cellulose from 0.05 M ammonium acetate buffer, better at pH 4.8 than at pH 3.9. Dilution and dialysis were then compared for lowering the ionic strength of supernatant (glutamate medium) far enough to allow efficient adsorption. Supernatant was dialysed overnight against running tap water, and the pH was adjusted to 4.8. On passage through a pad of CM-cellulose equilibrated with 0.01 M ammonium acetate, pH 4.8, 98% was adsorbed, of which 96% could be recovered by elution with 0.1 M ammonium acetate, pH 8.5. Forty-fold dilution with water was necessary to obtain the same conductivity, and after pH

adjustment/

adjustment to 4.8 no more than 62% could be adsorbed even by repeated passage.

It is difficult to compare directly the capacity of CM-cellulose with that of cellulose phosphate (since the latter loses most of its capacity if it is washed to the pH at which the adsorption occurs). However in one experiment 36 ml. (column vol.) of CM-cellulose adsorbed 6.6×10^6 units. Compared with the figures in the last Section the capacity per ml. column volume is sevenfold higher. Also an average recovery of 87% was obtained from CM-cellulose compared with 46% from cellulose phosphate (Table 7.8.1.).

In exo-enzyme preparations from PP9 onward, the culture supernatant, grown on glutamate medium was dialysed overnight against running tap water in 1½" diameter Visking dialysis tubing in a large laboratory sink. The volume increased up to 40%, and the conductivity was lowered to 1.0 - 1.5 mmhos/cm. The pH was then adjusted to 4.8 with acetic acid. It was then passed through a pad of CM-cellulose equilibrated (pH above 4.0) with 0.01 M ammonium acetate, pH 4.8, in a funnel on an evacuated filter flask. Assay before and after showed how much had been adsorbed. In one experiment, 38 litres of dialysed supernatant was passed once through a pad 9 cm. x 20 cm. diam. In one passage the activity was reduced from 3,300 units/ml. to less than 40 units/ml. After 24 hr. at 4° the material was reformed into a column

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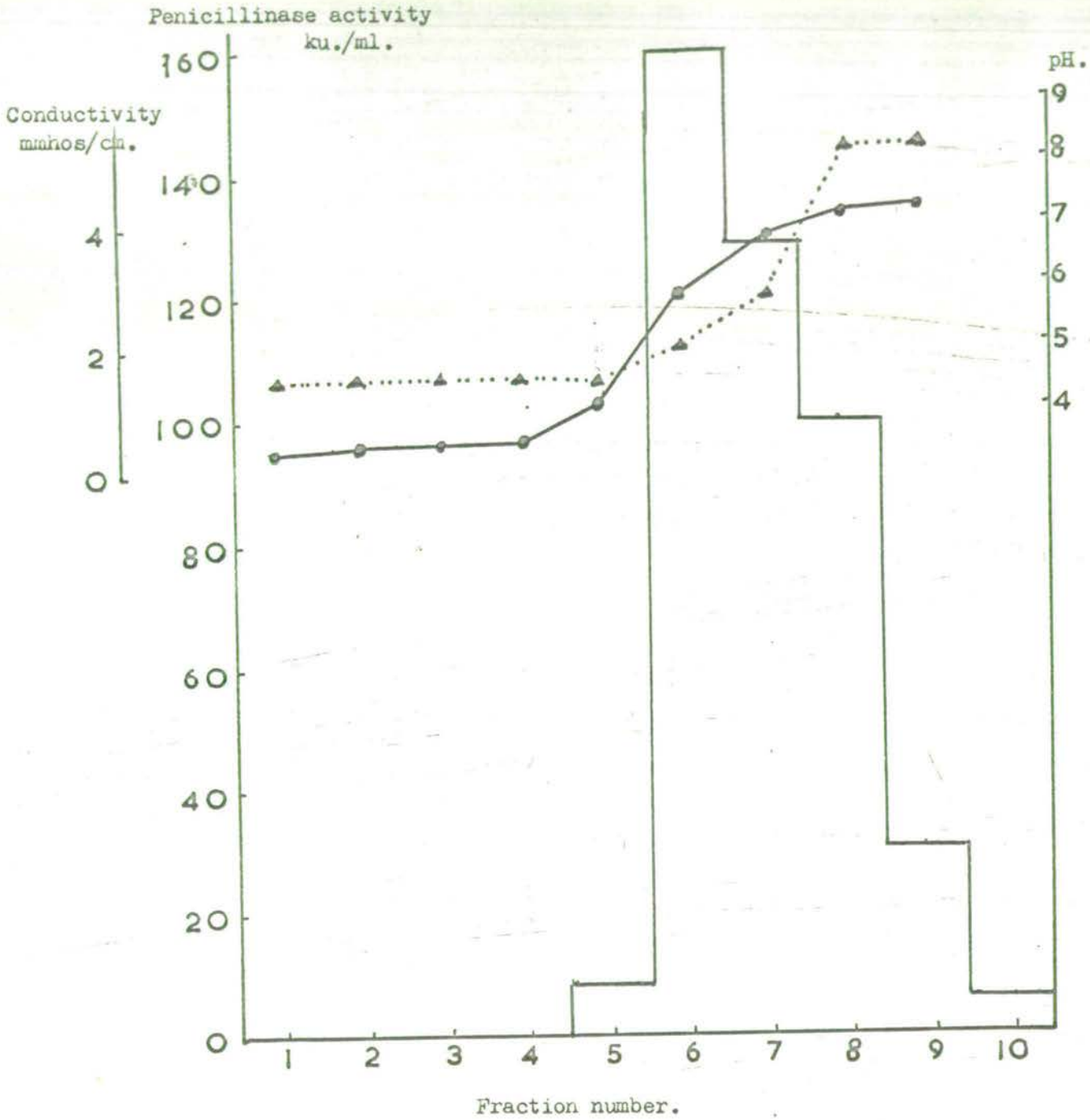


Figure 7.2.1. Elution of exo-penicillinase (histogram) from CM-cellulose (53 cm. x 5 cm. diam.) in 0.01 M ammonium acetate, pH 4.8, with 0.1 M ammonium acetate, pH 8.5. Each fraction was about 250 ml.

·····▲····· pH; —●— Conductivity.

42 cm. x 7.6 cm. diam. The column was carefully eluted with 0.1 M ammonium acetate, pH 8.5. The properties of the fractions eluted in a similar experiment are shown in Figure 7.2.1. 90.5% of the activity adsorbed was eluted, of which 98.3% was found in two fractions, comprising 570 ml., which were kept.

7.3. Concentration, de-salting and freeze-drying.

Solutions of penicillinase were concentrated by vacuum rotary evaporation, at 37°. No evidence of inactivation under these conditions was found. A little octan-2-ol was added to the solution to reduce foaming, and with an efficient water pump water was removed at up to 500 ml./hr. This treatment made precipitates back down very well on subsequent centrifugation.

To remove salt, preparations were dialysed, against tap water at early stages and, later in the purification, against distilled water.

Before freeze-drying, preparations were dialysed to reduce the salt concentration to about 0.01 M. Freeze-drying was employed both before and after gel-filtration. Although no good evidence of denaturation was found, in even the purest penicillinase preparations a little material failed to redissolve after freeze-drying.

7.4./

7.4. Trypsin release of enzyme.

The activity in the frozen cells (Section 5.4.) was found to be about 250,000 - 500,000 units/g. (wet weight). The cells were thawed overnight at room temperature, and blended with 1 litre/kg. of 0.01 M sodium phosphate, pH 7.0. Lysozyme, 200 mg./kg. cells, ribonuclease, 50 mg./kg. and deoxyribonuclease, equivalent to 40 mg. pure material/kg. were dissolved in a small quantity of buffer and added. Difficulty was found when less deoxyribonuclease was used. After one hour at 30°, the viscosity was much decreased.

50 mg./kg. trypsin was then added in a small volume of buffer, and the mixture was incubated at 37° overnight. The cell debris was then removed by centrifugation (16,000g, 4°, 40 min.). As shown in Table 7.8.1., about 90% of the total activity remains in the supernatant.

7.5. Ammonium sulphate fractionation.

The crude trypsin released enzyme was fractionated with ammonium sulphate. 4.65 volumes of 85% satd. ammonium sulphate were made by dissolving 465 g./l. of ammonium sulphate in 760 ml./l. water giving a final volume of 1 litre (Dixon, 1953). The pH was adjusted to about 7.5 with NaOH. The enzyme solution, in a dialysis sac, was floated on the solution overnight at 4° and equilibration was ensured by vigorous stirring. The solution

was/

was then centrifuged (16,000g, 4°, 2 hr.). After assays to check the location of the activity, the precipitate was discarded. The supernatant was then desalted by exhaustive dialysis together with rotary evaporation to reduce the volume (Section 7.3.). This generally precipitated sufficient protein to make re-centrifugation (20,000g, 4°, 1 hr.) necessary.

Preparation PP17 was inadequately equilibrated at this stage and as a result the preparation was impure after gel filtration (Table 7.7.1.), and impurity could be seen on starch gel electrophoresis. Further ammonium sulphate fractionation was employed after gel filtration. The approximate saturation required to precipitate the enzyme at the concentration used was calculated (Pollock, 1965; Dixon & Webb, 1961). After an abortive fractionation at 83% saturation, the fractions precipitated at 75% and soluble at 86% were discarded. Table 7.5.1. shows the properties of the various fractions.

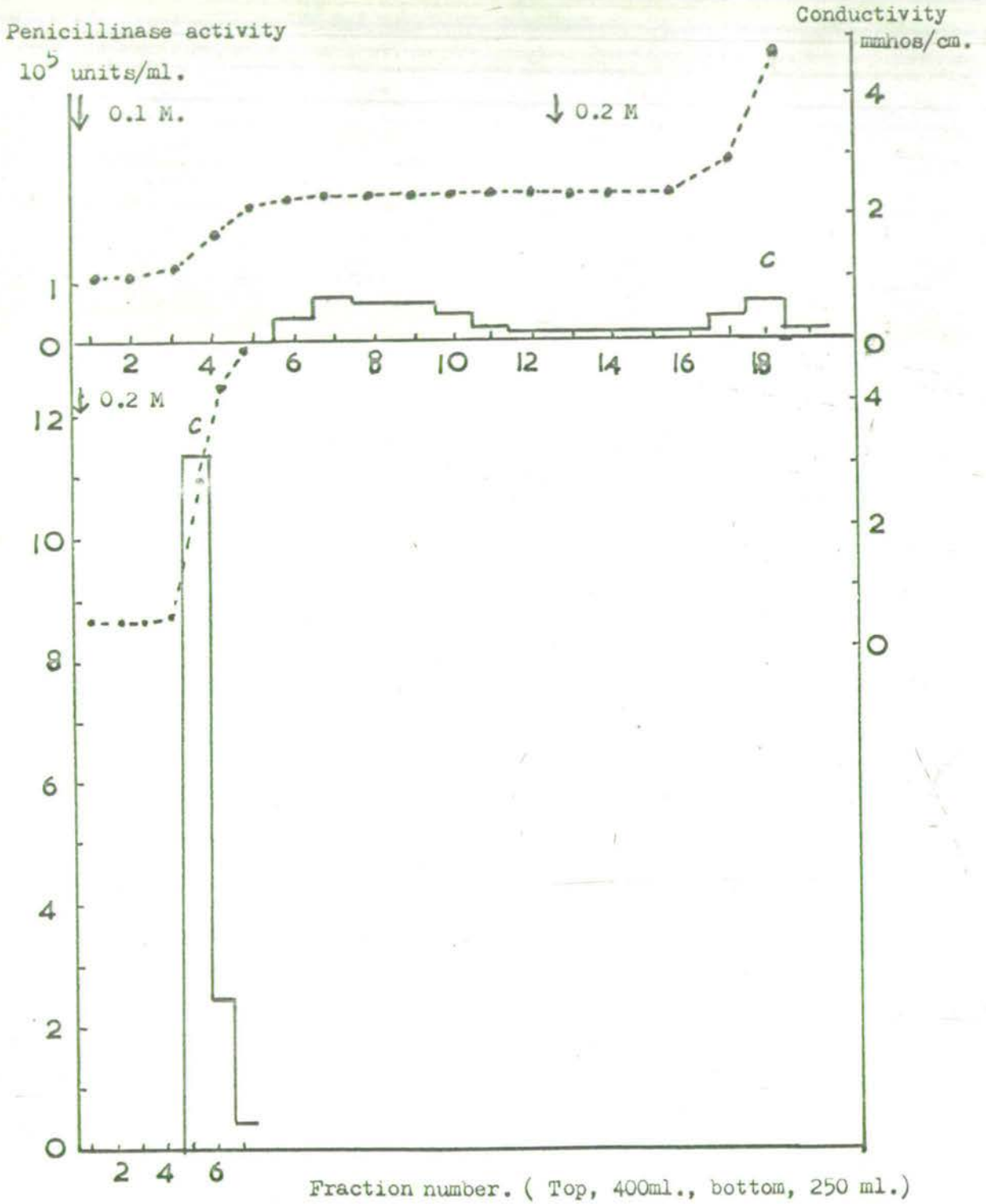
Table 7.5.1.

Properties of fractions obtained from PP17 with ammonium sulphate.

Fraction	Volume ml.	Activity ku./ml.	$E^{0.2cm.}_{280nm.}$	Sp. act. ku./ml./$E^{1.0cm.}_{280nm.}$	Total activity Mu.
83% supernatant	66	233	0.172	271	15.3
83% precipitate	73	1177	0.79	298	85.9
75% precipitate	28	53	0.55	19	1.5
75% supernatant	80	851	0.45	377	68.1[a]
86% supernatant	65	78	0.19	93	5.1
86% precipitate	17	482	0.27	357	8.2

[a]: About 10% of this fraction was accidentally lost.

It is evident from these figures that considerable purification was obtained. The fractions kept were dialysed exhaustively and freeze-dried. The overall recovery was 55%.



Figures 7.6.1. (top) and 7.6.2. (bottom). Elution of penicillinase (histogram) from DEAE-cellulose columns (top, 34 cm. x 7 cm. diam; bottom, 63 cm. x 5 cm. diam.). Ammonium acetate buffers, pH 8.5, initially 0.01 M. Eluting buffer added as shown. The total activity in the second experiment was about twice that in the first, and areas are proportional to activity. Cytochrome was present in the peaks marked C.

7.6. DEAE-cellulose chromatography.

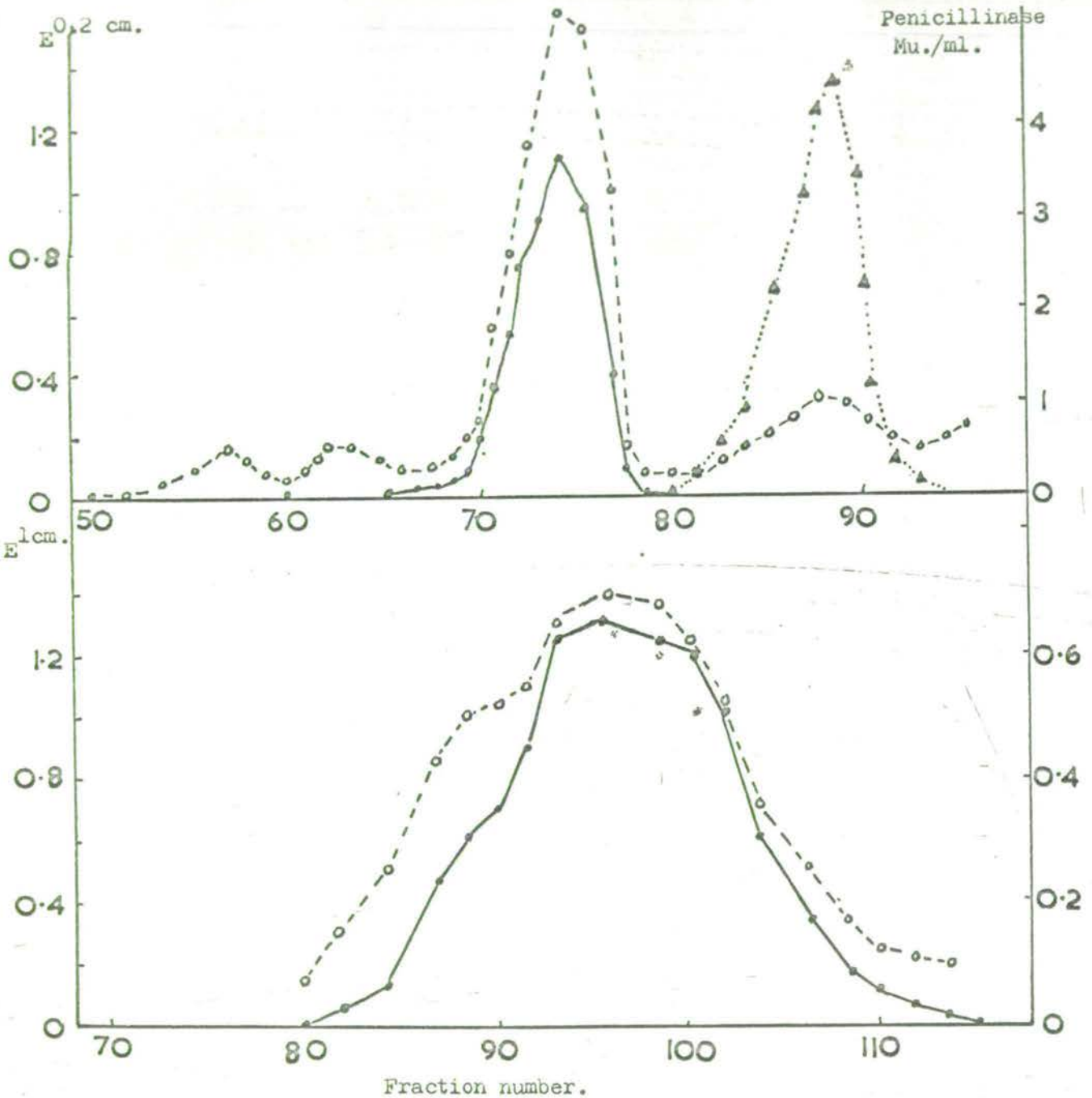
The preparation and re-use of DEAE-cellulose is described in Section 3.3. On one occasion a column 32 cm. x 7.6 cm. diam. sufficed for the product from 1 kg. cells. The column was treated with 0.01 M ammonium acetate pH 8.0, and was considered equilibrated when the effluent pH rose above 7.0.

The desalted ammonium sulphate supernatant was adjusted to match the equilibrating buffer in pH and specific conductivity. The solution was pumped on to the column at 1.5 litres/hr. A red band formed and moved down the column, stopping about three-quarters of the way down when all the sample was on. The sample was washed on with a few litres of equilibrating buffer.

The penicillinase and cytochrome were either partially separated by stepwise elution from the column with increasing salt concentrations (Figure 7.6.1.) or eluted together with 0.2 M ammonium acetate, pH 8.5 (Figure 7.6.2.). The latter technique gave a more concentrated penicillinase solution and the cytochrome could be removed subsequently by gel filtration.

7.7. Gel filtration:

The final step in most penicillinase preparations has been gel filtration on Sephadex G100. The preparations of the columns is described in Section 3.4. The buffer was 0.025 M ammonium acetate/



Figures 7.7.1. (top) and 7.7.2. (bottom). Chromatography of trypsin-released enzyme (top) and exo-enzyme (bottom) on Sephadex G100 in 0.025 M ammonium acetate, pH 8.5. Column and fraction sizes, 147 cm. x 2.5 cm. diam; 7.0 ml. (top); 82 cm. x 5 cm. diam; 11.7 ml. (bottom).

Penicillinase —●— . $E_{280nm.}$ – –○– – . $E_{410nm.}$ ·····▲····· .

acetate, pH 8.5, generally containing 1 mM sodium azide to prevent growth of micro-organisms.

The sample was dissolved in 7 - 15 ml. of this buffer and centrifuged (20,000g, 4°, 20 min.). A small volume of 5 mg./ml. ε-DNP-lysine was added. After excess buffer had been removed, the sample was carefully applied to the top of the column with a Pasteur pipette, and when it had all percolated in, a few ml. of buffer were used to wash it on. The column was then re-connected to the pump and fractions of eluate were collected by time or by drop counting.

Most of the fractions were assayed for penicillinase by the Citri method (Section 4.4.), and extinctions at 280 nm. were determined with a Unicam SP500 spectrophotometer. Where red protein (subsequently shown to be cytochrome) was present, extinctions at 410 nm. were also determined. Figure 7.7.1. shows a typical result with trypsin-released enzyme, while Figure 7.7.2. was obtained with exo-enzyme.

Fractions were then selected which contained substantially pure penicillinase. Over 90% of the activity eluted was generally kept. Some of the values are shown in Table 7.7.1. All the necessary data was not recorded for the earlier preparations. The chosen fractions were dialysed and freeze-dried. The freeze-dried material was stored at -20°.

Table 7.7.1.

Properties of fractions eluting from Sephadex G100 kept as pure penicillinase.

Preparation	ku./ml./$E^{1.0cm.}_{280nm.}$		
	Minimum	Maximum	Mean
PP8	518	719	632[a]
PP10	394	542	465
PP11	308	545	451
PP14	312	570	458
PP15	444	696	610[a]
PP16	[b]		547
PP17	334	411	386[c]
PP18	362	536	470

[a]: These values are discussed in Section 7.8.

[b]: Values were not determined for each fraction because the fraction size was very variable. The mean value was obtained with pooled fractions containing 90% of the total activity.

[c]: This preparation has been discussed in Section 7.5.

7.8. Summary and discussion.

Table 7.8.1. summarises the course of the main penicillinase preparations with the yields at each stage. The missing numbers in the series PP1 ..... correspond to minor or abortive preparations. The order of figures for a preparation shows how it was purified. The yield for each preparation is an overall figure.

Some attempts were made to measure losses at different stages. Not more than 9% of the material eluted from G100 was discarded. On one occasion 3.5%, on another 1%, of the activity present was left in glassware at the stage when the material was applied to G100.

Table 7.8.1.

Purification of penicillinase preparations.

Preparation	Medium	Millions of units								Yield %
		Initial	PC	CMC	TR	After ASP	DEAE	G100	ASF	
PP2(E)	CH/S	13	9					1[a]		8
PP3(E)	CH/S	116	41					22		19
PP4(E)	CH/S	154	68					22[a]		14
PP5(E)	CH/S	162	57					33		20
PP6(E)	4AA	b	42					44		-
PP7(T)		263			228	198	135	53[d]		20
PP8(T)		204			194	133	124	104		51
PP9(E)	G	328		295				114[c]		35
PP10(T)		249			255	172	146	116		47
PP11(T)		275			207	175	119	75		27
PP14(T)		376			316	235	175	140		37
PP15(T)		638			663	444	341	279		44
PP16(E)	G	529		442				285		54
PP17(T)		402			296	264	185	139	76	19
PP18(E)		126		111				91		72
Average yield%			46	87	89	77	79	75	55	
Lowest yield%			35	83	74	67	68	54		
Highest yield%			69	90	104	89	93	105		

T:/

Table 7.8.1. Contd.

T: Trypsin-released enzyme E: Exo-enzyme

CH/S and 4AA (Amino acids) media : Section 5.2. G : Glutamate medium, Section 5.3.

PC: Adsorption to and elution from cellulose phosphate. (Section 7.1.).

CMC: Adsorption to and elution from CM-cellulose (Section 7.2.).

TR: Trypsin release and centrifugation (Section 7.4.).

ASP: Ammonium sulphate fractionation (Section 7.5.).

DEAE: Adsorption to and elution from DEAE-cellulose (Section 7.6.).

G100: Freeze-drying, gel filtration and selection of fractions (Section 7.7.).

ASF: Final ammonium sulphate fractionation (Section 7.5.).

Table 7.8.1. Legend continued.

[a]: Considerable loss occurred through abortive ammonium sulphate fractionation.

[b]: Assay not performed (Section 5.2.).

[c]: Activity lost through freeze-drying in the presence of excessive salt.

[d]: Mechanical losses in application to G100.

The results obtained by estimating protein by the Lowry method (Section 3.1.) at various stages are summarised in Table 7.8.2.

Table 7.8.2.

Specific activities (Units/ microgram protein)

	PP7	PP8	PP11
Crude lysed cells		3	
Trypsin-released supernatant	7	5	8
Ammonium sulphate supernatant	22	33	28
After DEAE-cellulose step	147	268	121
After gel filtration	342	339	337

The loss with cellulose phosphate was through irreversible adsorption, since the activity was removed from the supernatant (Section 7.1.). It was never possible to obtain the recoveries of about 90% obtained by Pollock (1965). It is possible that Whatman P11 differs in some significant respect from the P40 used formerly. The CM-cellulose method seems to be as effective as the cellulose phosphate method was in the work of Pollock (1965).

The technique of dialysing ammonium sulphate into the protein solution is advantageous, since it decreases the volume substantially. The DEAE-cellulose chromatography is very effective; 80% of the total protein is not recovered, presumably either because it does not stick at all, or because it is not eluted under the conditions used.

The loss found on gel filtration is not accounted for by the activity/

activity deliberately discarded and that found in glassware etc. It was noted that all the material was not soluble after freeze-drying, and it may be that the missing activity was lost through denaturation in the course of freeze-drying.

Although protein determinations have not been performed regularly the data allows us to summarise the purification of trypsin-released enzyme in the form recommended by Dixon & Webb (1964, p.31). This is done in Table 7.8.3.

Table 7.8.3.

Purification of penicillinase from 1 Kg. (wet weight) cells.

Fraction	Vol. ml.	Concn.enz. ku./ml.	Total enz. Mu.	Concn. protein mg./ml.	Spec.act. units/µg.	Yield %	Purifn.
Crude lysate	1800	220	400	73.3	3	100	1
Trypsin released	1600	223	356	32.0	7	89	2.3
Amm. Sulph. supernatant	800	342	274	12.2	28	69	7.3
After DEAE step	1100	196	216	1.3	150	54	50
After G100	200	810	162	2.4	337	40	113

The shape of the elution curve from Sephadex G100 (Figure 7.7.1.) has been found repeatedly. The skewness, with the activity falling more rapidly than it rose, was found to increase at higher penicillinase concentrations. It has been shown (Edmond et al., 1968) that this phenomenon is predicted by the "osmotic" theory of gel filtration (Laurent & Killander, 1964) which explains gel filtration in terms of exclusion from a three-dimensional network of polymer fibres. Similar phenomena were produced by chromatographing dextrans on Sephadex.

No evidence of high molecular weight forms of penicillinase (Sargent, Ghosh & Lampen, 1969) has been found on assaying material eluting/

eluting at the void volume of G100 columns.

Three main criteria for purity of these preparations are available. The specific activities (PP7, PP8 & PP11, Table 7.8.2.) agree with that found by Pollock (1965) who showed his preparation to be pure by ammonium sulphate re-fractionation and ultra-centrifugation. Starch gel electrophoresis (PP9, PP11, PP15, PP16, PP17) showed no non-penicillinase bands. PP14 was pure by polyacrylamide disc electrophoresis (Figure 8.2.1.).

Using the specific activity of 440 units/µg. protein (Section 8.6.) and the $E^{1\ cm.}_{280\ nm.}$ figure 0.87 (Section 8.8.) we can calculate that the ku./ml./E for purified material should be 506. Most of the values in Table 7.7.1. are within experimental error of this value, but those from PP8 (632) and PP15 (610) are too high and if correct would show that all the other preparations are comparatively impure. The extent of this difference was not realised when the experiments were performed and the figures were not investigated immediately.

However, a new batch of iodine for assay was made up after the figure 632 had been determined and before the properties of the pooled material were examined. The same material proved to have a specific activity of 468. It seems that the iodine used earlier had been weaker than expected, in spite of the checking procedure used (Section 4.4.). At a very late stage in the work some thiosulphate which had been stored in a plastic bottle was found to have only

84%/

84% of its theoretical strength against iodine. It is thus very possible that the anomalous activity of PP15 was also due to undetected weak iodine.

The evidence shows that the later preparations on which most of the work was done were pure by the criteria used. The extent to which structural studies are evidence of purity is discussed in Chapter 18.

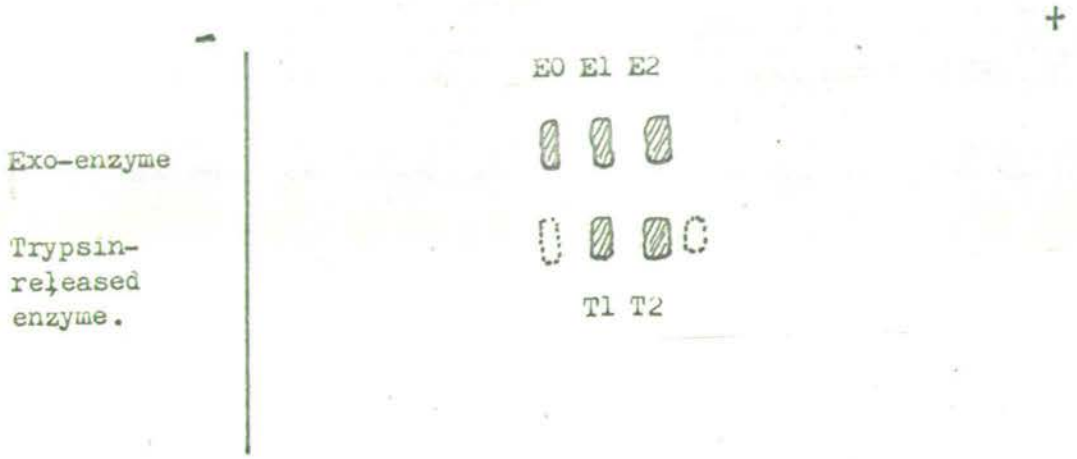


Figure 8.1.1. Numbering of bands produced by starch-gel electrophoresis of penicillinase at pH 8.5. The minor bands differed from experiment to to experiment. See Figure 9.3.2. for a photograph.

Figure 8.2.1. Bands produced by polyacrylamide gel disc electrophoresis of trypsin-released penicillinase. Magnification, about x4. The bands had moved about three cm. into the gel.

CHAPTER 8

PENICILLINASE PROPERTIES

8.1. Starch gel electrophoresis.

Pollock (1965) illustrated the multiple bands obtained from B. licheniformis penicillinase. The present technique (Section 3.2.) gave similar results. A photograph of typical results is shown as Figure 9.3.2. The numbering employed for the principal bands is shown in Figure 8.1.1. The minor bands have varied from preparation to preparation; the extra bands with trypsin-released material are comparatively strong in Figure 9.3.2. Similar patterns have been obtained by staining for penicillinase, and preparations have been routinely examined to see that no non-penicillinase bands were present. Band T2 was much weaker in material treated with trypsin for 30 min. at 30° than in that treated for 18 hr. at 37°.

8.2. Polyacrylamide disc electrophoresis.

Purified penicillinase has been examined in the presence and absence of 8M urea by polyacrylamide disc electrophoresis (Ornstein, 1964). The gels were prepared by photopolymerisation with riboflavin. It has been reported (Brewer, 1967) that ammonium persulphate (a chemical initiator) may interact with urea giving rise to artefacts.

Stock solutions had the composition shown in Table 8.2.1. (Davis, 1964)/

Table 8.2.1.

Solutions for polyacrylamide disc electrophoresis.

For each solution, the quantities shown were made up to 100ml., with (U) or without 48 g. urea.

	A	B	C	D	E	AU	BU	CU	DU	EU
12 M-HCl ml.	4.0					2.0				
Tris g.	36.6	5.7				18.3	5.7			
M-H_3PO_4 ml.		25.6					25.6			
Acrylamide g.			30	10				30	10	
BIS g.			0.8	2.5						
Riboflavin mg.					4					4
pH	8.9	7.1								

Volumes made up to 16 with water or 8 M urea respectively

	A	B	C	D	E	AU	BU	CU	DU	EU
Small pore no urea	2		4		1					
Large pore no urea		2		4	4					
Small pore urea						4		4		1
Large pore urea							2		4	4

1964) and the volumes mixed to make small pore (7½% acrylamide) and large pore (2½% acrylamide) solutions are shown in the same Table. The gels were made in precision glass tubes 7.5 x 0.5 cm. internally, fitted with plastic closures at the bottom. The tubes were filled up to 1.2 cm. from the top with small pore solution, and a few millimetres of water was layered on top. The gel was polymerised by 30 min. exposure to a fluorescent lamp. The water was removed, 1.0 cm. of large pore solution was put in, and again covered with water. This was also polymerised with fluorescent light and the water again removed.

Penicillinase (trypsin-released; PP14) was dissolved in 20% (w/v) sucrose at 4 mg./ml. (Riesfeld, Lewis & Williams, 1962). 0.05 ml. of this solution was added to the top of the gel. The tubes were then placed in the electrophoresis apparatus, in which two electrode vessels are placed one above the other, and the tubes are held in holes in the base of the upper by grommets, dipping into the lower vessel. The vessels were filled with pH 7.9 buffer (3g./1. tris. & 14.4g./1. glycine) with which the top of the tubes was also filled. The closures were removed and bubbles in the bottom of the tubes were removed with a bent pipette. The positive electrode was connected to the bottom and 5 ma. per tube was passed for two hours.

Some of the gels were stained for protein with Amido Black (Section 3.2.). Excess stain was removed electrolytically. A polyacrylamide solution was made by exposing 6 g. acrylamide and 0.5 mg. riboflavin in 100 ml. water to fluorescent light for one hour and diluting with an equal volume of 2 M-acetic acid. The gels were placed in 0.8 cm. diam. tubes drawn out to 0.2 cm. at the bottom, sealed with a small plug of small pore gel. The polyacrylamide solution filled the rest of the tubes. The electrode vessels were filled with M-acetic acid and the negative electrode was connected to the bottom. Electrolytic destaining was complete in about 2 hr.

Staining for penicillinase was performed with N-phenyl-1-naphthylamine-azo-o-carboxybenzene (Pollock, 1965). The gel was soaked for one hour in a saturated solution of the indicator, prepared by diluting a saturated solution in dimethylformamide three times with 0.1 M sodium phosphate, pH 7.0, and filtering. The gel was then transferred to a mixture of 10 ml. of the indicator solution with 10g. sodium benzylpenicillin. After 30 min. bands were seen and were intensified by washing in the pH 7.0 buffer and then dipping in M-acetic acid. Pale bands on a deep violet background were then seen.

In the absence of urea, two protein bands were seen, about 0.3 cm. apart after 3 cm. migration. (Figure 8.2.1.). Activity was seen in the same position on gels stained for enzyme, but the sharpness was insufficient for two bands to be resolved. With 8 M urea/

urea, one very diffuse band was seen.

8.3. Isoelectric focusing.

Isoelectric focusing is a powerful technique for separating proteins with different isoelectric points (Vesterberg & Svensson, 1966). On stationary electrophoresis a mixture of good carrier ampholytes (i.e. compounds with appreciable conductance in their isoelectric state) will form a pH gradient, as compounds with low isoelectric points will migrate toward the positive electrode, while those with high isoelectric points go toward the negative electrode. A protein placed in such a system will migrate and stay in its isoelectric point in the pH gradient.

The ampholytes used were a mixture of low molecular weight aliphatic polyaminopolycarboxylic acids (Ampholine). A fraction giving a gradient from pH 3 to 10 was used. The column used (110 ml. capacity) has been illustrated (Vesterberg et al., 1967). It consists of three concentric glass cylinders mounted in a vertical position. The central cylinder contains an electrode, which is connected at the bottom to the next cylinder, where the electrolysis occurs. This permits gas to escape without disturbing the column. The central cylinder can be closed off when required. The outer cylinder is a water jacket. The other electrode is connected with the liquid in the middle cylinder at the top. The gradient is stabilized with a sucrose concentration gradient. The composition of

the/

the various solutions is shown in Table 8.3.1. The gradient was made from mixtures of the dense and less dense solutions. The first fraction comprised 4.6 ml. of dense solution, the second 4.4 ml. of the dense and 0.2 ml. of the less dense, etc.

Table 8.3.1.

Composition of solutions for isoelectric focusing.

	Dense solution	Less dense solution	Cathode solution	Anode solution.
2% Ampholine ml.	9.2	3.0		
Water ml.	32.8	57.0	14.0	10.0
Sucrose g.	28		12	
Ethanolamine ml.			0.4	
Phosphoric acid ml.				0.1

10mg. exo-penicillinase (PP6) was added to two of the middle tubes. With the central compartment closed off, the twenty-four fractions were poured into the column successively, starting with the densest, and allowed to mix evenly round the column. The anode solution was added at the top, the cathode solution to the central compartment, and the connection at the bottom opened. The water jacket was circulated/

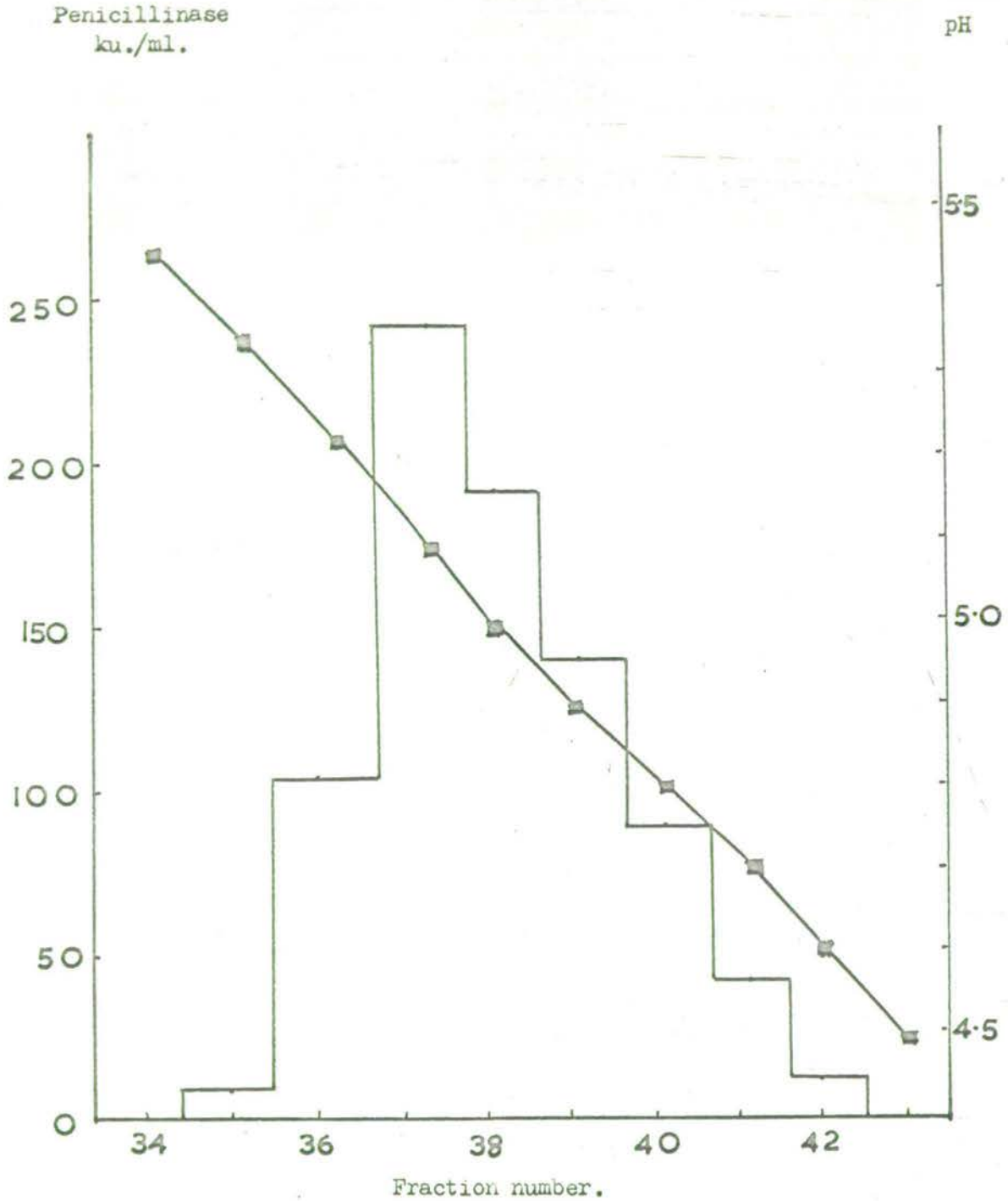


Figure 8.3.1. Iso-electric focussing of exo-penicillinase.

Fraction size, 2ml. Penicillinase: histogram; pH —■— .

circulated with cold water, and 500 v. was applied for 48 hr.

After the run, the central compartment was sealed off again. The contents of the column were pumped out at 20 ml./hr. and five minute fractions were taken. The pH and penicillinase activity (Citri method, Section 4.4.) of each fraction were determined. Figure 8.3.1. shows the result. Samples were examined by starch gel electrophoresis (Section 3.3.) and the bands compared with those normally found in exo-enzyme (Section 8.1.). Fraction 36 contained only E0, which was also found in 37 & 38, strongly, and 39 & 40, more weakly. E1 was strongest in 39 & 40, in which E2 was also observed.

Thus band E0 has a pI of about 5.05. More separation might have been obtained using a fraction of Ampholine giving a gradient between pH 3 & 6, but this would have required at least 1000v. which was not conveniently available.

8.4. Amino acid analysis.

Amino acid analysis was performed in parallel on trypsin-released enzyme (T, PP15) and exo-enzyme (E, PP16). The techniques are discussed in Sections 3.10 & 3.12. The protein solutions used were about 1.5 mg./ml. Six samples, 0.35 ml., of each were taken. Hydrolyses were in duplicate at 25, 50 & 100 hours. The analyser was re-calibrated at the same time. Two complete analyses and a few other peaks were lost by machine failure, but one value was obtained for/

for each amino acid at each time for both T & E.

The micromoles found were normalised to 0.2000 micromoles internal standard per column, which only involved corrections of a few percent. Each analysis was then normalised to the same amount of protein by summing a few reliable amino acids and adjusting this figure to be the same for each T analysis, and separately for each E analysis. The greatest difference then found between replicates was 13%, but only 8 cases were more than .0100 micromoles different. Values for serine and threonine were plotted against time, a straight line was found and the best value was determined by extrapolation to zero time. The values for valine and isoleucine were plotted against the reciprocal of time, but a straight line was not found, so the 100 hr. values were taken as best values. All the other amino acids were found to be independent of time, so all the values were averaged. The best values are shown in Table 8.4.1. It is clear that taking histidine as one residue/mole would give a molecular weight in agreement with that determined by gel filtration (Section 8.7.). On this basis the nearest integers for methionine, tyrosine and phenylalanine are 5, 6 & 7 respectively. Therefore for T & E the quantities of these amino acids were summed and divided by 19. Each best value was divided by the resultant figure and the figures are shown as residues/mole.

The amide ammonia was determined by comparing the ammonia content of samples with and without hydrolysis for 2 hr. at 105° in

2/

[c]: Including glutamine.

[d]: Mean of two methods used.

[e]: .0313 micromoles protein gave .579 micromoles ammonia; blank was .032 micromoles.

[f]: 24 Aspartic acid; 13 asparagine.

[g]: 20 Glutamic acid; 7 glutamine.

The differences between E & T do not seem significant, and are better regarded as a measure of the repeatability of the analysis. The largest difference is 4.3%. The differences between the sequence and the analysis are discussed in Chapter 18.

Table 8.4.1.

Amino acid analysis of penicillinase

Amino acid	Best Values		Residues/mole.		Nearest integer to mean	Sequence[a]
	E	T	E	T		
Lysine	.1450	.2080	24.2	24.1	24	24
Histidine	.0060	.0090	1.0	1.0	1	1
Arginine	.0896	.1252	15.0	14.5	15	15
Aspartic acid[b]	.2323	.3254	38.8	37.7	38	37 [f]
Threonine	.1245	.1825	20.8	21.1	21	21
Serine	.0685	.1030	11.4	11.9	12	11
Glutamic acid[c]	.1832	.2535	30.6	29.3	30	27 [g]
Proline	.0687	.0986	11.5	11.4	11	11
Glycine	.0942	.1331	15.7	15.4	16	15
Alanine	.1600	.2249	26.7	26.0	26	26
Valine	.0976	.1370	16.3	15.9	16	15
Methionine	.0291	.0410	4.9	4.7	5	5
Isoleucine	.0877	.1248	14.7	14.4	15	14
Leucine	.1641	.2308	27.4	26.7	27	27
Tyrosine	.0372	.0536	6.2	6.2	6	6
Phenylalanine	.0414	.0606	6.9	7.0	7	7
Tryptophan[d]			2.8		3	3
Amides[e]			17.5		18	20

[a]: See Figure 16.2.1.

[b]: Including asparagine.

c/

2 M-HCl. (Chibnall, Mangan & Rees, 1958). A third sample was hydrolysed normally and the lysine content determined. The protein content was calculated on the basis of 24 residues of lysine.

The tryptophan content was determined in two ways (Section 3.13.). The method of Goodwin & Morton (1946) gave 6.24 residues tyrosine and 2.82 residues tryptophan, the protein content being determined by the first dry weight determination (Section 8.5.). With the same protein solution the method of Spies & Chambers (1948) gave 2.86 residues, which was not increased by prior digestion of the protein with pepsin (Harrison & Hofmann, 1961). The reasons for rejecting the result obtained by the method of Barman & Koshland (1967) are given in Section 15.5.

8.5. Dry weight and carbohydrate content.

The first attempt to reconcile the dry weight and amino acid analysis of penicillinase was made on the milligram scale. Five glass tubes with screw caps were dried and weighed twice on an analytical balance (differences about 0.1 mg.). Penicillinase was desalted on Sephadex G25 into dilute formic acid (pH 5) and 5 ml. was pipetted into three while the same volume of water was placed in the other two. After drying over P_2O_5 at 60°, cooling and equilibrating, the protein tubes were 14.1 ± 0.3 mg. heavier, so the protein concentration was 2.81 ± 0.07 mg./ml. The protein from one tube was treated with 5 ml. 6 M-HCl at 37° for 24 hr., after which portions were hydrolysed and analysed in the usual way. The amounts of amino acid found corresponded on average to 0.404 micromoles which gives 2.37 mg./ml. protein (M.W. 29,400). Hence the recovery of amino acids is about 84%. It was considered that the dry weight was reasonably accurate, but that the protein had been inadequately solubilized.

Another attempt was made on a smaller scale, using different portions for amino acid analysis and for drying. Amino acid analysis as above showed 1.69 mg./ml. protein in a solution which had been desalted into 0.1 M-ammonia. The dry weight determination was done on an electrobalance of limited capacity which it was not possible to dry internally. A small boat of aluminium foil was made to be, when dry, 0.810 mg. lighter than a standard weight.

0.35 ml./

0.35 ml. of the protein solution was dried in the boat over H_2SO_4 at 60°. When the desiccator was cool, the weight was determined against the standard weight as the sample took up water. The weight became constant after 30 min. and the logarithm of the weight difference from that finally attained was plotted against time (Hanania, Yeghiayan & Cameron, 1966). The log weights were linearly related to time over the first few minutes, and it was estimated by extrapolation that 0.064 mg. water had been taken up. The boat was finally 0.730 mg. heavier than before, so the dry weight was considered to be 0.666 mg. This procedure is not precise, since half the water uptake occurred before the first weighing. This figure corresponds to a recovery of 89% in the amino acid analysis.

In view of the technical difficulty of dry weight determination (Chibnall, Rees & Williams, 1943), this was not considered to be evidence for the presence of non-amino acid components in the protein. Nevertheless, the protein was analysed for sugar. The method of Devor (1950) which detects pentose and hexose, but not amino-sugars, was used.

About 10 mg. trypsin-released penicillinase (PP11) was dissolved in 2 ml. 0.2 M ammonium acetate, pH 4.0. 0.1 ml. was hydrolysed and analysed for amino acids, showing that there was 0.194 micromoles protein/ml. In parallel with standards containing up to 0.1 mg. D-glucose, 1.5 ml. protein was diluted to 2 ml./

2 ml. with water. A solution of 0.4 g 1-naphthol/100 ml. conc. H_2SO_4 was kept in the dark for several hours before use. 5 ml. was added to each sample and after 10 min. at 100° the samples were cooled and their extinctions at 575 nm. were determined. This was found to be the maximum in the standards. The protein gave a straw colour instead of the usual red, and its extinction at 575 nm. corresponded to about 12.5 micrograms glucose, i.e. 0.07 micromoles. Thus there were less than 0.25 residues sugar per molecule of protein.

Galactosamine and glucosamine were looked for on the long column run on the amino acid analyser. An analysis with 0.0575 micromoles penicillinase was permitted to continue for two hours past phenylalanine. In the region occupied by the amino sugars there was no peak higher than .005 absorbance units. Knowing that the colour yields are about the same as amino acids, and estimating peak widths very roughly, it was estimated that no more than .006 micromoles of hexosamine were present. Thus even allowing for extensive destruction on hydrolysis, it was apparent that much less than one residue hexosamine was present.

Acid hydrolysates of penicillinase contain very little humin. In view of the fact that tryptophan is present, this agrees with the absence of sugars, since, when both are present, humin formation is extensive.

8.6./

8.6. Specific activity.

Protein determination by the Lowry method (Section 3.1.) does not agree well with amino acid analysis of penicillinase solutions. The Lowry method gives a value of about 340 units/µg. (Table 7.8.2.), in agreement with the findings of Pollock (1965). Some specific activities calculated more directly are shown in Table 8.6.1. In another experiment, protein determinations by the Lowry method were compared, over a range of concentrations, with measurement of extinction at 280 nm., converted to protein using 0.87 for the extinction of a 1 mg./ml. solution (Section 8.8.). The ratios obtained varied between 1.16 and 1.34, the Lowry values being higher. The mean value was 1.29. If the figure 340 units/microgram is multiplied by this factor, a value of 438 units/microgram is obtained.

Table 8.6.1.

Specific activity of penicillinase

Protein concentration. mg./ml.	Penicillinase activity units/ml.	Specific activity. units/microgram
2.81 (Dry weight)	1,240,000 (Perret method)	440
0.94 (Amino acid analysis)	415,000 (Citri method)	442

Figures of 340 and 440 units/microgram have been adopted for the specific activity using protein determination by the Lowry method/

method and by amino acid analysis respectively. It is not surprising that these values differ, since part of the colour in the Lowry method is due to aromatic groups, which are present in penicillinase to a greater extent than they are in serum albumin.

8.7. Molecular weight.

The fact that, in gel filtration, the elution volume of a globular protein from a particular gel is inversely proportional to the molecular weight has been well established (Andrews, 1964; Determann & Michel, 1966, quote a further 22 investigations).

The ratio of the elution volume of protein to that of ε-DNP-lysine was therefore determined for penicillinase, cytochrome c, myoglobin, ovalbumin and serum albumin. Two columns of Sephadex G100 were used, with different buffers. The details and results are shown in Table 8.7.1.; the technique of gel filtration is discussed in Section 3.4. Particular care was taken to standardise the procedure for measuring elution volumes. The molecular weights used were taken from Andrews (1964). The results were plotted semi-logarithmically and the two values for penicillinase corresponded to 27,000 & 33,000.

Table 8.7.1.

Elution volumes for various proteins from Sephadex G100.

	Buffer	Tris-HCl	Ammonium acetate	Molecular Weight
	pH	7.8	8.5	
	Molarity	0.05	0.025	
	Diameter of column cm.	1.0	1.5	
	Height of column cm.	78	117	
Ratio of elution volume to that of ε-DNP-lysine	Cytochrome c	.746	.723	12,400
	Myoglobin	.670	.667	17,800
	Ovalbumin	.457	.469	45,000
	Serum albumin	.402	.388	67,000
	Penicillinase (PP4)	.530	.571	

The data in the next Chapter do not rule out an hypothesis in which penicillinase consists of two different chains whose molecular weights total 30,000. It is interesting therefore to note (Davies, 1969) that activity elutes in the same position in the presence of 8 M-urea from Sephadex G200 as it does in its absence. Although there are proteins which are not entirely unfolded by this solvent (e.g. pepsin; Perlmann, 1959), it seems reasonable to regard a single chain hypothesis as one to be proved by further structural investigation.

8.8./

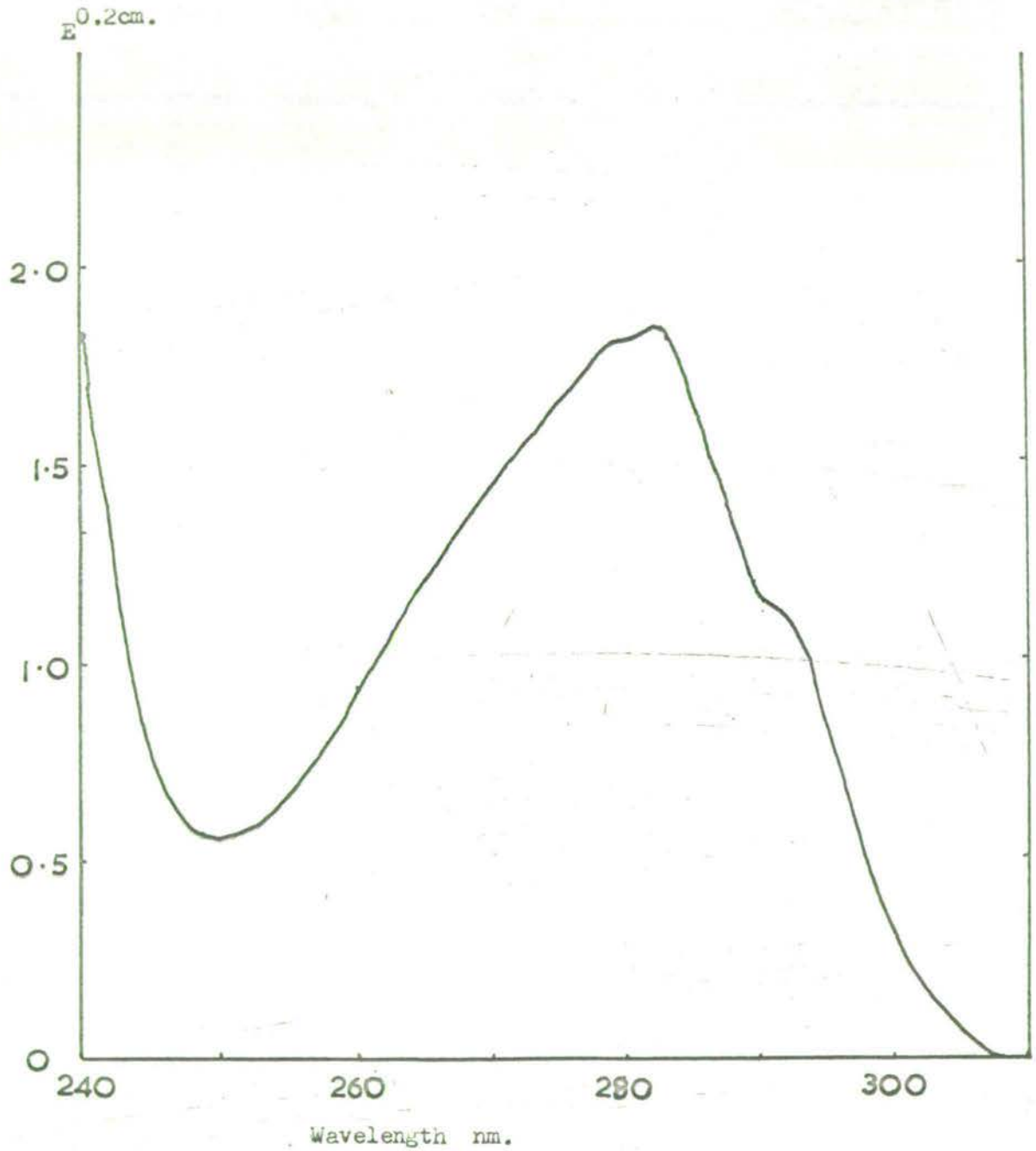


Figure 8.8.1. Ultra-violet absorption spectrum of penicillinase.

8.8. Other properties.

Figure 8.8.1. shows the u.v. spectrum of penicillinase, determined in a Unicam SP800 after elution from Sephadex G100. No absorption was found in the visible spectrum. The shoulder at 291 nm. and the flattish peak with its maximum at 281 nm. and an inflexion at 278 nm. are normally visible if the protein is concentrated enough. Table 8.8.1. shows two determinations of the extinction co-efficient. The mean value for $E^{1mg./ml.}_{280nm.}$ is 0.87. Using the known molar extinction coefficients of tryptophan (5,500) and tyrosine (1,200) at 280 nm. (Beaven & Holiday, 1952) we may calculate the value expected for a protein of molecular weight 30,000 containing three residues of tryptophan and six of tyrosine.

Table 8.8.1.

U.v. absorption of penicillinase

Material	$E^{0.2\ cm.}_{280nm.}$	Protein	$E^{1\ mg./ml.}_{1\ cm.}$
Pooled material	1.20	6.74mg./ml. (amino acid analysis)	0.89
PP11	0.484	2.85mg./ml. (dry weight)	0.85

The calculated value is 0.79. The agreement is satisfactory since 280 nm. is not a singularity in the amino acid spectra and the extinction co-efficients were taken from the spectra.

Denaturation/

Denaturation of penicillinase by trichloracetic acid is reversible. After precipitation with 2.5% (w/v) trichloracetic acid, extraction with ether, dissolution in 0.1 M-ammonia and adjustment to pH 8.5, 65% of the original activity was recovered.

The rate of recovery of activity on dilution of 8 M-urea solutions was explored, as a preliminary to exploiting the stability of chymotrypsin in 4 M-urea (Harris, 1956; Section 12.3). Davies (1969) showed that activity could be partially recovered. A solution of penicillinase was incubated for 10 hr. in 8 M-urea. Portions were then diluted with solutions calculated to reduce the urea concentration to various levels. The solutions were assayed (Section 4.4.) after a few minutes at room temperature. The results are shown in Table 8.8.2. When trypsin (one-tenth the weight of penicillinase) was added simultaneously with dilution to 2 M only 13% instead of 85% of the activity could be found.

Table 8.8.2.

Activity of penicillinase after dilution from 8 M-urea

Final urea conc. M.	Time min.	Activity restored %
0.5	5	99
1.0	5	82
2.0	5	85
4.0	20	1
4.0	5	less than 1
8.0	5	less than 1

CHAPTER 9

STUDIES ON THE TERMINAL REGIONS.

9.1. The N-terminus.

About 15 mg. protein was needed to determine the N-terminus with fluorodinitrobenzene. (Section 3.14.). Amino acid analysis showed that 0.20 to 0.25 micromoles DNP-protein was made. The quantities of DNP-amino acids found are expressed as molar percentages of the amount of DNP-protein in Table 9.1.1.

Table 9.1.1.

Yields of DNP-amino acids formed from penicillinase.

	PP16	PP14
	Exo-enzyme	Trypsin-released enzyme
DNP-threonine	16	27
bis-DNP-lysine	8	-
DNP-glutamic acid	-	4
DNP-glycine	7	1

N-termini were also examined by the DNS-method (Section 3.15.). A preliminary experiment showed that the use of urea in the reaction mixture/

mixture (Gray, 1967a) made no difference to the results.

Exo-enzyme showed DNS-glutamic acid and bis-DNS-lysine, sometimes with a trace of α-DNS-lysine. The main spots were of similar intensities. There was also a smear where DNS-threonine runs at pH 4.38, but this material did not behave authentically on electrophoresis at pH 2.0.

Trypsin-released enzyme showed DNS-threonine, which was confirmed by electrophoresis at pH 2.0. There was a trace of bis-DNS-lysine.

DNS-glycine was not detected in either preparation.

9.2. The C-terminus.

Qualitative experiments (Section 3.17.) showed that little digestion of oxidised protein was obtained with CPA. CPB gave extensive digestion, alone or with CPA. After 30 min. at 25° (enzyme:substrate 1:50 by weight) CPB released principally lysine from trypsin-released enzyme. On more extensive reaction (37°, 18 hr.) glycine, alanine, aspartic acid and leucine or isoleucine were released in comparable amounts, with traces of other amino acids.

The rate of release of lysine was followed quantitatively with CPB. The enzyme:substrate ration was 1:40 and the digestion was performed at 30°. The released reached 0.56 moles lysine/mole protein/

protein after 1.5 to 2 hours. The results were corrected for protein and enzyme blanks. The principal other amino acid observed was valine (0.20 moles/mole).

Hydrazinolysis was employed as described in Section 3.16. About 5 mg. each of PP15 (trypsin-released) and PP16 (exo-enzyme) was used. The results (Table 9.2.1.) are given as moles/100 moles protein.

Table 9.2.1.

Amino acids detected after hydrazinolysis of penicillinase as moles/100 moles protein.

	Trypsin-released enzyme	Exo-enzyme
Lysine	46	47
Histidine	6	8
Aspartic acid	5	7
Serine	18	18
Glycine	12	14
Alanine	7	7

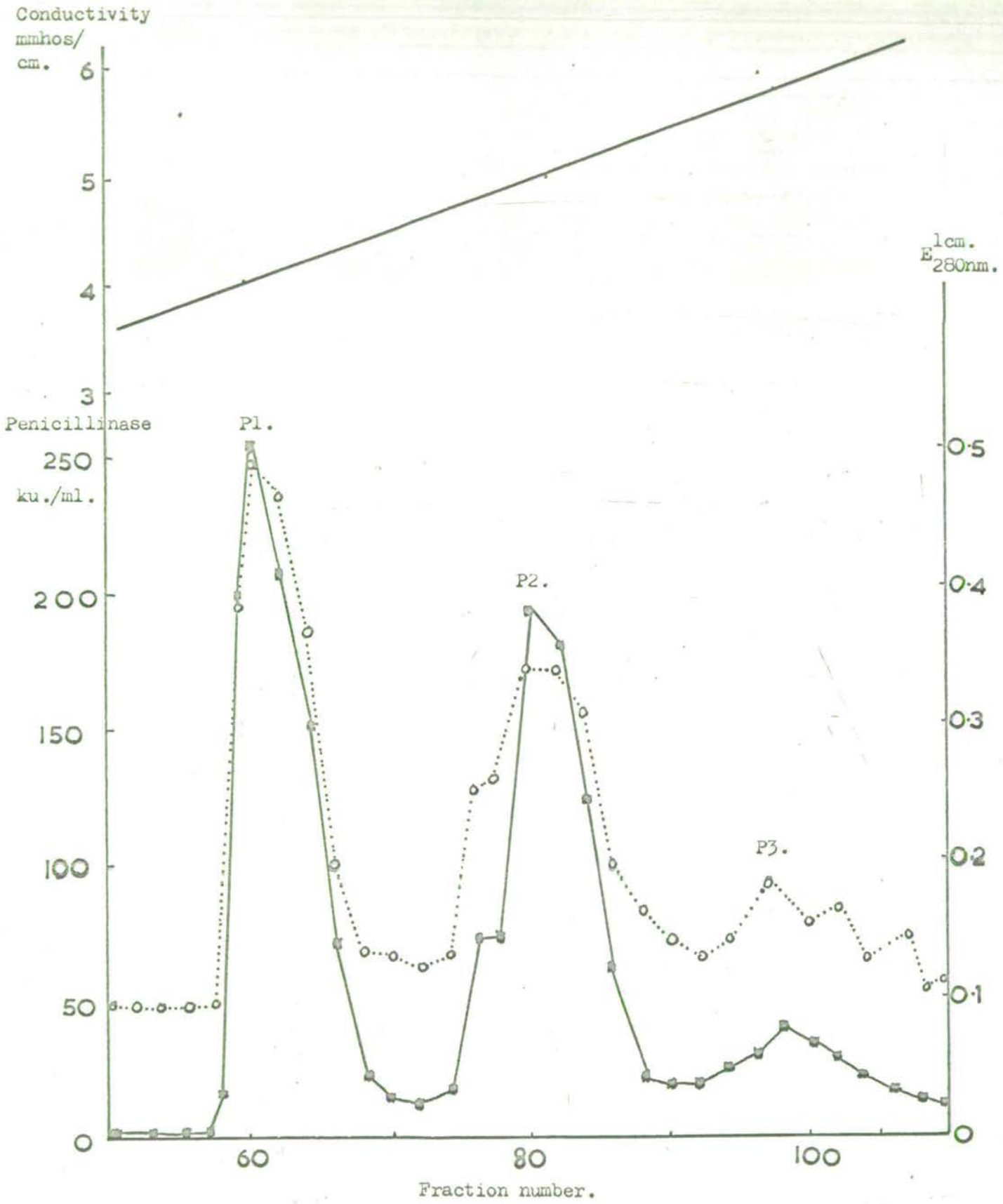


Figure 9.3.1. (Experiment 1 of Table 9.3.1.). Chromatography of exo-penicillinase on DEAE-cellulose. Penicillinase —●—. $E_{280nm.}$ ·····o····· .

9.3. Fractionation of penicillinase on DEAE-cellulose.

The electrophoretic separation at pH 8.5 (Section 8.1.) and the tailing elution profile obtained on elution from DEAE-cellulose by Pollock (1965) suggested that the material responsible for the bands on starch gel might be separated under suitable conditions.

Exo-enzyme was therefore adsorbed on to a column of DEAE-cellulose from a concentrated solution in starting buffer and eluted with a gradient. The elution profile obtained in one experiment is shown in Figure 9.3.1. The technical details are shown in Table 9.3.1., which shows the conditions and results for a similar experiment using a different ion-exchanger. The material eluted in three fractions, which will be referred to as P1, P2 & P3.

The material in the peaks was examined by starch gel electrophoresis. The result is shown in Figure 9.3.2. This gel was prepared after two months storage of the material at -20°; the proportion of minor bands had somewhat increased. When the material was fresh the minor bands were almost imperceptible.

Trypsin-released enzyme

Exo-penicillinase

P1

P2

P3

Figure 9.3.2. Starch gel electrophoresis of fractions separated by ion-exchange chromatography of exo-penicillinase.

Table 9.3.1.

DEAE-chromatography of exo-penicillinase.

Ion-exchanger.		DE11	DE52
Column height cm.		28	55
Diameter cm.		2.3	1.0
pH		7.5	8.2
Molarity of start and finish of tris-HCl gradient.		0.02 - 0.15	0.005 - 0.125
Volume in each vessel ml.		1000	1200
Penicillinase mg.		121	145
Specific	P1	4.0	3.2
Conductivities	P2	5.0	3.8
at peaks	P3	5.8	4.4
mmhos./cm.			

Thus it is clear that P1 contains chiefly E0, P2 contains E1 and P3 contains E2. In order to clarify the position, the material in the peaks from the second experiment (the result of which was generally similar to that in the Figure) was pooled, dialysed extensively against water, freeze-dried and rechromatographed on similar columns. The results of the experiments are shown in Table 9.3.2. Where necessary the peaks were identified by comparison of conductivities.

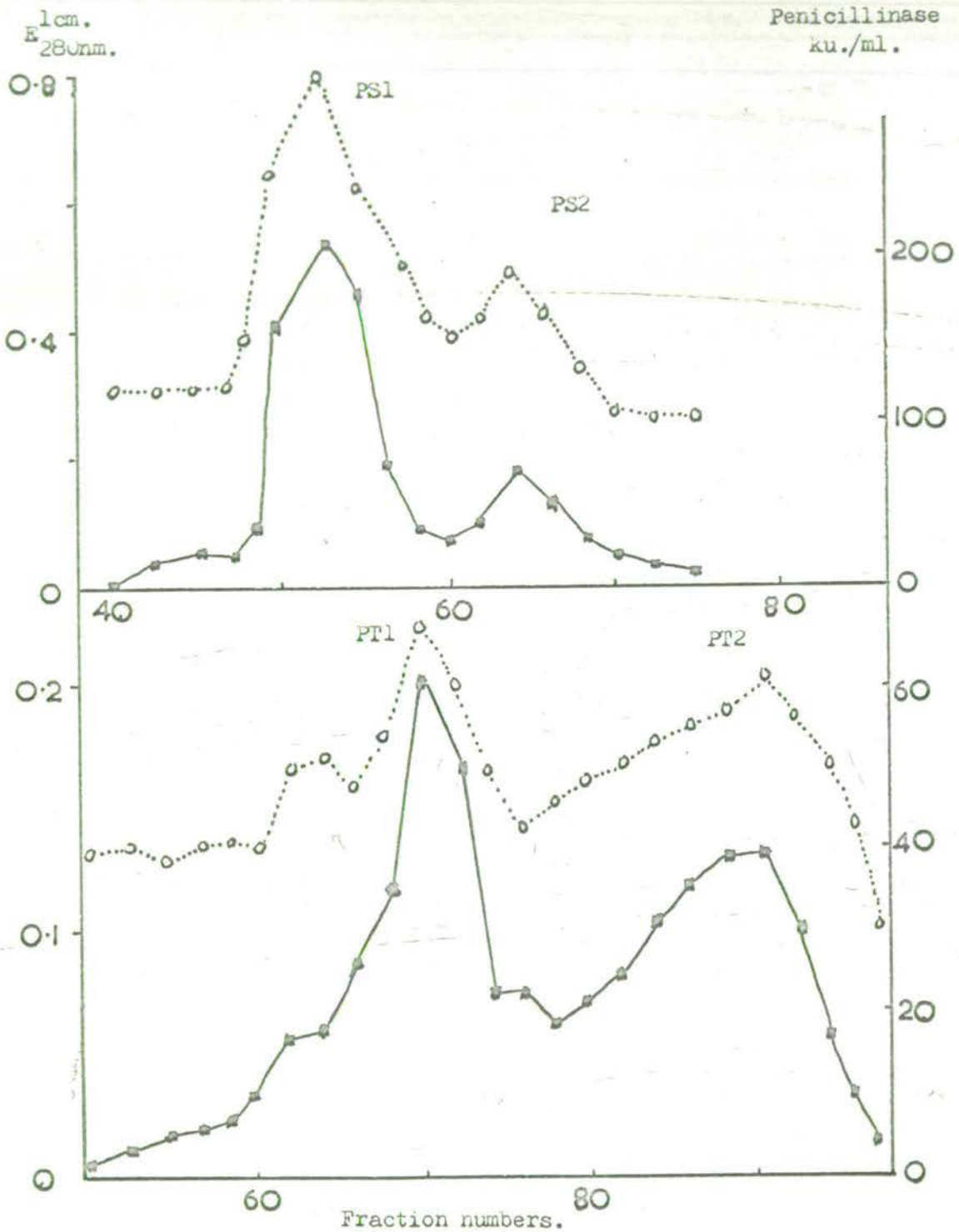
Table 9.3.2.

Re-chromatography of peaks on DEAE-cellulose.

Activities ku./ml.	First max. P1	First min.	Second max. P2	Second min.	Third max. P3
Original Sepn.	226	26	146	26	36
Rerun P1.	101	5	36	2	3
Rerun P2	6	2	105	10	28
Rerun P3			8	5	29

On starch gel electrophoresis a similar result to Figure 9.3.2. was obtained.

The separation of material prepared from cells by protease release by this method was also investigated. A batch of cells (about 120 x 10^6 units) was lysed and treated with nucleases as described in Section 7.4. It was then divided in two and one half was treated with trypsin as described there, while the other half was treated with an identical amount of subtilisin B. The two batches were dialysed against ammonium sulphate (Section 7.5.) and centrifuged in parallel. The supernatants were dialysed against water, concentrated by rotary evaporation and adjusted to about 500 ml., pH 8 and the same specific conductivity as 0.02 M tris-HCl, pH 8.0. Each was applied to a column of DE11, about 25 cm. x 3.2 cm. diam. equilibrated with the same buffer. The columns/



Figures 9.3.3. (top) and 9.3.4. (bottom). Chromatography of penicillinase released by subtilisin B (top) and trypsin (bottom) from cells. Elution with a salt gradient from DEAE-cellulose (DE11). Conditions generally similar to Experiment 1, Table 9.3.1.

columns were eluted with a gradient from 1200 ml. 0.02 M buffer to the same volume of 0.25 M buffer. Fractions were collected and their penicillinase activities and extinctions at 280 nm. are shown in Figures 9.3.3. & 9.3.4.

The material comprising the four peaks (PT1 & 2, PS1 & 2) was dialysed and freeze-dried. The purification of each fraction was completed by gel filtration on Sephadex G100 (Section 7.7.). The purified material was compared with the exo-enzyme fractions by starch gel electrophoresis and the first peaks from both separations were indistinguishable from E1 and the second peaks were indistinguishable from E2.

9.4. Properties of exo-enzyme fractions.

Various properties of the material eluting in the different peaks were compared. The relative activities of the enzyme with various substrates were measured. The formulae of the substrates are shown in Table 1.1. The assays were performed by the Perret method (Section 4.3.). The results are shown in Table 9.4.1.

Table 9.4.1.

Activities of exo-enzyme fractions against various substrates.

	Activity against benzylpenicillin put equal to 100.			
Substrate	P1.	P2.	P3.	Exo-enzyme
6-APA	4.7	4.6	4.3	5.0
Methicillin	0.5	0.5	0.5	0.5
Ampicillin	73	74	64	68
Cephalosporin C	0.36	0.36	0.33	0.33
Benzylcephalosporin	1.0	1.1	0.9	1.0
Cephaloridine	19.8	19.5	17.8	18.0

The titration of E0, E1 & E2 with rabbit anti-penicillinase antiserum was compared. Methods have been described by Pollock (1964). Enzyme from the peak fractions of P1, P2 & P3 (Figure 9.3.1.) was diluted with 0.5% (w/v) gelatin to 15 units/ml. Portions were then mixed with different dilutions of antiserum in 0.9% NaCl and allowed to stand for one hour at room temperature. The solutions were then assayed for penicillinase with benzylpenicillin by the Perret method (Section 4.3.). In Table 9.4.2. the amount of antiserum is expressed relative to the amount of enzyme, and the activities are expressed relative to the control put equal to 100.

Table 9.4.2.

Titration of exo-enzyme fractions against anti-penicillinase.

ml. antiserum per unit enzyme	Fraction.		
	P1	P2	P3
0	100	100	100
1.1×10^{-6}.	126	122	114
2.2×10^{-5}.	130	124	106
1.1×10^{-4}.	144	122	116

The material from peaks P1, P2 & P3 was examined for N-terminal amino acids by the DNS-method (Section 3.15.). The result is shown in Table 9.4.3. where the number of symbols is a measure of intensity.

Table 9.4.3.

N-termini of exo-enzyme fractions.

	P1	P2	P3
DNS-glutamic acid.	-	+	+++
α-DNS-lysine.	++	+	-

9.5. Cyanogen bromide digestion of exo-enzyme fractions.

The last traces of salt were removed from about 10 mg. penicillinase from each peak by gel filtration on Sephadex G25 in 0.1 M-ammonia, and the material was freeze-dried again. It was then digested with cyanogen bromide (Section 3.6.) in parallel with the same amount of trypsin-released enzyme (T) and exo-enzyme (E). The large peptides were removed by precipitation with trichloroacetic acid (Section 10.1.).

The soluble peptides were dissolved in 0.25 ml. 5% formic acid. A small sample was examined by electrophoresis at pH 6.5 and no acidic peptides were seen. The rest of the digests were put on 2 cm. bands of 3 MM paper in parallel, with the origin close to the anode. After electrophoresis (pH 6.5, 65v./cm., 1 hr.) the paper was allowed to dry, and the five strips were cut apart. Each was sewn to another sheet of 3MM paper and electrophoresed at pH 3.5, the red "Pentel" marker allowing the running times to be closely matched. Figure 9.5.1. shows the peptides which were detected with ninhydrin, while Table 9.5.1. shows the relative strength of the spots in the different samples. The scale for strengths is not linear, varying from '+' for very faint to '++++' for extremely strong. Four minor spots which were only detected on one sample each have been omitted. Further details of the peptides will be found in Chapter 10, which contains the data by which/

which the peptides were identified.

The most notable features are:

N-terminal' threonine only found in T.

α- & β-Asp-Gly-Lys are weak in P1 but strong in P2 & P3.

Asn-Gly-Lys is almost restricted to P1 & P2.

Lys-Thr-Glu-Hsl is found in P1 & P2.

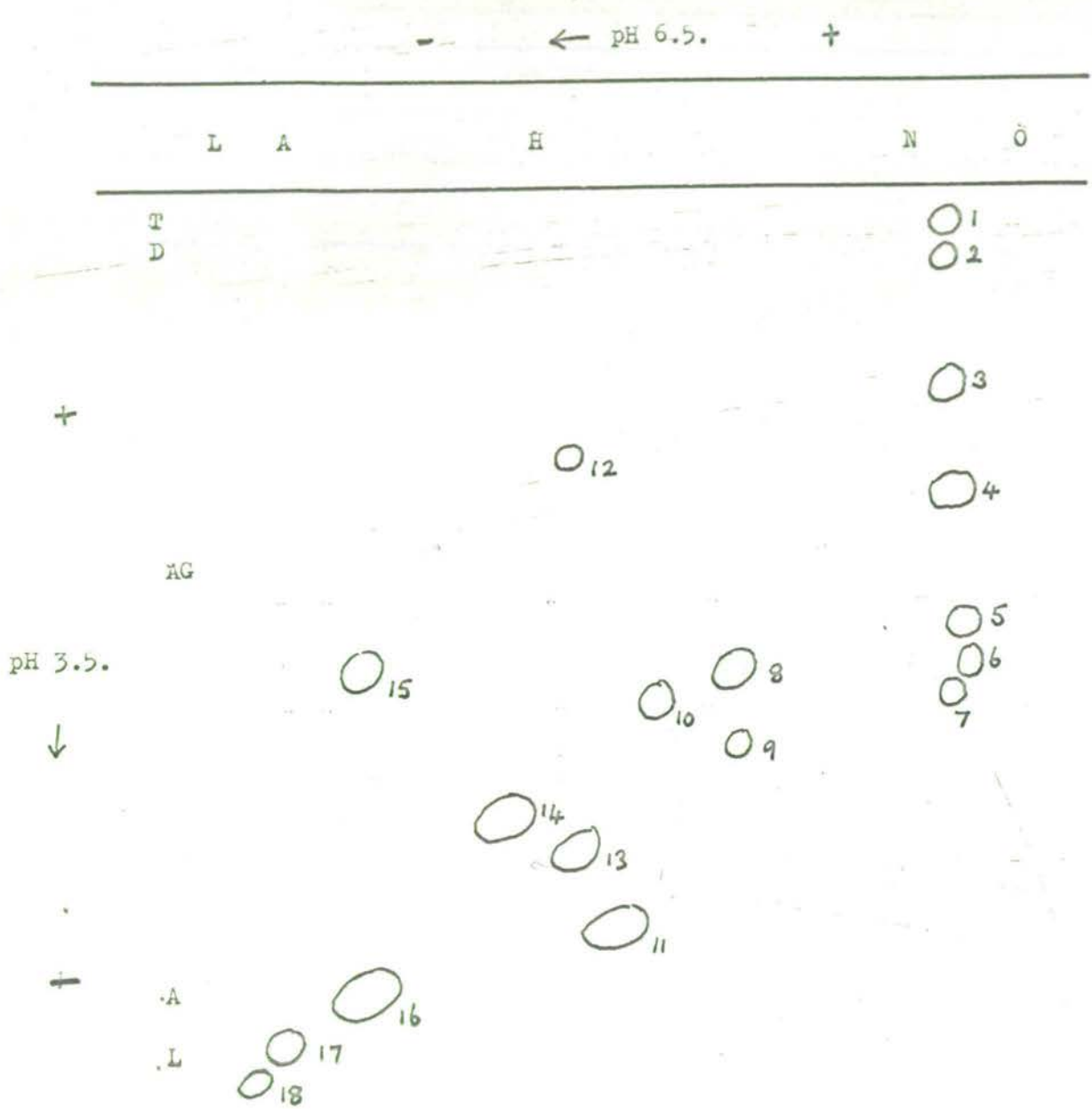


Figure 9.5.1. Electrophoresis (two dimensional) of soluble peptides from cyanogen bromide digestion of penicillinase.

O: origin.

Positions of standard substances:

N: monoaminomonocarboxylic acids; H: histidine; A: arginine; L: lysine; AG: alanylglycine; T: taurine; D: ε-DNP-lysine.

Table 9.5.1.
Tentative identification of
Peptides formed by cyanogen bromide digestion
of exo-enzyme fractions.

Spot	T	E	P1	P2	P3	Identification	Notes
1	+++	+++	+++	++++	++	Free amino acid.	?
2	++	+++	++	+++	++	Free amino acid.	?
3	++++	+++	++	++++	++++	β-Asp-Gly-Lys. (X3b)	a
4	+++	+++	++	++++	++++	α-Asp-Gly-Lys. (X3a)	
5	++++	-	-	-	-	Thr-Glu-Hsl. (TX1)	b
6	-	++	-	+++	+++	Lys-Ala-Leu-Asp-Met-Asp-Gly-Lys.	?
7	+	++	++	++	+++	Lys-Ala-Leu-Asp-Hsr. (X2).	?
8	++	+	+	++	++	Lys-Ala-Leu-Asp-Met-Asp-Gly-Lys.	?c
9	++	+	-	++	++	Lys-Ala-Leu-Asn-Met-Asp-Gly-Lys.	?
10	++++	++	++++	+++	+++	Lys-Ala-Leu-Asp-Hsl. (X2)	d
11	+	++++	++++	++++	+	Asn-Gly-Lys (X3c)	
12	+	+	++	+	-	Lys-Ala-Leu-Asp-Hsr (X2)	? cd
13	++	+++	++++	++	-	Lys-Ala-Leu-Asn-Hsr (X2)	? d
14	+	++	++++	+	-	Lys-Thr-Glu-Hsl (EX1)	
15	+	+	+	+	+	Lys-Ala-Leu-Asp-Hsl (X2)	? c
16	++++	++++	++++	++++	++++	Lys-Ala-Leu-Asn-Hsl (X2)	
17	+	++	+	+	-	?	
18	-	+	+	++	+	?	

a: This spot gave a bluish tint after 30 min. at 105°.

b: This spot initially gave a brown colour.

c: Possibly these spots arose through deamidation in the course of electrophoresis.

d: The possible forms of the last two residues are such that it is not possible to be sure of the exact structures.

?: The evidence does not permit these peptides to be identified with

CHAPTER 10

CYANOGEN BROMIDE DIGESTION

10.1. Removal of large peptides.

In preliminary experiments the large cyanogen bromide peptides were successfully separated from the small ones by dissolution of the digest from 20 mg. penicillinase in 1.5 ml. 66% formic acid followed by gel-filtration on Sephadex G25 in 5% formic acid in a column 100 cm. x 1.0 cm. diam. When an attempt was made to repeat the experiment with the digest from 100 mg. in 3 ml. 50% formic acid, the material set to a thick gel in the course of application to the column and the experiment had to be abandoned. It was also impossible to remove the large peptides by dissolving the digest in a little conc. formic acid, diluting twenty-fold and keeping overnight at 4°.

The large peptides were eventually removed by precipitation from 5% formic acid with 2.5% trichloroacetic acid. After 15 min. the precipitate was centrifuged (15 min., 4°, 25,000g) and the supernatant extracted three times with two volumes of ether to remove the precipitant. The residual ether was blown off with nitrogen and the solution freeze-dried. The precipitate was stored at 4° until required (Sections 10.6. & 10.8.).

10.2./

10.2. Separation of soluble peptides.

100 mg. scale digestions (Section 3.6.) were performed in parallel and the large peptides were removed by precipitation (Section 10.1.). The freeze-dried material was brown, and was dissolved in 0.75 ml. 5% formic acid and extracted twice with three volumes of ether to remove the last traces of trichloroacetic acid. Each mixture was separated by electrophoresis at pH 6.5 on 25 cm. 3MM. paper, with the origin toward the anode, as preliminary experiments showed no detectable acidic peptides. Further purification was performed at pH 3.5. The properties of the peptides found are shown in Table 10.2.1. 'T' & 'E' distinguish fractions coming from trypsin-released enzyme and exoenzyme respectively.

10.3. The X1 peptides.

This group contains the only difference found between T & E. The properties of TX1 were consistent with Thr-Glu-Hsl, and since EX1 had an extra lysine residue at the N-terminus it was apparently Lys-Thr-Glu-Hsl. An attempt was made to re-purify the rest of this peptide, but it could not be detected after electrophoresis.

These structures are confirmed by the sequences Thr-Glu-Mes-Lys- and Lys-Thr-Glu-Mes- found by the DNS-PTC method for the N-terminal peptides from digests of the two types of material. (Section 11.21.).

10.4./

Table 10.2.1.

Properties and composition of soluble cyanogen bromide peptides

Peptide	N-t	m	m'	Lys	Asp	Thr	Ser	Glu	Gly	Ala	Leu	Hsr.
TX1	Thr	0	+0.44	-	0.10	0.85	-	1.16	0.19	-	-	0.83
TX2	Lys	+0.86	+1.50	0.92	1.08	-	-	-	0.16	1.08	1.04	0.90
TX3a	Asx[a]	0	+0.90				b					
TX3b	Asx	0	+0.65	0.91	0.98	-	0.27	0.18	1.12	-	-	-
TX3c	None	+0.55	+1.92				b					
TX3d	None	+0.55	+0.83	0.85	0.93	-	0.22	-	1.22	-	-	-
EX1[c]	Lys	+0.55	+1.41	0.91	-	0.95	-	1.15	0.57	-	-	0.95
EX2	Lys	+0.89	+1.45	0.85	1.11	-	-	-	-	1.10	1.02	0.95
EX3a	Asx[a]	0	+0.92				b					
EX3b	Asx	0	+0.67				b					
EX3c	Asx[a]	+0.54	+1.86				b					
EX3d	None	+0.54	+0.83	0.93	0.92	0.15	0.24	0.15	1.15	-	-	0.15

[a]: Very weak.

[b]: Analyses not significantly different from TX3b and TX3d.

[c]: Less than 0.01 micromoles per column analysed.

10.4. The X2 peptides.

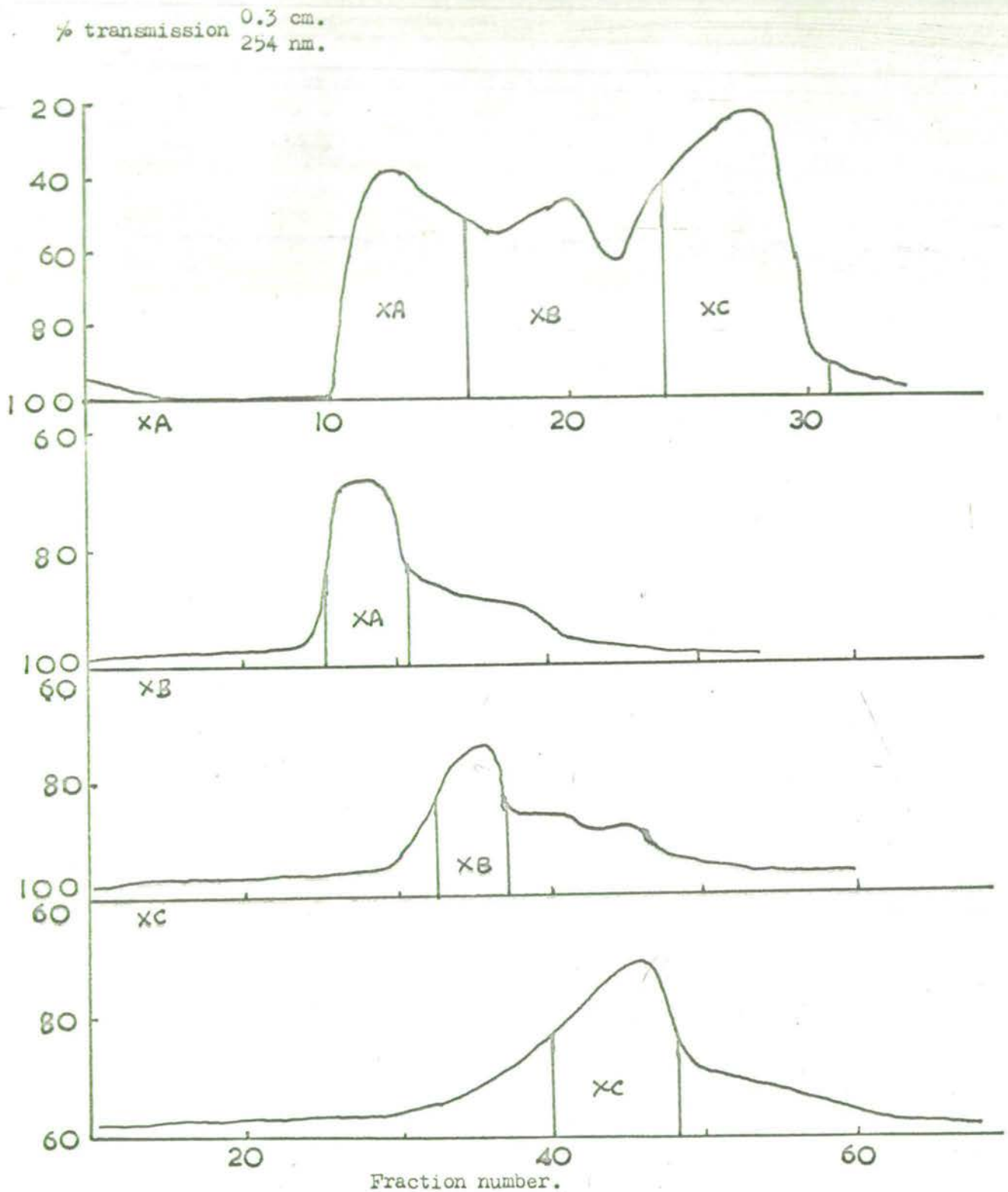
Peptides EX2 and TX2 were similar in all respects. The very basic spots found in Section 9.5. show that the aspartic acid residue can be amidated, while the existence of this peptide in neutral forms suggests that the amide is labile. The DNS-PTC method showed Lys-Ala-Leu- and then failed, possibly because of the form adopted by the asparagine residue. Peptide CA15 (Section 12.20.) has an N-terminal sequence Asn-Met-, and the evidence is consistent with a sequence -Met-Lys-Ala-Leu-Asn-Met- giving rise to this group of peptides. The various forms observed in Figure 9.5.1. are most easily explained by homoserine and homoserine lactone forms, with deamidation occurring in the course of electrophoresis.

10.5. The X3 peptides.

Four spots with composition Asp, 1; Gly, 1; Lys, 1 were isolated from each digest, apparently identical in each. They were each in very low yield, and difficulty was found removing all serine from them.

Peptides 3a and 3b, were neutral at pH 6.5, so they did not contain asparagine. They separated at pH 3.5, and the slower (3b) gave a brown colour with ninhydrin, which became slightly bluish after 30 min. at 105°. These findings are consistent with the peptides being α- & β-Asp-Gly-Lys, in support of which 3a gave

Asx/



Figures 10.o.1. (top) and 10.o.2. (rest). Chromatography and rechromatography of large cyanogen bromide peptides from penicillinase on Sephadex G75.

Asx-Gly-Lys by the DNS-PTC method, while 3b failed to work although the amount of ε-DNS-lysine showed that there was adequate material.

3c behaves at pH 6.5 & 3.5 as would be expected for Asn-Gly-Lys. The failure to react with DNS-chloride was probably due to further deamidation. The properties of 3d are consistent with its formation from 3c during purification. It is apparently α-Asp-Gly-Lys from its pH 3.5 mobility. No peptide was observed in the position taken up by β-Asp-Gly-Lys.

10.6. Gel-filtration of large peptides.

It was found that the large fragments were much more soluble in alkaline than acid conditions. Large peptides from 200 mg. penicillinase were dissolved in 10 ml. 2 M-ammonia with the aid of a little solid urea. The material was applied to a column of Sephadex G75 in 0.1 M-ammonia (110 cm. x 1.5 cm. diam.) and 10 min. fractions (4.0 ml.) were collected. The extinction of the eluate was recorded continuously and is shown in Figure 10.6.1. The three fractions indicated were pooled, freeze-dried, and redissolved in 3 ml. 12 M-ammonia each. Small portions were digested with trypsin and examined by electrophoresis. There appeared to be differences and each fraction was rechromatographed on another column of Sephadex G75 in the same solvent (130 cm. x 2.5 cm. diam.). 10 min. fractions (8.0 ml.) were collected, and the extinction was recorded again./

again. The result is shown in Figure 10.6.2. and the fractions marked (XA, XB & XC) were pooled and freeze-dried. Each was re-dissolved in 3 ml. 12 M-ammonia, and samples were digested with trypsin at pH 8.5 and separated by electrophoresis at pH 6.5. The neutral band was also separated at pH 3.5. The ninhydrin patterns of XA & XB were indistinguishable, but XC was completely different. The peptides were then stained with 1-nitroso-2-naphthol. (Section 3.3.). The mobilities of the peptides observed were compared with those for the tyrosine and tryptophan peptides (Table 11.6.3.). XC contained TB5 and TA9 which were absent from XA and XB, and these two fractions contained about equal amounts of TB8 and TN6 which were absent from XC. TA8 and TA14 are indistinguishable at pH 6.5 and all three fractions contained one or the other.

Thus it was considered that XC was the large cyanogen bromide peptide nearest the C-terminus in a reasonably pure form and it was further examined (Section 10.7.).

Although the possibility has not been excluded, there is no evidence of acid catalysed cleavage of the type shown by Hofmann (1964).

10.7. Peptide XC.

Samples of XC were analysed for amino acids and the result is shown in Table 10.7.1. Most of the ammonia was removed from the rest by evaporation and the pH was adjusted to about 9 with M ammonium/

ammonium acetate, pH 8.5. The material was digested with 0.5 mg. DPCC-trypsin for 1.5 hr. and freeze-dried. The digest (XCT) was dissolved in conc. formic acid and the material precipitated by twenty-fold dilution was collected by centrifugation (30 min., 4°, 25,000g.) and is referred to as XCTP. The soluble material was concentrated by rotary evaporation and de-salted on Sephadex G25 in 5% formic acid (125 cm. x 1.5 cm. diam.) and freeze-dried. This fraction was called XCTS.

XCTP was dissolved in a small volume of 12 M-ammonia. Its N-termini were glycine, alanine and aspartic acid. It was separated for 1 hr. at pH 9.5 (65 v/cm.) on 13 cm. 3MM paper. Three fractions were found, one still at the origin, and the others about 4 cm. in each direction. After elution, the fractions were examined qualitatively. The cathodic fraction showed no amino acids, but the others showed similar amino acids. Only the anodic fraction (XCTP3) gave N-termini, aspartic acid and glycine. The results of amino acid analysis of XCTP3 are shown in Table 10.7.1.

XCTS was separated on 13 cm. 3MM at pH 6.5 in the usual way. The neutral band was directly chromatographed in BAWP. The peptides were then purified at pH 3.5, and some at pH 2.0 also. During the purification unusual quantities of free amino acids were found; they included lysine, arginine, alanine and glutamic acid, as well as at least three other neutral amino acids which were not identified. There was only sufficient peptide for qualitative characterisation. The/

Table 10.7.1.

Amino acid analyses of XC & XCTP3.

Analyses are shown as micromoles found after 24 hr. Where 96 hr. values were substantially different they are shown as percent of the 24 hr. value.

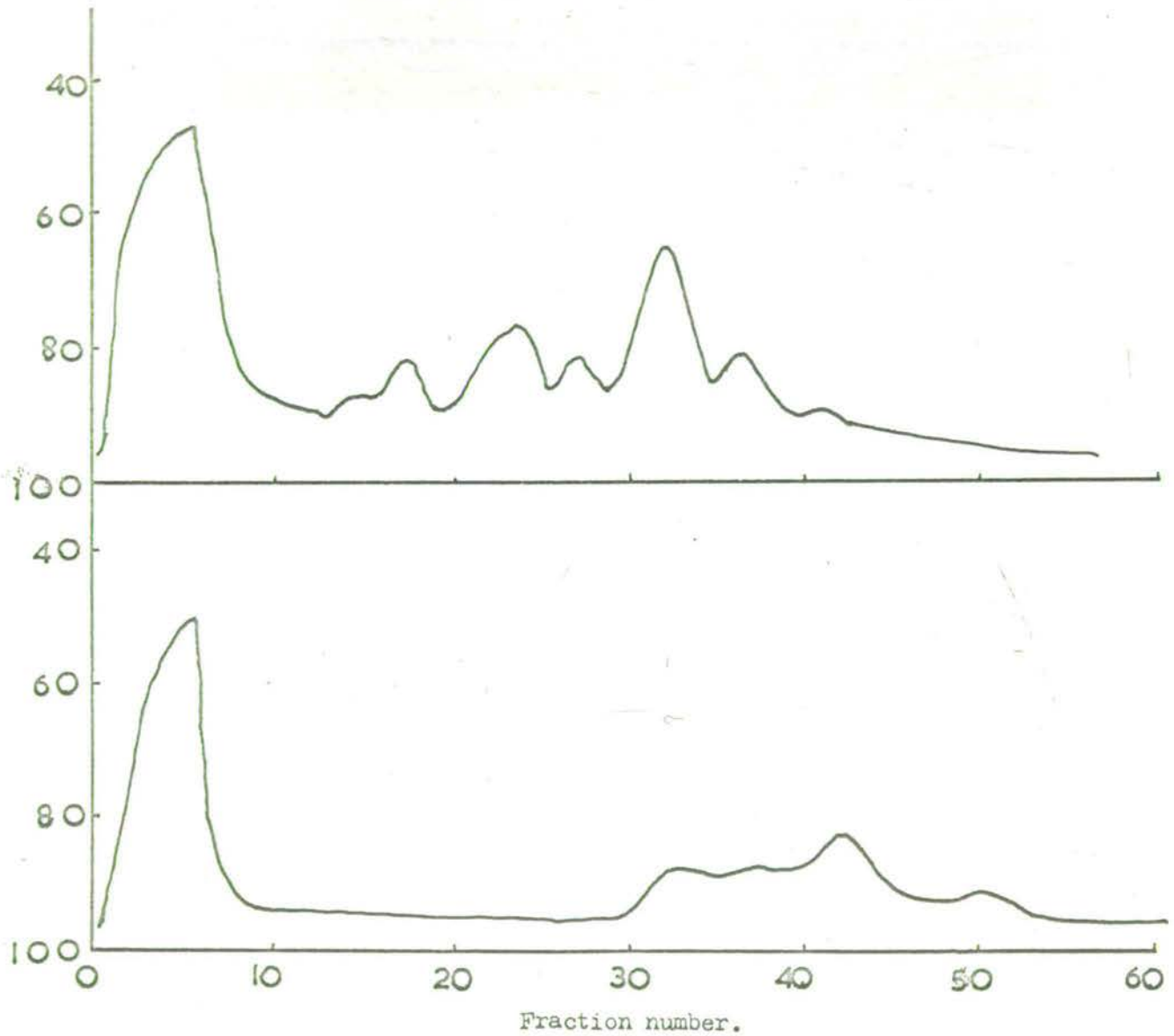
	XC	XC 96 hr. %	Moles/mole homoserine.	Residues from sequence (d)	XCTP3	XCTP3 96 hr. %
Lysine	.1025		6.28	8	.0222	
Arginine	.0502		3.07	4	.0178	
Aspartic acid[a]	.1917		11.86	11	.0363	
Threonine	.0858[b]		5.26	5	.0148	
Serine	.0645[b]		3.96	3	.0342	
Glutamic acid	.0680		4.17	2	.0095	
Proline	.0676		4.15	4	.0271	
Glycine	.0998		6.12	6	.0269	
Alanine	.1658		10.16	10	.0328	
Valine	.0923	123	6.98[c]	7	.0173	183
Isoleucine	.0663	115	4.69[c]	5	.0142	167
Leucine	.0821		5.04	4	.0184	123
Tyrosine	.0335		2.18	2	.0048	
Phenylalanine	.0087		0.53	0		
Homoserine	.0163		(1.00)	1		

[a]: Including asparagine.

[b]; By extrapolation to zero time.

[c]: From 96 hr. value.

[d]: Residues 184 to 259 (Figure 16.2.1.).



Figures 10.8.1. (top) and 10.8.2. (bottom). Chromatography of large cyanogen bromide peptides from penicillinase on CM-cellulose in 0.01 M ammonium acetate, pH 4.8, 8M-urea. With (bottom) and without (top) pretreatment with trifluroacetic acid. Elution with a salt gradient.

The peptides Asp-Lys-Lys, Leu-Ile-Ala-Glu-Ala-Thr-Lys, Val-Val-Hsr, Asp-Ala-Lys, Tyr-Asp-Asp-Lys and Ala-Gly-Val-Pro-Asp-Gly-Trp-Glu-Val-Ala-Asp-Lys were identified, and there were also fractions which might have been TB5 & TN7. There were also other peptide fractions in low yield. The implications of these findings are discussed in Section 16.2.

10.8. CM-cellulose chromatography of large peptides.

It is possible to separate these peptides on columns of CM52 in 8 M-urea, but this has not been fully exploited. Columns 8 cm. x 1.0 cm. diam. equilibrated with 0.01 M ammonium acetate, pH 4.8, 8 M-urea were used. The large peptides from 100 mg. penicillinase were dissolved in 3 ml. 8 M-urea, and equilibrated with the starting buffer by dialysis. The effluent extinction was monitored directly at 254 nm. A considerable amount of u.v. absorbing material eluted directly. This was not adsorbed to another similar column either, and had the normal u.v. spectrum of a protein. The column was then eluted with a gradient from two vessels, each containing 165 ml. starting buffer, the second being 0.15 M in sodium chloride. Figure 10.8.1. shows the elution pattern obtained.

The separation was also tried after pretreatment of the peptides with trifluoroacetic acid (Ambler, 1965), to convert all the peptides to their homoserine lactone forms. Drying the acid off *in vacuo* left the peptides intractably insoluble. The peptides from/

from 100 mg. penicillinase were therefore dissolved in 1 ml. trifluoroacetic acid and transferred to the starting buffer for chromatography by gel filtration on Sephadex G25 (41 cm. x 1.0 cm. diam.). The CM-cellulose chromatography was then carried out as above. The elution pattern is shown in Figure 10.8.2. The pattern is simpler and it is possible that less peptide failed to adsorb at all.

CHAPTER 11

TRYPTIC DIGESTION

11.1. Conditions.

A preliminary experiment was performed with oxidised penicillinase and different amounts of DPCC-trypsin. This showed that 1% by weight left some peptides appreciably weaker, as judged by paper electrophoresis, than did 4%.

The first experiment was performed with penicillinase denatured only by trichloroacetic acid precipitation. The details are in Table 11.1.1. The yields of peptides were very low and there was a large precipitate after digestion. After this, and the finding (Section 8.8.) that most of the activity can be recovered after such precipitation, the conditions necessary for digestion were more thoroughly investigated. In a preliminary experiment with a tryptic digest of oxidised lysozyme, it was shown that digestion overnight with 3% by weight of CPB gave substantially complete liberation of C-terminal lysine. Small portions (about 1 mg.) of penicillinase were precipitated and redissolved in 0.1 M-ammonia and adjusted to pH 8.5 with acetic acid, while others were oxidised with performic acid (Section 3.5.) freeze-dried and redissolved in the same manner. Each was digested overnight at 37°/

37° with 4% by weight of DPCC-trypsin. Each digest was divided in three portions. One was analysed directly for lysine, and showed very little. Another was totally hydrolysed to give a measure of total lysine. The third was digested with CPB as above, and then analysed for lysine. Whereas 103% was found with oxidised material, only 75% was recovered with the precipitated protein. It was concluded that trichloroacetic acid precipitation does not denature penicillinase adequately.

Table 11.1.1. summarises the conditions used in the other digests.

Table 11.1.1.

Conditions for tryptic digestion

Digest	mg. penicillinase	Denaturation	%(w/v) trypsin	Hours	Temp.	Section
1	220	Precipitation	2.7	2	37	11.2
2	145	Oxidation	3.5	2.5	37	11.3
3	225	Oxidation	2.7	6	37	11.4
4	60	Oxidation	3.3	1	37	11.5

11.2./

11.2. Digest 1.

The material was a mixture of exo-enzyme and trypsin-released enzyme, activity 93 x 10^6 units, corresponding to about 7 micro-moles. It was precipitated from 10 ml. water with 2.5% (w/v) trichloroacetic acid, and after centrifugation, the protein was extacted twice with three volumes of ether. The residual ether was blown off with nitrogen, and the protein dissolved in 15 ml. 0.5 M-ammonia and adjusted to pH 8.5 with acetic acid. 0.6 ml. 1% DPCC-trypsin was added and after 2 hr. at 37° the digest was freeze-dried. It was redissolved in 6 ml. 50% formic acid, and a precipitate (T1P) was removed. The solution was applied to a column of Sephadex G25 in 5% formic acid (100 cm. x 1.5 cm. diam.). 7 ml. fractions were collected with the column flowing under gravity. Electrophoresis at pH 6.5 of small fractions showed that the earliest fractions contained material which did not move from the origin. This material was removed and freeze-dried (T1P'). The other peptides were ill-separated so they were all pooled and freeze-dried.

The soluble peptides were redissolved in 1 ml. 5% formic acid and rechromatographed on the same column. The column was pumped at 30 ml./hr. and 3 ml. fractions were collected. Fractions were electrophoresed as described in Section 11.4., and a similar result to that there was obtained. The fractions were dissolved in 5% formic acid (T1P in 70% formic acid). Samples were hydrolysed and analysed/

analysed for amino acids. T1P and T1P' had compositions very similar to whole protein. No more than 28% of any amino acid was recovered. It was considered that the missing material either was not precipitated by trichloroacetic acid, or became adsorbed to the column of Sephadex G25 on dilution from 50% to 5% formic acid. Subsequent experiments (Section 11.1.) confirmed that trichloroacetic acid precipitation was an unsuitable method of denaturation.

The soluble peptides were divided into four fractions on the basis of size. Each was separated on 25 cm. 3MM paper by electrophoresis at pH 6.5. Those peptides shown to contain methionine (Section 3.9.) were oxidised by the first technique in Section 3.5. All the peptides were purified by chromatography in BAWP and electrophoresis at pH 3.5 as detailed in Table 11.6.1.

11.3. Digest 2.

The material was part of PP10 (trypsin-released enzyme). It was oxidised with performic acid (Section 3.5.). After dissolution in 12 ml. 3 M-ammonia it was diluted twelve fold with water. Amino acid analysis of a portion showed 145 mg. protein (about 5 micromoles). The solution was adjusted to pH 8.5 with acetic acid and digested with 5 mg. DPCC-trypsin for 2.5 hr. at 37°. It was then freeze-dried four times to remove some of the salt.

The digest was dissolved in 20 ml. 5% formic acid and a small precipitate/

precipitate was removed. The peptides were separated on a column of Sephadex G25 (144 cm. x 2.5 cm. diam.) in 5% formic acid which was pumped at 60 ml./hr. 11 ml. fractions were collected. 0.2 ml. samples were dried in a dessicator and examined by electrophoresis at pH 6.5 as described in Section 11.4., and 5 size fractions were pooled. Each was dissolved in 1 ml. 5% formic acid and separated at pH 6.5 on 25 cm. 3MM paper. Fractions 2 & 3 did not dissolve well and a certain amount of solid was transferred to the paper. The rest was dissolved in conc. formic acid and kept. The papers with fractions 2 & 3 dried out and charred near the origin after about 30 min. and the basic, neutral and acidic groups were re-run. The peptides were purified by chromatography in BAWP and electrophoresis at pH 3.5 (Section 11.6.).

The fractions of the insoluble material which dissolved in ethanol: 5% formic acid (equal vols.) was separated by gel filtration on a column of Sephadex LH20 (90 cm. x 1 cm. diam.) flowing under gravity at 1.7 ml./hr. 1 hr. fractions were collected and their extinctions at 254 nm. were recorded. Chymotryptic digestion of samples showed a lot of neutral material and very few acidic or basic peptides. Two fractions eluting in different positions were pooled and analysed for amino acids. Though variable, the compositions resembled whole protein rather than peptides. All amino acids were represented in each fraction, and no fraction was particularly enriched with valine, isoleucine or phenylalanine. One fraction/

fraction was all digested with chymotrypsin. Two of the most distinct bands were analysed after separation at pH 6.5, but the band was still very heterogeneous. It was concluded that no fraction had a particular peptide as a significant proportion of the total material.

11.4. Digest 3.

Part of PP16 (exo-enzyme) was oxidised and redissolved after freeze-drying in 40 ml. 3 M-ammonia solution. After dilution with 200 ml. water amino acid analysis of a small portion showed a total of 7.6 micromoles. The solution was adjusted to pH 8.5 with acetic acid and incubated at 37° with 6 mg. DPCC-trypsin for 6 hr. The digest was then taken to near dryness several times in a rotary evaporator to remove some of the salt, after which it was freeze-dried.

The digest was dissolved in conc. formic acid and diluted to 5%. 5% (w/v) trichloroacetic acid was added and after 30 min. at 4° the precipitate (t0) was centrifuged off and then redissolved in 50% formic acid. The solution was extracted thrice with 2.5 volumes ether and freeze-dried. It was then dissolved in 3 ml. 5% formic acid and applied to a column of Sephadex G25 in the same solvent (120 cm. x 1.5 cm. diam., pumped at 40 ml./hr.). 50 drop (2.9 ml.) fractions were collected and samples were applied to 3MM paper. 0.01 ml. samples were applied successively to dots 0.6 cm. apart/

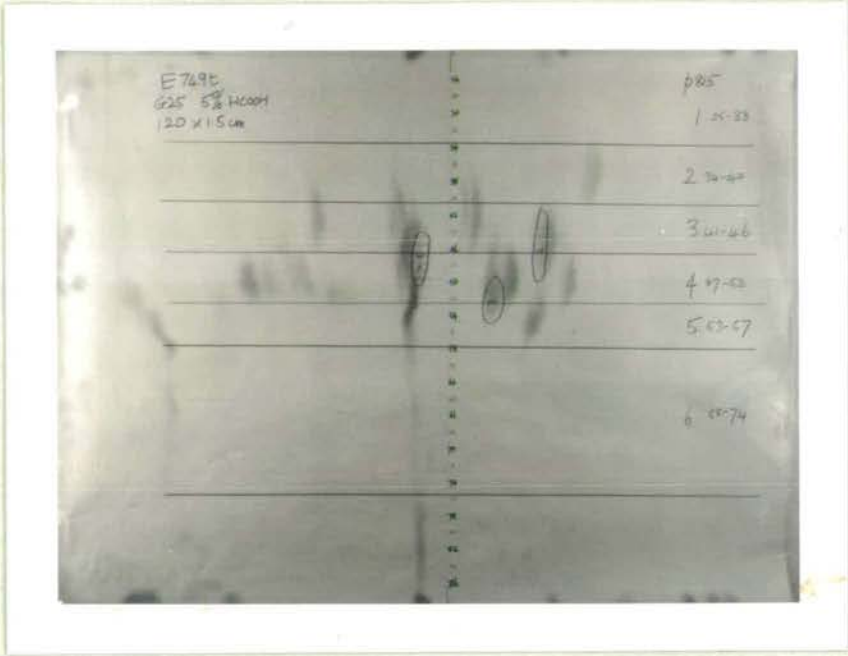


Figure 11.4.1.

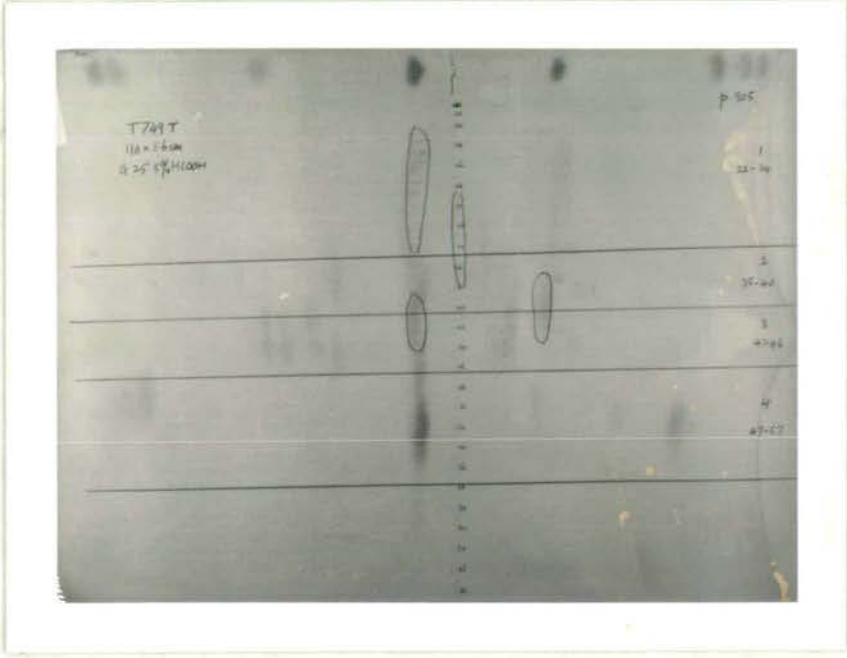


Figure 11.5.1.

Electrophoresis at pH 6.5 of tryptic peptides separated on Sephadex G25.

apart on the origin. Drying was accelerated with cold air from a hair drier and by the time the last sample was applied the first spot was, dry enough to receive another 0.01 ml. In this manner 0.03 ml. per fraction was applied (though up to 0.08 ml. may be applied if necessary), and the peptides were separated at pH 6.5 (65 v/cm.) for 50 min. The dry paper was examined under u.v. (365 nm.) light and fluorescent spots were marked. The result is shown in Figure 11.4.1. The peptides were then pooled into fractions t1 to t6 as shown in the Figure, separating so far as possible peptides of similar mobility and minimising the splitting of peptides into two fractions.

The fractions were freeze-dried, but t6 was very salty. It was dissolved in 10 ml. water and the peptides were adsorbed on sulphonated polystyrene (Permutit ZK225, SRC 13, H^+. form) by adding beads until the pH fell to 2.5. The resin was washed twice with two resin volumes of water, and the peptides were eluted with two volumes of 2 M-ammonia four times. The solution was then rotary evaporated to dryness.

Fractions, t1, 2 & 5 were dissolved in 1 ml. 5% formic acid, and t6 in 1 ml. 2 M-ammonia. t3 & t4 were insoluble in 50% pyridine and also in pH 6.5 electrophoresis buffer. They were dissolved in the latter with the aid of solid urea which was removed by gel filtration over Sephadex G25 equilibrated with the same buffer. The solutions were partly concentrated by rotary evaporation. Samples from/

Table 11.4.1.

Amino acid analysis of fractions of digest 3.

The total quantity of each amino acid was compared with that expected for 7.6 micromoles of material of the known composition of penicillinase. (Section 8.4.). The recoveries did not differ much from amino acid to amino acid. The average was 82%. The amount of amino acid in each fraction was then divided by 7.6 x 0.82. By this method a peptide containing one residue of, say, proline, and wholly present in fraction t4 should contribute 1.0 to the corresponding figure.

	t0	t1	t2.	t3	t4	t5	t6	Total	Composition
Lys	0.80	0.51	3.95	4.74	9.15	1.62	1.14	21.9	24
His	0.13	0.08	0.73	0.69	0.19	0	0.09	1.9	1
Arg	0.31	0.23	2.52	1.18	5.75	2.31	1.50	13.8	15
Asp	1.37	1.11	10.39	10.96	12.19	2.32	1.82	40.2	38
Mes	0.06	0.18	1.17	1.64	2.40	0.12	0.09	4.6	5
Thr	0.72	0.61	7.59	4.38	5.75	1.67	1.11	21.8	21
Ser	0.85	0.43	3.47	2.18	4.83	0.74	0.66	13.2	12
Glu	1.22	1.11	13.89	7.20	9.01	1.10	1.42	35.0	30
Pro	0.71	0.53	5.54	3.25	2.26	0.15	0.38	12.8	11
Gly	1.08	0.70	5.13	4.65	3.61	0.63	0.91	16.7	16
Ala	1.23	0.71	4.86	5.75	10.51	1.06	1.29	25.4	26
Val	1.30	0.53	4.41	3.65	3.40	0.26	0.78	14.3	16
Ile	0.81	0.34	1.87	4.12	3.90	0.84	0.90	12.8	15
Leu	1.72	0.60	5.00	5.94	9.24	1.07	1.42	25.0	27
Tyr	0.28	0.28	0.40	1.35	1.69	1.46	0.47	5.9	6
Phe	0.34	0.17	2.22	0.57	2.70	0.39	0.44	6.8	7
Cys	trace	-	-	-	-	-	-	-	0

from each fraction were analysed for amino acids (24 hr. hydrolyses) and the results are in Table 11.4.1. t0 apparently contained principally the trypsin used for digestion, while t1 also contained very little material.

Fractions t2 to t6 were separated by electrophoresis at pH 6.5 on two 25 cm. bands (except t6 on one). t3 and t4 were not allowed to dry but wetted immediately after application with buffer for electrophoresis.

The peptides were purified by chromatography in BAWP and electrophoresis at pH 3.5. Peptides TB10 (Thr-Arg) and TN15 (Ile-Thr-Tyr) were located chiefly by the chlorination method. Part of the neutral material from t3 & t4 remained at the origin and was examined separately. Part of the material, with an R_x in BAWP of about 0.7, reacted very strongly with the o-tolidine-KI reagent after chlorination. After a few minutes a bright orange precipitate appeared in the centre of the spot. On electrophoresis at pH 3.5 the material remained with the free amino acids (m' 0.0). This material was partly responsible for the fluorescence at the origin at pH 6.5, but did not appear to be peptide. Possibly it originated from the trichloroacetic acid used as a precipitant.

In the course of this purification another fluorescent peptide was found. After purification of the fluorescence there was little amino acid associated with it. There was a leucine N-terminus but a completely unstoichiometric amino acid analysis. In particular the contents of proline and isoleucine were quite unremarkable.

11.5/

11.5. Digest 4.

Digest 3 contained two peptides (TB11 & TN12) which apparently originated by chymotrypsin-like cleavage. It was hoped to find larger peptides from these regions by gentler digestion conditions. Since trichloroacetic acid had failed to precipitate peptides in digest 3 it was hoped to remove a fraction insoluble in 5% formic acid (compare XCTP, Section 10.7.).

The material available was PP17, and the amount of material was rather small for such an experiment. The performic acid oxidised protein was dissolved in 5 ml. 8 M-urea and transferred to 0.2 M-ammonium acetate, pH 8.5 by gel-filtration on a small column of Sephadex G25. The solution (38 ml.) contained 1.94 micromoles penicillinase, as determined by analysis. 2 mg. DPCC-trypsin was added and the digest was freeze-dried after 1 hr. at 37°.

The digest was dissolved in 1 ml. conc. formic acid, and when the solution was diluted twenty-fold a precipitate was formed, which increased after 1 hr. at 4°. It was centrifuged off and not examined further. The solution was concentrated somewhat by rotary evaporation and applied to a column of Sephadex G25 in 5% formic acid (110 cm. x 1.5 cm. diam.), pumped at 20 ml./hr. 50 drop fractions (2.9 ml.) were collected and 0.05 ml. was examined by electrophoresis at pH 6.5 as described in Section 11.4. The result is shown in Figure 11.5.1. Four fractions were pooled as shown, freeze-dried, and separated by electrophoresis at pH 6.5.

The/

The examination concentrated on certain parts of the digest. TA1 (the large acidic peptide) was redigested with 0.2 mg. DPCC-trypsin overnight at 37° to convert it to TA2 which was purified at pH 6.5 and pH 3.5. A peptide which stuck at the origin at pH 6.5 was also particularly examined (Section 11.20.).

The largest neutral fraction was not entirely eluted from paper with 0.1 M-ammonia, and most of the fluorescent material was eluted with 50% formic acid. The amino acid analysis was quite unremarkable, with small, unstoichiometric quantities of lysine, aspartic acid, serine, glutamic acid, glycine, alanine, valine and leucine. It evidently did not originate from the PN4 region of the molecule. The neutral, fluorescent peptides from fractions 2 & 3 was purified by chromatography in BAWP and at pH 3.5. It was also impure, but not from the PN4 region.

11.6. Tryptic peptides - summary.

Table 11.6.1. shows which peptides were found in each digest. The Sephadex fraction is the largest fraction in which the peptide was found, with the numbering of Figure 11.4.1.

Table 11.6.2. summarises the amino acid analyses and N-termini of the tryptic peptides, while Table 11.6.3. shows the mobilities on electrophoresis at pH 6.5 and 3.5 and the R_x on chromatography in BAWP. Most of this data was obtained from preparative separations, and so is not particularly accurate.

Table 11.6.1.

Purification of tryptic peptides.

- : Not present I : present but considerably impure. Blank : not examine

Purification methods in order:

6 : pH 6.5 electrophoresis. 3 : pH 3.5 electrophoresis.

9 : pH 9.5 electrophoresis. B : Chromatography in BAWP.

Peptide	Sephadex fraction	Digest 1	2	3	4	Peptide	Sephadex fraction	Digest 1	2	3	4
TB1	4	6B3	6B3	6B3		TN13	4	6B3	-	6B3	
TB2	4	6B3	6B39	6B3		TN14	2	-	-	6B3	
TB3	4	I	6B3	6B3		TN15	6	-	-	6B3	
TB4	4	6B3	6B3	6B3		TN16	4	-	-	-	63
TB5	4	-	6B3	6B3		TN17	4	6B3	-	6B3	
TB6	4	I	6B3	-		TA1	1	-	6B3	-	6[a]
TB7	4	6B3	6B3	-	6B3	TA2	2	63	6B3	6B3	63[a]
TB8	5	I	-	6B3		TA3	3	-	6B3	6B3	
TB9	4	6B3	-	-		TA5	2	63	6B3	6B3	
TB10	4	I	6B3	6B3		TA6	2	-	6B3	-	
TB11	4	6B3	-	6B3		TA7	4	I	6B3	6B3	
TN1	2	6B3	6B3	6B3		TA8	3	63	6B3	6B3	
TN3	2	6B3	6B3	6B3		TA9	3	63	6B3	6B3	6B3
TN4	3	6B3	6B3	6B3		TA10	2	63	6B3	-	
TN6	3	6B3	6B3	6B3	6B3	TA11	4	63	6B3	6B3	
TN7	4	6B3	6B3	6B3	6B3	TA12	3	63B6	6B3	6B3	
TN8	4	-	6B3	6B3	6B3	TA13	4	63B6	6B3	6B3	
TN9	3	6B3	6B3	6B3	6B3	TA14	5	63B6	6B3	6B3	
TN10	4	6B3	6B3	6B3	6B3	TA15	5	63B6	I	6B3	
TN11	5	6B3	6B3	6B3	6B3	TA16	4	636	6B3	6B3	
TN12	4	6B3	-	6B3		TA17	2	63	6B3	6B3	

[a]: Peptide TA1 was converted to TA2 by further tryptic digestion.

Table 11.6.2.

Amino acid analyses of tryptic peptides

Peptide	TB1	TB2	TB3	TB4	TB5	TB6	TB7	TB8	TB9	TB10	TB11
Lys	1.97	-	0.79	1.00	-	-	0.89	-	-	-	-
His	-	-	-	-	-	-	-	-	-	-	-
Arg	-	1.03	-	-	1.07	0.97	-	0.91	1.08	1.18	0.82
Asp	1.03	-	2.06	-	-	-	-	-	-	-	-
Mes	-	-	1.05	-	-	-	-	-	-	-	-
Thr	-	0.95	-	-	1.80	0.32	1.05	2.26	1.07	0.82	0.35
Ser	-	0.98	-	-	1.09	1.03	1.29	-	-	-	2.00
Glu	-	-	-	-	-	-	-	-	-	-	-
Pro	-	-	-	-	-	-	-	-	-	-	-
Gly	-	-	1.27	-	1.90	0.27	-	-	0.86	-	-
Ala	-	0.98	0.79	-	2.11	-	2.06	-	-	-	1.00
Val	-	1.05	-	1.97	-	-	-	-	-	-	1.06
Met	-	-	-	1.03	-	-	-	-	-	-	-
Ile	-	-	-	-	-	-	0.93	1.07	-	-	0.26
Leu	-	2.01	1.06	-	-	-	-	-	-	-	1.12
Tyr	-	-	-	-	1.03	-	-	0.66	-	-	-
Phe	-	-	-	-	-	-	1.77	-	-	-	-
Trp	-	-	-	-	-	-	-	-	-	-	-
N-t.	Asx	Ala	Ala	Val	Thr	Ser	Phe	Ile	Gly	Thr	Ala
Notes				a							
Section	11.7.	11.8.	11.9.	11.7.	11.10.	11.11.	11.12.	1113.	11.10.	11.13.	11.11.

[a]: 96 hr. hydrolysis.

Table 11.6.2. cont'd.

Peptide	TN1	TN3	TN4	TN6	TN7	TN8	TN9	TN10	TN11	TN12	TN13
Lys	1.79	0.90	0.91	0.88	-	0.13	0.95	1.08	-	-	0.95
His	-	c	-	-	-	-	-	-	-	-	-
Arg	-	-	-	-	0.89	1.73	-	-	1.05	1.13	-
Asp	0.16	1.06	-	3.33	1.89	0.98	2.00	0.95	-	2.03	-
Mes	-	1.02	-	-	-	-	1.15	-	-	-	-
Thr	-	1.96	1.01	-	1.90	0.90	-	-	-	2.03	-
Ser	0.97	-	-	0.82	-	-	-	-	-	-	0.97
Glu	2.01	-	1.13	0.93	0.17	1.23	-	-	0.90	-	1.12
Pro	0.97	-	-	0.21	-	1.22	-	-	-	-	1.06
Gly	2.05	1.05	-	-	1.07	-	1.05	-	-	0.93	-
Ala	-	-	2.10	2.26	1.09	0.99	0.93	0.97	-	0.87	-
Val	-	1.02	-	-	-	0.96	-	-	-	-	-
Met	-	-	-	-	-	-	-	-	-	-	-
Ile	1.04	-	1.00	1.12	1.02	-	-	-	-	-	-
Leu	1.17	0.99	0.86	2.00	1.14	0.15	0.94	-	1.05	1.01	0.88
Tyr	-	-	-	0.69	-	0.74	-	-	-	-	-
Phe	-	-	-	-	-	-	-	-	-	-	-
Trp	-	-	-	-	-	-	-	-	-	-	-
N-t.	None	c	Leu	Tyr	Asx	Thr	Ala	Asx	Glx	Ala	Leu
Notes	b					d					
Section	11.14.	11.15.	11.16.	11.17.	11.18.	11.19.	11.9.	11.7.	11.7.	11.20.	11.7.

[b]: Slight amounts of other N-termini.

[c]: See text.

[d]: Together with a little leucine.

Table 11.6.2. cont'd.

Peptide	TN14	TN15	TN16	TN17	TA1	TA2	TA3	TA5	TA6
Lys	2.64	-	0.22	0.17	0.85	-	1.01	0.94	1.91
His	-	-	-	-	-	-	-	-	-
Arg	-	-	0.91	1.89	2.00	0.99	-	1.04	-
Asp	2.03	-	2.12	0.97	5.18	2.88	-	1.96	2.00
Mes	0.92	-	-	-	-	-	-	-	1.01
Thr	1.04	1.02	1.90	-	4.03	2.95	-	0.95	0.80
Ser	0.29	0.34	0.50	0.20	1.38	1.12	0.99	-	-
Glu	1.31	0.26	0.47	1.12	7.12	4.91	2.16	2.20	1.07
Pro	-	-	-	1.00	2.90	2.03	0.86	0.91	-
Gly	-	0.25	2.02	0.17	1.77	1.13	2.14	1.01	-
Ala	1.10	-	1.17	-	0.92	1.07	-	-	1.13
Val	-	-	-	-	2.02	1.07	-	1.03	-
Met	-	-	-	-	-	-	-	-	-
Ile	-	0.98	1.00	-	0.84	-	0.91	0.97	-
Leu	-	-	1.88	-	1.00	1.00	0.94	-	-
Tyr	-	0.55	-	-	-	-	-	-	-
Phe	0.96	-	0.94	-	1.07	0.84	-	-	1.06
Trp	-	-	-	-	-	-	-	-	-
N-t.	Lys	Ile	Leu	Arg	Lys	Phe	none	Lys	Thr
Notes		b	c						
Section	11.21.	11.13.	11.20.	11.19.	11.22.	11.22.	11.14.	11.23.	11.21.

Table 11.6.2. (continued).

Peptide	TA7	TA8	TA9	TA10	TA11	TA12	TA13	TA14	TA15	TA16	TA17
Lys	0.86	0.94	1.03	-	0.95	-	-	0.98	0.99	0.82	1.64
His	-	-	-	-	-	-	-	-	-	-	-
Arg	-	-	-	f	-	1.08	0.90	-	-	-	-
Asp	1.15	3.95	2.27	2.03	1.01	1.07	2.13	2.13	2.08	0.99	1.12
Mes	1.00	-	-	-	-	-	-	-	-	-	-
Thr	-	0.99	0.17	0.98	-	-	-	-	-	-	-
Ser	-	-	-	-	-	0.94	0.77	-	-	-	1.16
Glu	0.94	1.06	1.20	2.22	1.10	0.86	2.04	-	-	3.10	2.28
Pro	-	1.08	1.08	1.01	-	-	-	-	-	-	1.04
Gly	0.10	0.12	1.61	0.96	-	-	-	-	-	-	0.15
Ala	0.11	-	1.74	-	1.90	2.14	-	-	0.93	0.93	1.74
Val	-	0.93	2.08	1.05	-	-	-	-	-	-	-
Met	-	-	-	-	-	-	-	-	-	-	-
Ile	1.00	1.03	0.14	0.71	-	-	1.03	-	-	-	-
Leu	2.04	1.05	-	-	1.03	1.90	1.15	-	-	0.92	2.12
Tyr	-	0.96	-	-	-	-	-	0.89	-	-	-
Phe	-	-	-	-	1.00	-	-	-	0.99	0.94	0.91
Trp [e]	+	-	+	-	-	-	-	-	-	-	-
N-t	Glx	Asx	Ala	Ile	Ala	Glx	Ser	Tyr	Asx	Leu	Ala

Notes

Section 11.24. 11.25. 11.26. 11.23. 11.27. 11.28. 11.29. 11.7. 11.21. 11.30. 11.27

[e]: Found by electrophoresis of hydrolysates, at pH 2.0.

[f]: Not calculable, but about 1 residue.

Table 11.6.3.

Chromatographic and electrophoretic properties of tryptic peptides.

	m (pH 6.5)	m' (pH 3.5)	R_x (BAWP)		m (pH 6.5)	m' (pH 3.5)	R_x (BAWP)
TB1	± 0.53	± 2.24	0.07	TN13	0	+ 1.52	0.39
TB2	+ 0.43	+ 1.41	0.97	TN14	0	+ 1.79	0.16
TB3	+ 0.35	+ 1.42	0.17	TN15	0	+ 0.62	1.09
TB4	+ 0.45	+ 1.82	0.43	TN16	stuck	+ 0.64	-
TB5	+ 0.30	+ 1.18	0.54	TN17	+ 0.03-0	+ 1.81	0.17
TB6	+ 0.54	+ 2.14	0.30	TA1	- 0.44	+ 0.57	0.24
TB7	+ 0.25	+ 1.21	0.97	TA2	- 0.53	+ 0.18	0.34
TB8	+ 0.40	+ 1.43	0.77	TA3	- 0.28	+ 0.84	0.58
TB9	+ 0.56	+ 2.14	0.35	TA5	- 0.17	+ 1.08	0.33
TB10	+ 0.48	+ 2.00	0.69	TA6	- 0.21	+ 1.10	0.21
TB11	+ 0.37	+ 1.40	0.59	TA7	- 0.24	+ 0.60	0.87
TN1	0	+ 0.92	0.62	TA8	- 0.41	+ 0.22	0.49
TN3	+ 0.10-0	+ 1.32	0.53	TA9	- 0.38	+ 0.57	0.34
TN4	0	+ 1.11	0.60	TA10	- 0.44	+ 0.63	0.34
TN6	0	+ 0.62	0.67	TA11	- 0.24	+ 1.00	0.73
TN7	0	+ 0.79	0.65	TA12	- 0.29	+ 0.63	0.22
TN8	0	+ 1.37	0.39	TA13	- 0.24	+ 0.69	0.54
TN9	0	+ 0.79	0.26	TA14	- 0.34	+ 0.81	0.28
TN10	0	+ 1.09	0.17	TA15	- 0.35	+ 0.28	0.28
TN11	0	+ 1.38	0.57	TA16	- 0.45	+ 0.72	0.46
TN12	0	+ 0.83	0.39	TA17	- 0.15	+ 1.07	0.58

11.7. Small tryptic peptides.

TB1. The mobility of this peptide shows that asparagine is absent. CPB released lysine and aspartic acid, and N-terminal lysine was found after one cycle of DNS-PTC degradation, so the sequence is

Asp-Lys-Lys.

TB4. CPB releases lysine. As well as DNS-Val, N-terminal determination gave a compound which behaved on electrophoresis and chromatography as DNS-Val-X. DNS-PTC degradation gave the sequence Val-Val-Mes- so the complete sequence is

Val-Val-Met-Lys.

TN10. The aspartic acid cannot be amidated since the peptide is neutral. CPB releases lysine, and alanine is found after one cycle of DNS-PTC degradation. The sequence is

Asp-Ala-Lys.

TN11. The glutamic acid cannot be amidated since the peptide is neutral. Leucine is found after one cycle of DNS-PTC degradation. The sequence must be

Glu-Leu-Arg.

TN13. The glutamic acid residue cannot be amidated since the peptide is neutral. The sequence of the peptide was determined by the DNS-PTC method as

Leu-Pro-Ser-Glu-Lys.

A/

A double peptide was also found with this and TA11 (Section 11.27.)

TA14. CPB released lysine from this peptide. The mobility shows that neither aspartic acid residue can be amidated. DNS-PTC degradation showed aspartic acid after one and two cycles. The sequence is

Tyr-Asp-Asp-Lys.

11.8. Peptide TB2.

CPB released arginine, as well as other amino acids, from this peptide. The results of DNS-PTC degradation are shown in Table 11.8.1.

Table 11.8.1.

DNS-PTC degradation of peptide TB2.

Peptide	F1	F2	F3	F4	F5	F6
N-terminus	Leu	Val	Thr	Ser	Leu	Arg
Substantial impurities.			Val			
Slight impurities.		Leu	Leu	Leu	Arg	Leu

Hence the sequence of the peptide is

Ala-Leu-Val-Thr-Ser-Leu-Arg.

11.9./

11.9. Peptides TB3 & TN9.

These two peptides have the same composition, although TB3 had a rather anomalous glycine/alanine ratio. On final purification at pH 3.5, TB3 showed a second component with the same mobility as TN9, and an identical composition from qualitative analysis. Both acid residues in TB3 must be asparagines, and the findings are consistent with TN9 arising through deamidation of one of them. CPB, alone, or mixed with CPA, failed to remove anything but lysine from either peptide. DNS-PTC degradation gave a partial sequence Ala-Leu-Asx-Mes- for TN9. Thus the evidence shows the partial sequence

Ala-Leu-Asx-Met-(Asx,Gly)-Lys.

The data on PB5 (Section 13.4.) and X3 (Section 10.5.) shows that the sequences are

TB3 : Ala-Leu-Asn-Met-Asn-Gly-Lys.

TN9 : Ala-Leu-Asn-Met-Asp-Gly-Lys.

11.10. Peptides TB5 & TB9.

TB5 was cleaved with chymotrypsin and two fragments were formed and separated by electrophoresis at pH 6.5. The properties of the fragments are shown in Table 11.10.1.

Table 11.10.1.

Chymotryptic degradation of peptide TB5.

Peptide	m	N-t.	Arg	Thr	Ser	Gly	Ala	Tyr
TB5CB1	±0.62	Gly	(1)[a]	0.94	-	1.06	-	-
TB5CN1	0	Thr	-	0.87	1.06	1.12	2.06	0.91

[a]: Arginine detected by qualitative analysis.

DNS-PTC degradation showed Gly-Thr-Arg for TB5CB1, while TB5CN1 showed a partial sequence Thr-Gly-Ala-Ala-. Direct DNS-PTC degradation of TB5 showed that the N-terminus of TB5F4 was serine. Assuming to some extent that tyrosine is C-terminal in TB5CN1, the sequence of TB5 is

Thr-Gly-Ala-Ala-Ser-Tyr-Gly-Thr-Arg.

TB9 is identical to TB5CB1 in analysis and mobility. It was only found in the first tryptic digest, and presumably originated through chymotrypsin-like cleavage at tyrosine.

11.11. Peptides TB6 & TB11.

These peptides have been isolated irregularly, impure, and in low yield. The DNS-PTC method showed that TB11 has the sequence

Ala-Val-Leu-Ser-Ser-Arg.

This is the only region of the molecule with which TB6, apparently Ser-Arg, can be reconciled. There is no other evidence, and/

and cleavage here has not been found in any other digests. The origin of TB6 cannot be regarded as certain.

11.12. Peptide TB7.

The yield of this peptide has been inexplicably low, and the DNS-PTC method only showed that the second residue is alanine. Lysine was released by CPB. So the peptide may be formulated

Phe-Ala-(Thr,Ser,Ala,Ile,Phe)-Lys.

The full sequence can be deduced from peptic peptides PB8 (Section 13.10.) and PN1 (Section 13.13.) and is

Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys.

11.13. Peptides TB8, TB10 & TN15.

Peptide TB8 reacted weakly with ninhydrin, but was easily detected by chlorination or with 1-nitroso-2-naphthol. The DNS-PTC method showed the sequence

.Ile-Thr-Tyr-Thr-Arg.

TB10 was apparently Thr-Arg, and a principal component of TN15 was Ile-(Thr,Tyr). These peptides presumably arose through chymotrypsin-like cleavage at the tyrosine residue in TB8.

11.14. Peptides TN1 & TA3.

These two peptides differ in analysis only by a lysine residue. A basic peptide of similar qualitative composition was also/

also observed, but in insufficient quantity for amino acid analysis. This had N-terminal glutamic acid/glutamine. Degradation of a chymotryptic peptide covering the whole region of sequence gave rise to a similar peptide (CA5TB4, Section 12.17.). The basic peptide showed in the course of final purification another peptide, with the same mobility as TN1, but no demonstrable N-terminus. The electrophoretic mobilities of TN1 and TA3 were those expected for peptides lacking glutamine.

These observations suggest that what is happening is the cyclisation of N-terminal glutamine to pyrrolidone carboxylic acid (Glp). This has been frequently reported in similar circumstances (e.g. Smyth, Stein & Moore, 1962). The chemistry of the modified form has been examined (Dekker, Stone & Fruton, 1949) and its occurrence reviewed (Blombäck, 1967). The observations suggest that tryptic digestion gives rise to Gln-.....-Lys-Lys and Gln-.....-Lys, which become converted to Glp-.....-Lys-Lys (TN1) and Glp-.....-Lys (TA3).

The N-terminal pyrrolidone carboxylic acid has hampered sequence investigation. Table 11.14.1. shows the fragments found after degradation with thermolysin and electrophoresis at pH 6.5.

Table 11.14.1.

Products of digestion of TN1 with thermolysin.

Peptide.	m_1	N-t.	Detection	Lys	Ser	Glu	Pro	Gly	Ile	Leu
TN1HB1	+0.96	Leu	Ninhydrin	1.88	-	-	-	-	-	1.1
TN1HA1	-0.71	None	Chlorine	-	0.99	2.06	0.98	2.01	0.96	-

This showed that the C-terminal sequence of TN1 was -Leu-Lys-Lys. TN1 was also degraded with subtilisin B. The digest was separated by electrophoresis at pH 6.5, and a lot of free amino acid was observed. The only peptide isolated had a slight acidic mobility (-0.05), N-terminal serine and an analysis Ser, 0.98; Leu, 1.02. DNS-Leu was found after one cycle of DNS-PTC degradation. Thus the direct evidence for the sequence gives

Gln-(Glu,Pro,Gly_2,Ile)-Ser-Leu-Lys-Lys.

The sequence was proved by DNS-PTC degradation of CA5TB4 (Section 12.17.) and PA1 (Section 13.17.) and was (for unpyrrolidonised TN1)

Gln-Ile-Gly-Gly-Pro(-Glu)-Ser-Leu-Lys-Lys.

11.15. Peptide TN3.

This peptide shows no histidine on the analyser, and the short column trace is otherwise normal. On qualitative analysis at pH 2.0, instead of a spot in the normal histidine position, ninhydrin with collidine/

collidine shows a yellow spot slightly in front of glycine. On N-terminus determination there is a faint, smeary spot running slightly slower than DNS-histidine both at pH 4.38 and at pH 9.5.

This suppression of the α-amino group apparently occurred after the peptide was formed. It could still be detected after digestion of protein with trypsin (Table 11.4.1.), and peptide CA7TA2 (Section 12.18.) shows it can still be found, albeit with bad stoichiometry, after purification at pH 6.5. The analysis of the region is such that there is no doubt that CA7TA2 originates from the TN3 region. These findings suggest the N-terminal histidine residue is subject to some chemical modification in the course of purification. Schroeder (1967) has reported anomalies with N-terminal histidine reached in the course of Edman degradations.

The peptide released lysine and leucine with CPB, and addition of CPA also released threonine. The sequence of the peptide was examined by papain digestion. The fragments were separated by electrophoresis at pH 6.5 and pH 3.5. The results are shown in Table 11.15.1. Fragment TN3MA3 was detected by chlorination.

Table 11.15.1.

Qualitative analysis of products of digestions of TN3 with papain.

Peptide	m	m'	N-t.	Lys	Val	Leu	Thr	Gly	Mes	Asp	"Yellow spot"
TN3MB1	+0.66	+2.25	Leu	++	-	++	-	-	-	-	-
TN3MB2	+0.54	+1.98	Thr	+++	-	+++	++	-	-	-	-
TN3MB3	+0.37	+1.73	Mes	+++	-	+++	++	-	++	-	-
TN3MA1	-0.13	+0.87	Mes	-	-	+++	++	-	++	-	-
TN3MA2	-0.13	+0.73	Mes	-	-	-	++	-	++	-	-
TN3MA3	-0.48	+1.36	none	-	+++	-	-	-	-	+++	++
TN3MN1	0	+0.80	Thr	-	-	-	++	++	-	-	-

The acidic mobilities of those peptides with methionine sulphone N-termini and no lysine are due to the weakening of the α-amino group at the N-terminus (Ambler & Brown, 1967). TN3MA3 was unreactive with ninhydrin. Its histidine residue behaved like that of TN3 on N-terminal determination. Some fragments were examined by the DNS-PTC method; TN3MN1 gave Thr-Gly, TN3MA1 Mes-Thr-Leu and TN3MA2 Mes-Thr. The papain digest also contained free lysine. These data may be expressed as a partial sequence

(His,Val,Asp)-Thr-Gly-Mes-Thr-Leu-Lys

The results obtained with CA7TA2 (Section 12.18.) show the complete sequence is

His-Val-Asp-Thr-Gly-Met-Thr-Leu-Lys.

11.16./

11.16. Peptide TN4.

The glutamic acid residue cannot be amidated since the peptide is neutral. The products of digestions with thermolysin were separated by electrophoresis at pH 6.5, and are summarised in Table 11.16.1.

Table 11.16.1.

Products of thermolysin digestion of peptide TN4.

Peptide	m	N-t.	Lys	Thr	Glu	Ala	Ile	Leu	Ser	Gly
TN4HB1	+0.64	Ala	(1)[a]	1.03	-	0.97	-	-	-	-
TN4HA1	-0.39	Leu	-	-	1.04	1.08	0.92	0.96	-	-
TN4HA2	-0.48	Ile	-	-	0.98	1.00	1.02	-	0.24	0.29

[a]: Detected by qualitative analysis.

The results with DNS-PTC degradation were, TN4HA1, Leu-Ile-Ala-Glu and TN4HA2, Ile-Ala-Glu. TN4HB1F1 was lost, but TN4HB1F2 showed lysine. Hence the sequence of TN4 is

Leu-Ile-Ala-Glu-Ala-Thr-Lys.

11.17. Peptide TN6.

The mobility of this peptide shows that three of the four acid residues must be amidated. The peptide was degraded with chymotrypsin and the fragments were separated at pH 6.5, the neutral band being sewn to another sheet of paper for separation at pH 3.5. The/

The results are shown in Table 11.17.1.

Table 11.17.1.

Products of chymotryptic degradation of peptide TN6

Peptide	m	m'	N-t.	Lys	Asp	Ser	Glu	Gly	Ala	Ile	Leu	Tyr
TN6CB1	+0.54		Ile	(1)[a]	-	-	-	-	-	1.04	0.96	-
TN6CB2	+0.47		Leu	(1)[a]	-	0.3	0.2	0.2	0.2	1.07	1.93	-
TN6CN1	ca.0	+0.87	Asx	-	1.02	0.16	-	-	0.21	-	0.98	-
TN6CA1	-0.27		Tyr	-	3.02	0.95	1.07	-	2.03	0.24	1.19	0.75
TN6CA2	-0.32		Tyr	-	2.15	0.91	1.02	-	1.92	0.15	0.27	0.66

[a]: Lysine found on qualitative analysis.

TN6CB1F1 had leucine at its N-terminus. The two acidic peptides were pooled and subjected to DNS-PTC degradation, giving a partial sequence Tyr-Ser-Asx-Asx-; TN6CA1(&2)F3 had m -0.08, so an asparagine residue has been removed. The partial sequence is therefore

Tyr-Ser-Asp-Asn-(Ala_2,Gln)-Asn-Leu-Ile-Leu-Lys.

The sequence was completed by examination of chymotryptic peptides (Section 12.13.) and is

Tyr-Ser-Asp-Asn-Ala-Ala-Gln-Asn-Leu-Ile-Leu-Lys.

11.18./

11.18. Peptides TN7.

The mobility of this peptide shows that one residue of asparagine must be present. Peptide TN7F1 was detected by chlorination, and was neutral, Hence the N-terminal residue is asparagine. The results of DNS-PTC degradation are shown in Table 11.18.1. CPA and CPB together released arginine, leucine and isoleucine.

Table 11.18.1.

DNS-PTC degradation of peptide TN7.

Peptide	F1	F2	F3	F4	F5
N-terminus	Thr	Thr	Gly	Asx	Ala
Substantial impurities		Gly			
Slight impurities			Asx		

A small amount of TN7 was digested with chymotrypsin and a basic peptide (m +0.68) was isolated by electrophoresis at pH 6.5. Qualitative analysis showed arginine and leucine/isoleucine. The N-terminal determination showed DNS-Ile and DNS-Ile-Arg (Section 3.15.). Thus the sequence of TN7 is

Asn-Thr-Thr-Gly-Asp-Ala-Leu-Ile-Arg.

11.19./

11.19. Peptides TN8 & TN17.

This peptide has not been entirely purified from peptide TN13. The mobility shows that neither acid residue is amidated. CPB released arginine, together with traces of lysine, and the residual peptide had m -0.21, showing that only one of the arginine residues has been removed. The results of DNS-PTC degradation are shown in Table 11.19.1.

Table 11.19.1.

Results of DNS-PTC degradation of peptide TN8.

Peptide	F1	F2	F3	F4	F5	F6	F7	F8
N-terminus	Val	Ala	Tyr	Arg	Pro[a]	Asx	Glx	Arg
Substantial impurities	Thr				Arg		Asx	
Slight impurities	Ala				Asx			Glx

[a]: 18 hr. hydrolysis, so relatively low recovery.

The sequence is: Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg.

The amino acid analysis of TN17 shows that it originates from the C-terminus of TN8. It is presumably Arg-Pro-Asp-Glu-Arg arising from chymotrypsin-like cleavage at the tyrosine residue in TN8.

11.20. Peptides TN12 & TN16.

Peptide/

Peptide TN12 was isolated in low yield from two digests. Since it is neutral, one of the acid residues must be asparagine. The material was used for sequence determination by the DNS-PTC method, giving the result Ala-Leu-Asx-Thr-Gly- . This is the same sequence as the N-terminal part of peptide CA10 (Section 12.9.), so the sequence of TN12 is probably

Ala-Leu-Asp-Thr-Gly-Thr-Asn-Arg.

Peptide TN16 was found under conditions of gentle digestion. Amino acid analysis showed a peptide Arg, Asp_2, Thr_2, Gly_2, Ala, Ile, Leu, Phe, together with half a residue each of serine and glutamic acid. Repurification failed to give any detectable material after electrophoresis at pH 6.5. The N-terminus was leucine. The peptide is most easily explained as formed of the fragment Leu-Gly-Ile-Phe (Table 16.1.1.) attached to the N-terminus of TN12.

11.21. Peptides TN14, TA6 & TA15.

Neither acid residue in TA15 can be amidated. CPB released alanine and lysine. The DNS-PTC method showed that the sequence was

Asp-Asp-Phe-Ala-Lys.

Peptide TA6 was found in a digest of trypsin-released enzyme. With CPB it behaved as did TA15, while addition of CPA led to the release of phenylalanine also. Digestion of a small portion with trypsin/

trypsin gave a neutral peptide together with one with the mobility of TA15. The mobility shows that the glutamic acid residue cannot be amidated. The DNS-PTC method gave the partial sequence Thr-Glu-Mes-Lys- . Thus the sequence of TA6 is

Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys.

TN14, slightly impure, was found in a digest of exo-enzyme, in which TA6 was not found. A small portion was digested overnight with trypsin. After electrophoresis at pH 6.5, in addition to the fragments found from TA6, free lysine and a basic peptide (m +0.46) were seen, in a position compatible with Lys-Thr-Glu-Met-Lys. The DNS-PTC method gave Lys-Thr-Glu-Mes- . The sequence of TN14 is therefore

Lys-Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys.

Peptides corresponding to the first four or five residues respectively were not found.

11.22. Peptides TA1 & TA2.

Peptide TA2 was a very large acid peptide found in high yield. CPB alone released arginine and alanine, while addition of CPA also led to the release of threonine and serine. TA2F1 had N-terminal glutamic acid/glutamine. The total charge at pH 6.5 could not be accurately found from the data of Offord (1966) but was about four.

A peptic digest was performed and the properties of the fragments are in Table 11.22.1. The peptides were not completely pure/

Table 11.22.1.

Products of pepsin (P), thermolysin (H) and papain (M) digests of peptide TA2

Peptide	N-t.	' m	'm'	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Leu	Phe
TA2PB1	Thr	+0.44	-	(1)[a]	-	1.83	1.14	-	-	-	1.03	-	-	-
TA2PA1	Asx	-0.40	-	-	2.90	3.02	1.10	3.15	0.99	0.96	0.94	0.94	-	-
TA2PA2[b]	Phe	-0.63	-	-	0.45	0.27	-	2.33	0.85	-	-	-	0.90	0.92
TA2HB1	Ala	+0.73	-	0.96	-	-	-	-	-	-	1.04	-	-	-
TA2HA1	Leu	-0.54	+0.43	-	1.00	-	-	1.10	-	-	-	-	0.90	-
TA2HA2	Val	-0.54	+0.07	-	2.24	3.05	1.09	2.62	0.96	1.05	-	0.97	-	-
TA2HA3	Phe	-0.76	+0.33	-	-	-	-	2.13	1.01	-	-	-	-	0.86
TA2NB1	Thr	+0.54	+2.20	1.10	-	0.91	0.30	-	-	-	0.98	-	-	-
TA2MN1	Leu	0	+0.60	-	1.02	0.18	0.26	-	-	-	-	-	0.98	-
TA2MA1	Glx	-0.46	+0.43	-	0.99	-	-	1.02	1.12	0.98	-	0.90	-	-
TA2MA2	Glx	-0.57	+0.36	-	0.15	0.94	0.20	2.06	-	-	-	-	-	-
TA2MA3	Asx	-0.64	-0.37	-	0.95	0.91	1.14	-	-	0.18	-	-	-	-
TA2MA4	Glx	-0.75	-0.07	-	1.01	1.84	1.05	2.10	-	-	-	-	-	-
TA2MA5	Phe	-0.75	+0.37	-	-	-	-	2.08	1.08	-	-	-	-	0.84

The papain digest also contained free arginine.

[a]: Detected by qualitative analysis.

[b]: It was evident from the analysis that the peptide contained 0.09 moles/mole of TA2.

pure after electrophoresis at pH 6.5 but DNS-PTC degradation was performed on some. A thermolysin digest was then done, and the fragments are also shown in the Table. Subtilisin B. and papain digests were tried to split the region of the molecule toward the C-terminus inaccessible in the other digests, but the papain peptides were sufficient to finish the sequence, so they only are shown in the Table.

Peptide TA2PA2 comes from the N-terminus, and neither glutamic acid residue can be amidated. It gave the sequence Phe-Glu-Pro-Glu-Leu by the DNS-PTC method. Together with TA2PA1 the entire composition of TA2 is accounted for, so the N-terminus of TA2PA1 must follow TA2PA2. Table 11.22.2. shows the result of DNS-PTC degradation of TA2PA1. The composition and N-terminus of TA2PB1, together with the CPA & CPB experiments on TA2 show that the C-terminal sequence is -Thr-(Thr,Ser)-Ala-Arg.

Table 11.22.2.

DNS-PTC degradation of peptide TA2PA1.

Peptide	(F0)	F1	F2	F3	F4	F5	F6
N-terminus	Asx	Glx	Val	Asx	Pro	Gly	Glx
Slight impurities					Asx		
m	-0.40	-0.43	-0.22				

The mobility of MA1 shows that one of the two acid residues in it is amidated. Since the N-terminal glutamic acid is free, from the mobility studies on PA1, the aspartic acid residue must be asparaginyl. The neutrality of MN1 confirms that the N-terminal residue of PA1 must be asparagine.

The composition of MB1 shows that the serine residue must be located in a -Thr-Ser-Thr- sequence, and the composition and mobility of MA3 shows that this must be preceded by free aspartic acid. The remainder of the sequence can then be derived from MA2. After one cycle of DNS-PTC degradation this showed a neutral peptide with N-terminal threonine, and after two cycles DNS-Glu was observed, The material was insufficient to allow direct demonstration of free-glutamine at this point. Peptide MA4 is evidently MA2 + MA3 ; a partial sequence by DNS-PTC degradation showed Glx-Thr-Glx-Asx-.

Other peptides also gave confirmatory partial or complete sequences/

sequences by this method:

MA3 : Asx-Thr

MN1 : Leu-Asx, and free asparagine found by electrophoresis after one cycle.

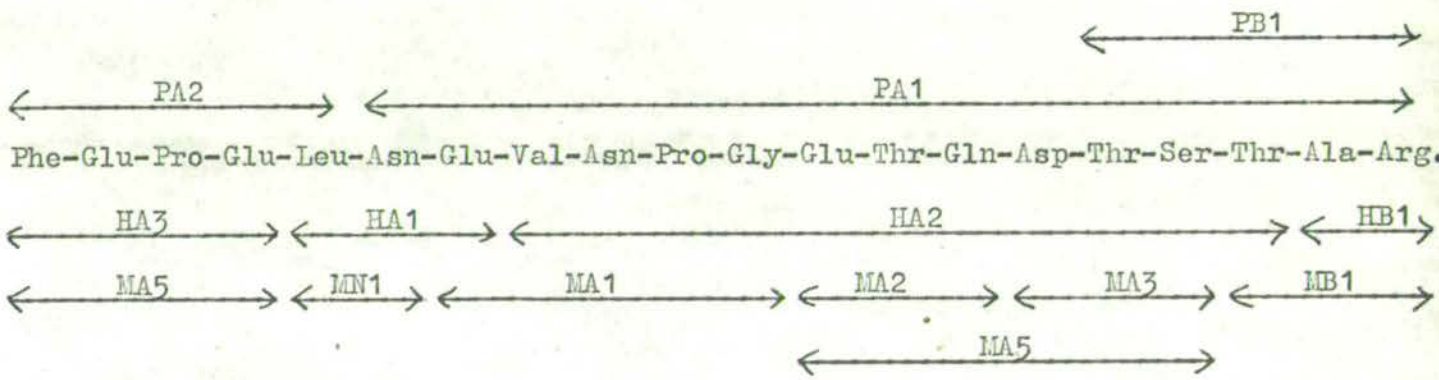
MB1 : Thr-Ala-Arg.

HA1 : Leu-Asx-Glx.

The complete sequence is shown in Figure 11.22.1., together with the peptides.

Figure 11.22.1.

Peptide TA2: the sequence.



Peptide TA1 corresponds in composition to the sum of TA2 & TA5, and the lysyl N-terminus suggests the structure TA5-TA2. This was confirmed by finding two peptides after further tryptic digestion with mobilities at pH 6.5 agreeing with those expected.

11.23./

11.23. Peptides TA5 & TA10.

These peptides differ only in a lysine residue. Peptide TA10, apparently lacking an N-terminal lysine, was found in lower yield. The mobilities of the peptides suggest that one of the acid residues is amidated. Direct DNS-PTC degradation of TA5 gave Lys-Ile-Gly-Asx-Glx-Val- . TA5 was degraded with thermolysin, and the fragments were separated by electrophoresis at pH 6.5 and pH 3.5.

Table 11.23.1.

Products of thermolysin digestion of peptide TA5.

Peptide	m	m'	N-t.	Lys	Arg	Asp	Thr	Glu	Pro	Gly	Val	Ile
TA5HN1	0	+1.30	Val	-	(1)[a]	1.07	0.92	1.16	1.00	-	0.84	-
TA5HA1	-0.38		Lys	(1)[a]	-	1.09	-	1.11	-	0.94	-	0.87

[a]: Detected by qualitative analysis.

It is evident from the mobilities that the amide is located in the neutral fragment. The DNS-PTC method showed that the sequence of TA5HA1 was Lys-Ile-Gly-Asp-Glu. The residue after four cycles was shown to be free glutamic acid by electrophoresis. The results of similar investigations of TA5HN1 are shown in Table 11.23.2.

Table 11.23.2.

Results of DNS-PTC degradation of peptide TA5HN1.

Peptide	F1	F2	F3	F4	F5
N-terminus	Thr	Asx	Pro	Glx	Arg
Slight impurities	Asx		Asx		Glx
m			+0.04[a]		

[a]: Detected with the isatin reagent.

Thus the sequence of TA5HN1 is Val-Thr-Asn-Pro-Glu-Arg. The sequence of TA5 is therefore

Lys-Ile-Gly-Asp-Glu-Val-Thr-Asn-Pro-Glu-Arg.

In gentle tryptic digests this peptide was found in a double peptide TA1 (Section 11.22.).

11.24. Peptide TA7.

There is no direct evidence that only one tryptophan residue is present in this peptide; however tryptophan has been found in three distinct regions of the molecule and analysis shows three residues of tryptophan. The mobility of the peptide shows the absence of amides. CPB released lysine, while addition of CPA also released methionine sulphone. The DNS-PTC method showed the apparent sequence Glu-Leu-Leu-Ile-Asp-Asp-Mes- , where the second/

second aspartic acid was very weak. The findings with peptide CA8TA3 (Section 12.16.) show that the second appearance of aspartic acid is where the tryptophan residue is. The sequence of the peptide is

Glu-Leu-Leu-Ile-Asp-Trp-Met-Lys.

11.25. Peptide TA8.

CPB released lysine from this peptide. The peptide was degraded with chymotrypsin and the fragments were separated at pH 6.5; the details are shown in Table 11.25.1. The mobility of the whole peptide shows that two amide residues are present, and one fell in each chymotryptic peptide.

Table 11.25.1.

Chymotryptic fragments from peptide TA8.

Peptide	m	N-t.	Lys	Asp	Thr	Glu	Pro	Val	Ile	Leu	Tyr
TA8CN1	0	Asx	(1)[a]	1.01	0.99	1.03	0.97	-	1.00	-	-
TA8CA1	-0.63	Asx	-	3.06	-	-	-	1.23	-	0.97	0.83

[a]: Detected by qualitative analysis.

The lysine residue in TA8CN1 made it impossible to determine the position of the amide by mobility determination after DNS-PTC degradation./

degradation. Instead of removing the lysine with CPB (Ambler, 1967b) the peptide was degraded with thermolysin giving two neutral peptides (Table 11.25.2.).

Table 11.25.2.

Thermolytic fragments of peptide TA8CN1.

Peptide	m	m'	N-t.	Lys	Asp	Thr	Glu	Pro	Ile
TA8CN1HN1	approx. 0	+1.48	Ile	0.98	-	0.92	1.12	-	0.99
TA8CN1HN2	approx. 0	+0.56	Asx	-	1.04	-	-	0.96	-

The first fragment showed Ile-Thr-Glu-Lys by DNS-PTC degradation, while the second after one cycle showed DNS-Pro (6 hr. hydrolysis) confirmed as free proline after electrophoresis at pH 2.0 by staining with isatin after ninhydrin and collidine. Hence TA8CN1 is Asn-Pro-Ile-Thr-Glu-Lys. The results of DNS-PTC degradation of TA8CA1 are shown in Table 11.25.3.

Table 11.25.3.

Results of DNS-PTC degradation of peptide TA8CA1.

Peptide	(F0)	F1	F2	F3	F4	F5
N-terminus	Asx	Asx	Leu	Val	Asx	Tyr
Slight impurities				Asx		
m		-0.63	-0.36	0		

Thus TA8CA1 is Asp-Asp-Leu-Val-Asn-Tyr, and TA8 is

Asp-Asp-Leu-Val-Asn-Tyr-Asn-Pro-Ile-Thr-Glu-Lys.

11.26. Peptide TA9.

The DNS-PTC method gave a partial sequence Ala-Gly-Val-Pro-Asp-Gly- . ' The mobility of the peptide rules out the presence of amides. The proline was at first considered to be alanine, but the findings with CPA & CPB and partial acid hydrolysis, as well as the analysis of PA15 make this untenable. Experiment with DNS-Pro that it was not easily distinguishable from DNS-Ala on electrophoresis at pH 2.0, and does not extract completely into the white spirit. Unfortunately there was no material available to repeat the degradation, but the experiments on PA15 (Section 13.18.) leave no doubt that this residue is proline. In agreement with this leucine amino peptidase (0.2 M ammonium acetate, pH 8.5, 2 mM Mg^{++}, 37°, overnight) removed only glycine and alanine.

CPB released lysine or lysine, alanine and aspartic acid under different conditions. Similarly, CPA + CPB released lysine and aspartic acid, together with alanine or alanine and valine, under different conditions. The peptide was resistant to chymotrypsin, and pepsin, Pronase and subtilisin B did not seem to give useful results in trial experiments.

TA9 was treated by partial acid hydrolysis (6 M-HCl, 100°, 15 min.) and the peptides were separated by electrophoresis at pH 6.5 and pH 3.5. The pure peptides observed were Ala-Gly, and a peptide containing valine (N-terminal), alanine, lysine and aspartic/

aspartic acid.

These data show the C-terminal sequence -Val-Ala-Asp-Lys. Combined with the data on peptide PA15 (Section 13.18.) the sequence of TA9 is

Ala-Gly-Val-Pro-Asp-Gly-Trp-Glu-Val-Ala-Asp-Lys.

11.27. Peptides TA11 & TA17.

The mobility of TA11 shows it has no amides. CPB released lysine. The results of DNS-PTC degradation are shown in Table 11.27.1.

Table 11.27.1.

Results of DNS-PTC degradation of peptide TA11.

Peptide	F1	F2	F3	F4	F5	F6
N-terminus	Phe	Ala	Leu	Glx	Asx	Lys
Slight impurities		Phe	Ala	Leu	Glx	Asx

Hence the sequence of TA11 is

Ala-Phe-Ala-Leu-Glu-Asp-Lys.

The composition of TA17 is the sum of TN13 and TA11. CPA and CPB released lysine and glutamic acid. It has N-terminal alanine. Therefore its structure is

TA11 - TN13.

11.28./

11.28. Peptide TA12.

The mobility of the peptide shows that amides are absent. CPB released arginine and leucine. DNS-PTC degradation gave the partial sequence Glu-Leu-Ala-Asp-Ala-Ser- so the sequence of TA12 is

Glu-Leu-Ala-Asp-Ala-Ser-Leu-Arg.

11.29. Peptide TA13.

The mobility of the peptide shows the presence of two amide residues. CPB released arginine, some asparagine and leucine, and possibly glutamine. The results of DNS-PTC degradation are shown in Table 11.29.1.

Table 11.29.1.

Results of DNS-PTC degradation of peptide TA13.

Peptide	F1	F2	F3	F4	F5
N-terminus	Ile	Glx[b]	Asx	Leu	Asx
Substantial impurities				Asx	Leu
Slight impurities	Mes[a]		Glx		

[a]: This spot is probably DNS-Ile-Glu, which runs in the same position as DNS-Mes at pH 4.38 (Gray, 1967a).

[b]: Very weak.

Preliminary/

Preliminary experiments showed that the peptide did not degrade well with chymotrypsin, so the fragments from thermolysin degradation were separated by electrophoresis at pH 6.5. The results are shown in Table 11.29.2.

Table 11.29.2.

Results of thermolysin digestion of peptide TA13.

Peptide	m	N-t.	Arg	Asp	Ser	Glu	Ile	Leu
TA13HB1	+0.44	Leu	0.99	0.99	-	1.08	-	0.94
TA13HA1	-0.71	Ser	-	1.04	0.82	1.21	0.94	-

Thus the two acid residues in TA13HA1 are free, while the two in the basic peptide are amides. The two peptides gave sequences Leu-Asx-Glx-Arg and Ser-Ile-Glx-Asx by the DNS-PTC method, so the sequence of TA13 is

Ser-Ile-Glu-Asp-Leu-Asn-Gln-Arg.

11.30. Peptide TA16.

CPB released lysine, while CPA & CPB also released aspartic acid, alanine and phenylalanine. The mobility of the peptide shows that one amide residue is present. The result of DNS-PTC degradation of TA16 is shown in Table 11.30.1.

Table 11.30.1.

Results of DNS-PTC degradation of peptide TA16.

Peptide	F1	F2	F3	F4
N-terminus	Glx	Glx	Glx	Phe
Substantial impurities.				Glx
Minor impurities.			Phe	

The peptide was degraded with chymotrypsin, and the fragments were separated at pH 6.5. The results are shown in Table 11.30.2.

Table 11.30.2.

Results of chymotryptic degradation of peptide TA16.

Peptide	m	N-t.	Lys	Asp	Glu	Ala	Leu	Phe
TA16CN1	0	Asx	(1)[a]	1.01	-	0.99	-	-
TA16CA1	-0.70	Leu	-	-	3.06	-	0.94	1.00

[a]: Detected by qualitative analysis.

TA16CN1 showed alanine after one cycle of DNS-PTC degradation. Hence the partial sequence of TA16 is

Leu-Glx-Glx-Glx-Phe-Asp-Ala-Lys.

The position of the glutamine was determined with peptide PA16 (Section 13.11.). The complete sequence of TA16 is

Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys.

CHAPTER 12

CHYMOTRYPTIC DIGESTION

12.1. Introduction.

The first digest was done in a way generally similar to that used for tryptic digests (Section 12.2.). Difficulty was found in separating the large neutral peptides and the oxidised state of tryptophan was a disadvantage.

For the second digest, 4 M-urea was used to denature, and peptides were separated on columns of sulphoethyl-Sephadex. The digestion was for a much shorter period, giving larger peptides (Section 12.3.).

12.2. Digest 1.

150 mg. trypsin-released enzyme (PP8) was oxidised (Section 3.5.) and freeze-dried. The material was dissolved in 10 ml. 2 M-ammonia, diluted to 100 ml. and adjusted to pH 8.5 with acetic acid. 3.6 mg. SBTI-chymotrypsin was added, and the mixture was incubated at 37° for 11 hr., after which it was acidified and freeze-dried twice, to remove a certain amount of salt. The digest was dissolved in 1.25 ml. 5% formic acid and a precipitate was centrifuged off.

The/

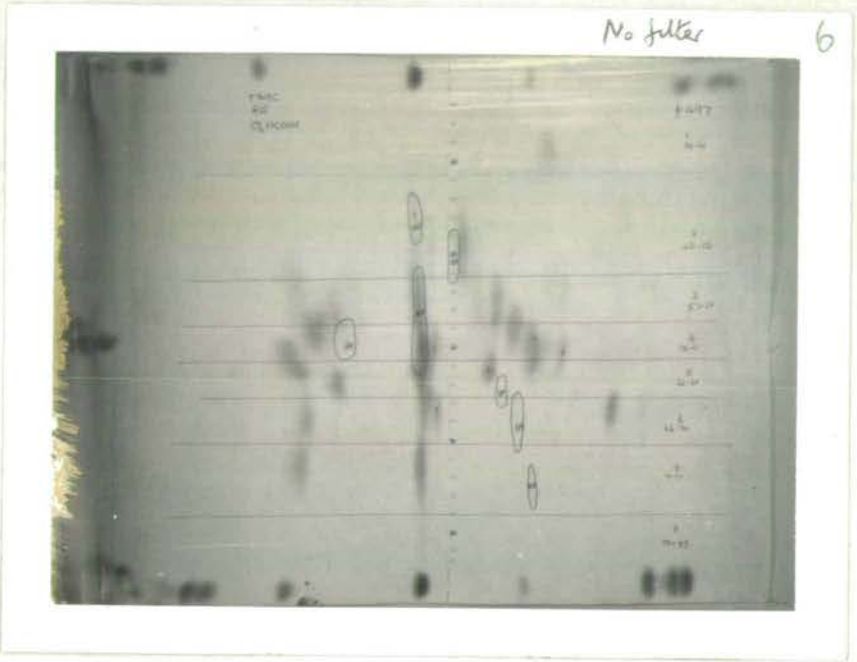


Figure 12.2.1.

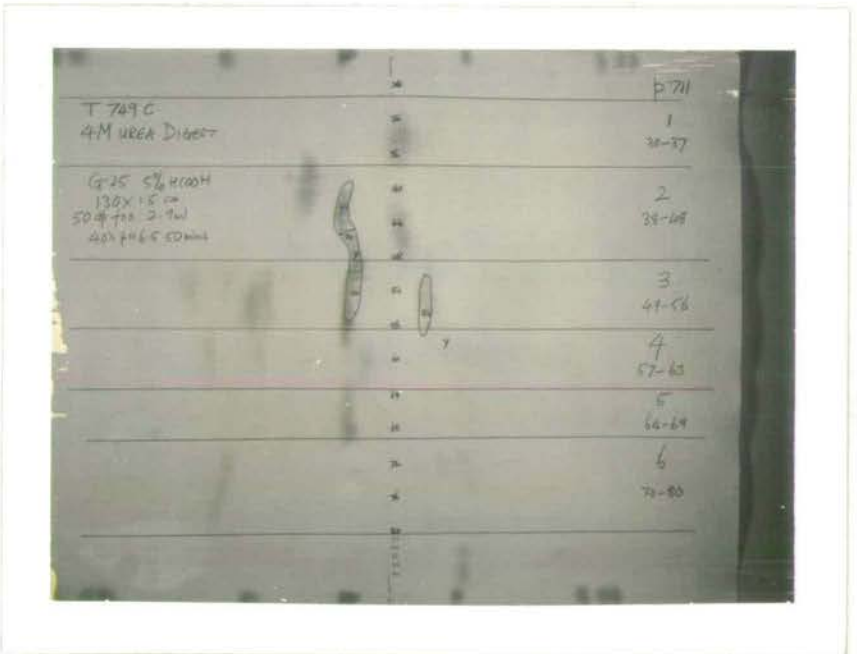


Figure 12.3.1.

Electrophoresis at pH 6.5 of chymotryptic peptides separated on Sephadex G25.

The solution was applied to a column of Sephadex G25 in 5% formic acid (112 cm. x 1.0 cm. diam.) and the column was pumped with the same solvent. 5 min. fractions (1.1 ml.) were collected and 0.02 ml. portions of each were separated by electrophoresis at pH 6.5 as described in Section 11.4. The result is shown in Figure 12.2.1., in which fractions were pooled and freeze-dried as indicated. The fractions were each dissolved in 0.5 ml. 5% formic acid, and amino acid analysis showed a recovery of about 80% of the total amino acids. Each fraction was separated on 25 cm. 3MM paper at pH 6.5. The peptides were purified by chromatography in BAWP and electrophoresis at pH 3.5 as detailed in Table 12.4.1. The properties of the peptides are in the two following Tables.

One particular peptide could not be obtained pure. It had an analysis with a lot of proline, valine and isoleucine, and released valine and leucine with CPA. It was not possible to find an N-terminus. It is possible the peptide originated from the same region of the protein as PN4.

The 5% formic acid insoluble fraction was dissolved in ethanol: conc. formic acid (equal volumes). It was separated on a column of Sephadex LH20 eluted with the same solvent at 25 ml./hr. Fractions of about 2 ml. were collected, and peptide was detected by developing with ninhydrin 0.02 ml. of each fraction spotted on 3MM paper, and by qualitative analysis of fractions for amino acids. Two groups of fractions were selected with similar compositions, pooled/

pooled and analysed quantitatively. No particular stoichiometry was evident in the results. Both fractions had principally alanine N-terminal. Both fractions were examined by electrophoresis after digestion with trypsin. There were many, faint, components and the neutral band was particularly heterogeneous. It was concluded that neither fraction represented any particular part of the molecule.

12.3. Digest 2.

Preliminary experiments on digestion in urea solutions are in Section 8.8. 11 micromoles of PP14 (trypsin-released enzyme) was dissolved in 3 ml. 8 M-urea. 6 mg. SBTI-chymotrypsin in 3 ml. 0.4 M-ammonium acetate, pH 8.5 was added, and the mixture incubated at 37° for 2 hr. The solution was made 5% (w/v) in trichloroacetic acid and kept at 4° overnight, after which a precipitate was centrifuged off. The solution was applied to a column of Sephadex G25 in 5% formic acid (130 cm. x 1.5 cm. diam.) and fractions of 50 drops (2.9 ml.) were collected. 0.04 ml. of each fraction was examined by electrophoresis at pH 6.5 (Section 11.4.), and the result is shown in Figure 12.3.1. The pooled fractions shown were freeze-dried.

The neutral region in fraction 6 showed a white spot, presumably due to low molecular weight compounds. The material was dissolved in the minimum possible water, and applied to a column of Sephadex G10 in 5% formic acid (110 cm. x 1.0 cm. diam.) eluted at 22 ml./hr.

3.7/

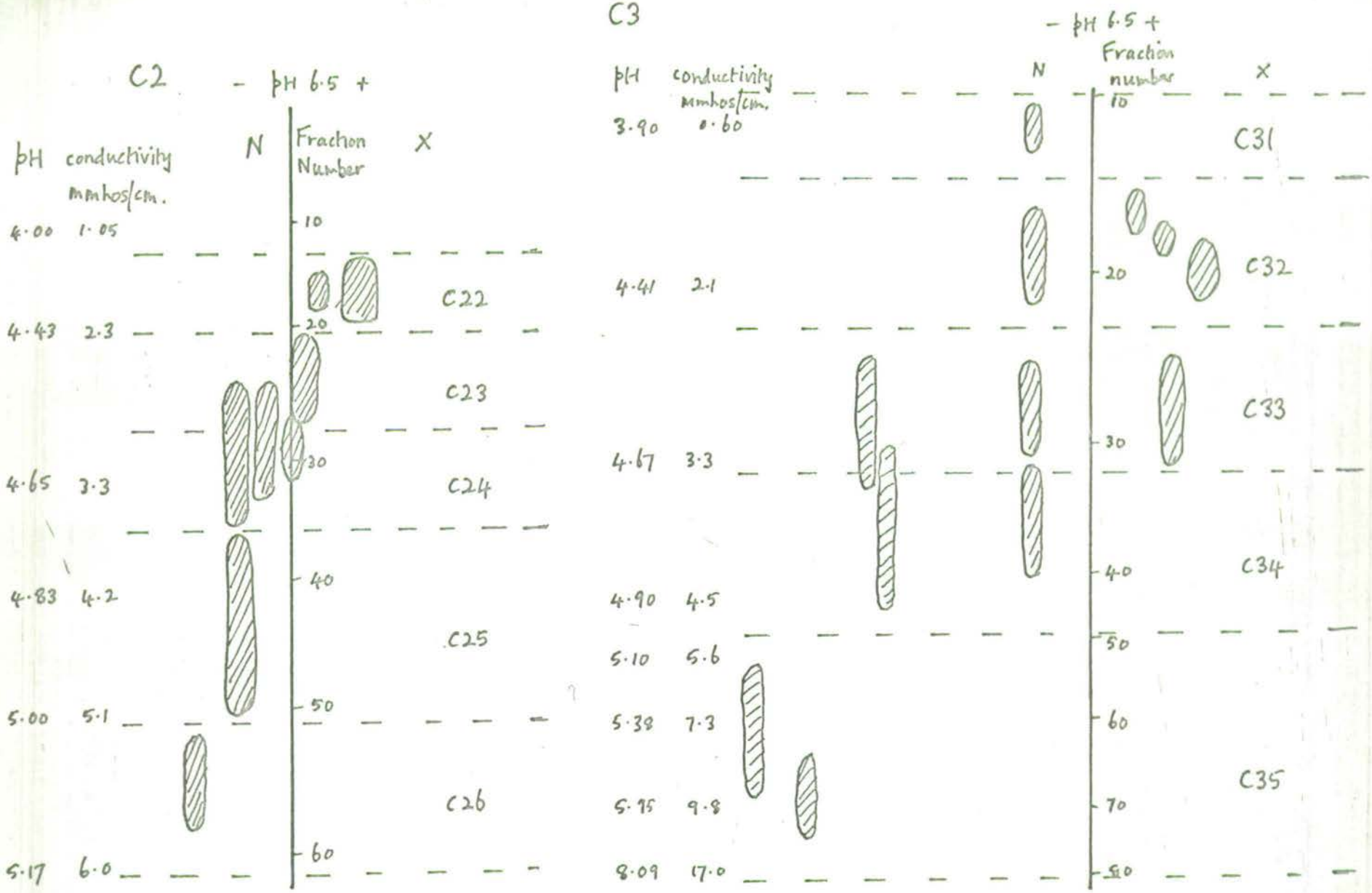


Figure 12.3.2. Electrophoresis of C2 and C3 at pH 6.5 after separation on SE-Sephadex.
N: position of monoaminomonocarboxylic acids. X: position of XCFF.

3.7 ml. fractions were collected and 0.05 ml. samples were examined by electrophoresis at pH 6.5 as before (Section 11.4.). The result showed two regions with peptides, each with neutral peptides and a basic one (m +0.48), separated by the white spot again. The basic components were lost in the course of purification.

Fractions 2 and 3 were separated on columns of SE-Sephadex (R.P. Ambler, unpublished). The exchanger (Section 3.3.) was equilibrated with 0.075 M ammonium acetate, pH 3.6 in a column (8 cm. x 1 cm. diam.). The peptides were applied dissolved in 2 ml. of the same buffer, and the column was eluted with a gradient between 160 ml. starting buffer and the same volume of 0.2 M ammonium acetate, pH 8.5. 3 ml. fractions were collected. When the effluent pH had reached about 5.7 the column was pumped directly with pH 8.5 buffer. The pH and specific conductivity of every tenth fraction were measured, and 0.05 ml. of each was examined by electrophoresis (Section 11.4.). Figure 12.3.2. shows the result. The fractions were pooled as shown and freeze-dried, though fractions 26 & 35 were first desalted on a column of Sephadex G25 in 5% formic acid.

These various fractions were separated by the methods shown in Table 12.4.1. Peptides shown to contain methionine but not tryptophan were oxidised with the vapour of performic acid (Section 3.5.) prior to elution.

The fraction precipitated with trichloroacetic acid was examined to see if the missing tryptophan peptide could be found. Nearly all the/

the u.v. absorbing material failed to stick at all to DE52 or CM52 in 0.01 M-ammonium acetate, 8 M-urea, pH 8.5 and 4.8 respectively. The material was recovered from urea solution by the method of Harris & Hindley (1965). Amino acid analysis showed that the material had the composition of whole protein, approximately.

12.4. Chymotryptic peptides - summary.

Table 12.4.1. summarises the purification of the various peptides. The fractions S_n & I_n refer to Figures 12.2.1., 12.3.1. & 12.3.2. Tables 12.4.2. & 12.4.3. summarise amino acid analyses and other properties of the chymotryptic peptides.

Table 12.4.1.

Purification of Chymotryptic Peptides.

S_n: fraction in which peptide eluted from Sephadex G25.

I_n: fraction in which peptide eluted from SE-Sephadex.

6: pH 6.5 electrophoresis. 3 : pH 3.5. electrophoresis.

B: Chromatography in BAWP. 2 : pH 2.0 electrophoresis.

Peptide	Digest 1	Digest 2	Peptide	Digest 1	Digest 2
CB1	$S_2$6B3		CN17	$S_6$6B3	
CB3	$S_3$6B3		CN18	$S_7$6B3	
CB4	$S_3$6B3		CA1	$S_1$6B3	
CB5	$S_3$6B3	$S_3I_3$6B3	CA2	$S_2$6B3	
CB6		$S_4$6B3	CA3	$S_2$6B3	
CB7	$S_4$6B3	$S_4$6B3	CA4	$S_2$6B3	
CB9	$S_4$6B3		CA5		$S_1$63
CB10	$S_5$6B3		CA6	$S_2$6B3	
CB12	$S_5$6B3		CA7		$S_2I_2$6B3
CB14	$S_7$6B3		CA8		$S_2I_3$6
			CA9	$S_3$6B3	$S_3I_2$6B3
CN2	$S_3$6B3		CA10	$S_3$6B3	
CN3		$S_3I_3$6B3	CA11	$S_3$6B3	$S_4$6B3
CN4		$S_4$63B3	CA13	$S_3$6B3	
CN5	$S_5$6B3	$S_4$63	CA15	$S_4$6B3	
CN6	$S_4$6B3		CA18	$S_4$6B32	
CN7	$S_4$6B3		CA19	$S_4$6B3	
CN8		$S_3I_3$6B3	CA20	$S_4$6B3	
CN9/					

Table 12.4.1. continued.

Peptide	Digest 1	Digest 2	Peptide	Digest 1	Digest 2
CN9		$S_4$63B3	CA21	$S_4$6B3	
CN10		S_2I_5B3	CA22	$S_5$6B3	
CN11	$S_4$6B3		CA23	$S_5$6B3	
CN12	$S_4$6B3	$S_5$6B3	CA24	$S_6$6B3	
CN13		$S_5$6B3	CA25	$S_6$6B3	
CN14	$S_5$6B3				

Table 12.4.2.

Amino acid analyses of chymotryptic peptides.

Peptide	CB1	CB3	CB4	CB5	CB6	CB7	CB9	CB10	CB12	CB14
Lys	3.05	0.92	1.98	0.88	-	0.84	-	-	-	-
His	-	-	-	-	-	-	-	-	-	-
Arg	1.06	-	-	-	(1)[b]	-	0.95	0.88	0.79	1.05
Asp	2.16	-	-	-	-	-	-	-	-	-
Mes	-	1.08	-	-	-	-	-	-	-	-
Thr	-	-	-	0.95	0.98	-	-	0.16	0.95	-
Ser	1.85	-	-	1.02	1.17	-	-	0.16	-	-
Glu	-	-	0.87	-	0.24	-	-	-	-	-
Pro	-	-	-	-	-	-	-	-	-	-
Gly	-	-	-	-	0.25	-	-	-	-	-
Ala	1.01	-	-	2.06	1.28	1.09	0.99	1.13	1.02	-
Val	-	1.98	-	-	0.91	-	-	-	1.23	-
Met	-	-	-	-	-	-	-	-	-	-
Ile	-	-	-	1.01	-	-	-	-	-	-
Leu	-	-	1.15	1.08	1.36	1.07	1.07	-	-	-
Tyr	0.87	-	-	-	-	-	-	-	1.01	0.95
Phe	-	-	-	-	0.64	-	-	0.99	-	-
Trp	-	-	-	-	-	-	-	-	-	-
N-t	none	Lys	Lys	Ala	Val	Lys	Arg	Arg	Arg	Arg
Section	12.7.	12.5.	12.5.	12.5.	12.8.	12.5.	12.5.	12.8.	12.9.	12.10
Notes		a								

[a]: 96 hr. hydrolysis; 24 hr. gave Val 1.42.

[b]: Detected by qualitative analysis.

Table 12.4.2.

Peptide	CN2	CN3	CN4	CN5	CN6	CN7	CN8	CN9	CN10	CN11
Lys	0.82	0.82	-	-	-	-	0.95	-	1.85	0.98
His	-	-	-	-	-	-	-	-	-	-
Arg	-	0.93	0.93	-	-	-	0.85	(1)[b]	1.71	-
Asp	0.28	2.10	2.05	-	-	-	1.11	2.88	4.22	0.95
Mes	-	-	-	-	-	-	-	-	-	-
Thr	-	1.03	2.91	-	0.83	0.98	-	-	2.88	-
Ser	0.94	1.01	-	-	-	1.12	1.11	1.13	1.12	-
Glu	2.09	4.25	-	-	-	-	1.20	1.07	1.31	-
Pro	1.00	-	-	-	-	-	-	-	0.94	-
Gly	1.92	-	1.08	-	1.08	-	-	-	3.88	-
Ala	-	-	2.02	0.99	-	-	1.90	1.87	4.95	1.05
Val	0.11	-	1.01	1.00	2.03	0.99	-	-	1.88	-
Met	-	-	-	-	-	-	-	-	0.55	-
Ile	1.12	1.87	-	-	-	-	-	-	1.02	-
Leu	1.10	1.99	1.01	1.01	1.06	0.91	1.88	0.99	1.19	1.05
Tyr	-	0.42	0.45	-	-	-	0.47	(1)[d]	0.80	-
Phe	-	-	-	-	-	-	-	-	-	-
Trp	-	-	-	-	-	-	-	-	0.72	-
N-t	Lys	Leu	Ala	Ala	Thr	Val	Lys	Arg	Lys	Asx
Section	12.11.	12.12.	12.9.	12.6.	12.6.	12.8.	12.10.	12.13.	12.14.	12.6.
Notes				c	c					

[c]: 24 hr. hydrolysis; result not changed by 96 hr. hydrolysis.

[d]: Tyrosine present but too little to calculate.

Table 12.4.2. cont'd.

Peptide	CN12	CN13	CN14	CN17	CN18	CA1	CA2	CA3	CA4
Lys	-	-	-	-	-	0.72	0.99	1.93	1.03
His	-	-	-	-	-	-	0.96	-	-
Arg	1.96	1.99	-	-	-	1.72	-	1.09	1.09
Asp	1.07	1.01	-	0.18	-	5.09	2.02	1.08	2.81
Mes	-	-	-	-	-	-	1.00	-	-
Thr	-	-	-	-	-	3.92	2.93	-	1.28
Ser	-	-	-	-	-	1.21	-	1.03	1.00
Glu	1.11	1.10	-	0.25	-	7.20	1.11	3.06	1.28
Pro	0.86	0.90	-	-	-	2.80	0.92	0.85	1.22
Gly	-	-	-	-	0.96	2.03	1.07	-	3.00
Ala	-	1.02	0.89	0.97	-	0.94	-	0.86	3.75
Val	-	-	-	-	-	2.36	0.95	-	2.13
Met	-	-	-	-	-	-	-	-	-
Ile	-	-	-	-	1.02	1.01	1.04	-	0.91
Leu	-	-	1.11	-	-	1.04	0.98	4.07	0.38
Tyr	-	-	-	-	-	-	-	-	0.81
Phe	1.00	1.99	-	1.03	1.02	0.96	-	-	-
Trp	-	-	-	-	-	-	-	-	f
N-t	Arg	Arg	Ala	Ala	Gly	Arg	Asx	Ala	g
Section	12.15.	12.15.	12.16.	12.15.	12.6.	12.17.	12.18.	12.16.	12.14.
Notes									e

[e]: Peptide contaminated with about 0.1 moles/mole of CA3.

[f]: Peptide contains oxidised tryptophan.

[g]: Faint spot compatible with DNS-Ile.

Table 12.4.2. cont'd.

Peptide	CA5	CA6	CA7	CA8	CA9	CA10	CA11	CA13	CA15
Lys	4.16	-	0.99	1.93	0.72	-	-	0.94	1.06
His	-	-	0.92	-	-	-	-	-	-
Arg	2.96	-	0.98	0.92	-	-	-	-	-
Asp	6.29	2.15	4.93	2.02	1.06	2.16	2.98	1.89	1.85
Mes	-	-	-	-	-	-	-	-	1.09
Thr	4.75	2.84	4.00	-	-	2.13	-	0.93	-
Ser	2.78	1.10	-	1.02	1.05	-	0.92	-	-
Glu	11.63	2.71	1.24	3.22	1.21	-	1.07	1.15	-
Pro	4.01	0.86	0.94	1.02	-	-	-	-	-
Gly	4.16	1.18	1.17	-	-	0.76	-	-	1.10
Ala	2.73	1.03	-	1.04	1.96	0.79	1.98	1.96	-
Val	2.59	1.14	1.98	-	-	-	-	-	-
Met	-	-	0.80	-	-	-	-	-	-
Ile	2.54	-	1.01	0.99	-	-	-	1.04	-
Leu	5.41	-	1.98	3.92	1.99	1.14	1.01	1.09	-
Tyr	-	-	0.61	-	-	-	-	-	-
Phe	0.98	-	-	-	-	-	-	-	-
Trp	-	-	-	0.75	-	-	-	-	-
N-t	Ile	none	none	Ala	Lys	Ala	Ser	Asx	Asx
Section	12.17.	12.17.	12.18.	12.16.	12.10.	12.9.	12.13.	12.19.	12.20.

Table 12.4.2. cont'd.

Peptide	CA18	CA19	CA20	CA21	CA22	CA23	CA24	CA25
Lys	-	0.85	-	1.03	0.81	-	-	-
His	-	-	-	-	-	-	-	-
Arg	-	-	-	-	-	1.00	-	-
Asp	0.93	2.12	2.12	2.19	-	3.11	1.03	-
Mes	-	-	-	0.94	-	-	-	-
Thr	-	-	-	0.69	-	0.88	-	-
Ser	1.11	-	0.81	-	-	-	-	-
Glu	0.35	-	1.06	1.16	3.30	-	-	2.99
Pro	-	-	-	-	-	-	-	-
Gly	0.24	-	-	-	-	-	-	-
Ala	1.79	-	2.00	-	0.78	-	-	-
Val	-	-	-	-	-	1.11	-	-
Met	-	-	-	-	-	-	-	-
Ile	-	-	-	-	-	-	-	-
Leu	1.17	1.03	-	--	1.04	0.98	0.97	-
Tyr	-	-	-	-	-	0.96	-	-
Phe	-	-	-	1.00	1.07	-	-	1.01
Trp	-	-	-	-	-	-	-	-
N-t	Ala	Asx	Ser	Thr	Ala	Thr	Asx	Glx
Section	12.10.	12.19.	12.13.	12.21.	12.22.	12.18.	12.13.	12.22.

Table 12.4.3.

Electrophoretic mobilities and R_x values in paper chromatography for chymotryptic peptides

Peptide	m (pH 6.5)	R_x (BAWP)	m' (pH 3.5)	Peptide	m (pH 6.5)	R_x (BAWP)	m' (pH 3.5)
CB1	+0.54	0.21	+2.50	CN17	0	0.95	+0.77
CB3	+0.46	0.72	+1.77	CN18	0	1.16	+0.77
CB4	+0.40	0.51	+2.36	CA1	-0.50	0.45	+0.65
CB5	+0.35	0.91	+1.61	CA2	-0.07	0.82	+1.07
CB6	+0.29	1.04	+1.41	CA3	-0.17	0.73	+1.30
CB7	+0.53	0.79	+2.44	CA4	-0.17	0.83	+0.80
CB9	+0.45	0.85	+2.37	CA5	-0.15	-	smear at abo +1.00 - +0.7
CB10	+0.46	0.88	+1.89	CA6	-0.63	0.40	-0.15
CB12	+0.32	0.90	+1.72	CA7	-0.31	0.86	+0.52
CB14	+0.47	0.80	+2.05	CA8	-0.27	-	-
				CA9	-0.26	0.42	+0.88
CN2	0	0.76	+1.06	CA10	-0.32	0.74	+0.12
CN3	0	0.79	+0.90	CA11	-0.32	0.60	+0.19
CN4	0	0.81	+0.73	CA13	-0.55	0.66	+0.07
CN5	0	1.22	+1.00	CA15	-0.04	0.30	+1.19
CN6	0	1.27	+0.79	CA18	-0.46	0.79	+0.60
CN7	0	1.05	+0.74	CA19	-0.46	0.57	+0.50
CN8	0	0.70	+1.10	CA20	-0.46	0.30	-0.04
CN9	0	0.56	+0.81	CA21	-0.53	0.54	+0.57
CN10	0	0.68	+1.00	CA22	-0.28	0.76	+1.07
CN11	0	0.67	+1.00	CA23	-0.28	0.76	+0.60
CN12	0	0.56	+1.51	CA24	-0.16	0.80	+0.92
CN13	0	0.75	+1.21	CA25	-0.72	0.58	-0.05
CN14	0	1.04	+1.00				

12.5. Small basic peptides.

CB3. This peptide contains a -Val-Val- sequence. After one cycle of DNS-PTC degradation valine was found. In view of the specificity of chymotrypsin the peptide is

Lys-Val-Val-Mes.

CB4. CPA released leucine, together with glutamic acid which increased after more vigorous digestion. DNS-PTC degradation showed nothing after one cycle (lysine is sometimes overlooked), glutamic acid after two and leucine after three. The sequence is

Lys-Lys-Glu-Leu.

CB5. This peptide released alanine and leucine/isoleucine with CPA. The complete sequence was determined by the DNS-PTC method and was

Ala-Ser-Thr-Ile-Lys-Ala-Leu.

CB7. CPA released leucine. After one cycle of DNS-PTC degradation the chief N-terminus was alanine, so the sequence is

Lys-Ala-Leu.

CB9. CPA released leucine. After one cycle of DNS-PTC degradation alanine was the N-terminus, so the sequence is

Arg-Ala-Leu.

12.6. Small neutral peptides.

CN5. CPA released leucine. The DNS-PTC method showed the sequence/

sequence was

Ala-Val-Leu.

CN6. CPA released valine and leucine, together with a little glycine. The analysis after 24 hr. showed that a -Val-Val- sequence was not present. The data tentatively suggest a sequence Thr-Val-Gly-Val-Leu, which is in agreement with the sequence found for PN12 (Section 13.4.).

CN11. The mobility of this peptide rules out the presence of asparagine. CPA released leucine, and DNS-PTC degradation showed the sequence

Asp-Ala-Lys-Leu.

CN18. CPA released principally phenylalanine. The DNS-PTC method showed the sequence

Gly-Ile-Phe.

12.7. Peptide CB1.

There was a faint N-terminus which might have been serine. CPA released tyrosine. The mobility of the peptide rules out the presence of amides. The peptide was digested with trypsin and the fragments were separated by electrophoresis at pH 6.5 & pH 3.5. The results are shown in Table 12.7.1.

Table 12.7.1.

Tryptic peptides from peptide CB1.

Peptide	m	m'	N-t.	Lys	Arg	Asp	Ser	Ala
CB1TB1	+0.52	+2.16	Asx	2.10	-	0.90	0.17	-
CB1TB2	+0.54	+2.05	Ser	0.27	1.08	-	1.92	-
CB1TN1	0	+1.11	Asx	1.10	-	0.99	-	0.91

Free tyrosine was also present, characterised with 1-nitroso-2-naphthol and by its mobility at pH 3.5. From these data it is evident that CB1TB1 is the tryptic peptide TB1, Asp-Lys-Lys, while CB1TN1 is the tryptic peptide Asp-Ala-Lys, TN10. The third fragment is Ser-Ser-Arg, and the tenuous agreement with the N-terminus of the whole peptide, together with its being the only fragment not a complete tryptic peptide, establish it as N-terminal in CB1. Hence the sequence of CB1 is

CB1TB2 - (CB1TB1, CB1TN1) - Tyr,

and the evidence does not permit us to order the internal peptides. The sequence may be expressed

Ser-Ser-Arg-Asp-(Ala)-Lys-Asp-(Lys)-Lys-Tyr

where the two possible positions of the bracketed residues cannot be distinguished.

12.8. Peptides CB6, CB10 & CN7.

CB10 is slightly impure. CPA apparently released slightly more/

more phenylalanine than alanine. The sequence

Arg-Ala-Phe

was obtained by DNS-PTC degradation.

One cycle of DNS-PTC degradation of CN7 showed threonine. CPA released leucine and serine. Hence considering the specificity of chymotrypsin, the likeliest sequence is

Val-Thr-Ser-Leu.

CB6 was isolated only in small quantities and not obtained pure. The easiest interpretation is that it is the sum of the other two peptides:

Val-Thr-Ser-Leu-Arg-Ala-Phe.

Its mobility at pH 6.5 is consistent with this.

12.9. Peptides CB12, CN4 & CA10.

From CB12, CPA releases tyrosine, alanine and valine extensively. DNS-PTC degradation gives a partial sequence Arg-Thr-Val-Ala-. Hence the sequence is

Arg-Thr-Val-Ala-Tyr.

The mobility of CA10 shows that one residue of asparagine is present together with one of aspartic acid. Threonine and asparagine were released with CPA. A complete sequence Ala-Leu-Asx-Thr-Gly-Thr-Asx was obtained by DNS-PTC degradation, so CA10 is

Ala-Leu-Asp-Thr-Gly-Thr-Asn.

The analysis of CN4 was consistent with its being the sum of the two/

two previous peptides. Thermolysin digestion gave only neutral fragments, and one was obtained pure by electrophoresis at pH 3.5. This had N-terminal valine and an analysis Ala, 1.14; Val, 1.01; Tyr, 0.86, together with aspartic acid and threonine at the 0.2 to 0.3 level. DNS-PTC degradation showed that valine was the second residue.

These data are consistent with CN4 being CA10 - CB12.

12.10. Peptides CB14, CN8, CA9 & CA18.

Peptides CN8 & CA9 have very similar compositions, differing only by one residue each of arginine and tyrosine.

Peptide CB14 is apparently Arg-Tyr.

Peptide CA9 gave leucine with CPA. Under stronger conditions alanine, serine and aspartic acid were also released. The DNS-PTC method gave a partial sequence Lys-Glu-Leu-Ala-Asp- (the mobility of the peptide rules out amide groups). Hence we may write a partial sequence

Lys-Glu-Leu-Ala-Asp-(Ser,Ala)-Leu.

Insufficient CN8 was found to permit more than amino acid analysis. Its lysine N-terminus suggest that it is CA9 - CB14, and its mobility is consistent with this.

Peptide CA18 was also found only in small quantities. It is Ala-(Ala,Asp,Ser,Leu). It is most easily reconciled with the sequence/

sequence as part of CA9, presumably arising by partial cleavage at the leucine residue.

So we may write the sequence of the region

Lys-Glu-Leu-Ala-Asp-(Ser,Ala)-Leu-Arg-Tyr.

These peptides are shown in Section 12.13.

12.11. Peptide CN2.

This peptide released only leucine with CPA. Its mobility requires the presence of a residue of glutamine, so we may write

Lys-(Ser,Glu,Gln,Pro,Gly_2,Ile)-Leu.

A DNS-PTC degradation was performed defectively, but gave DNS-glutamic acid at F1 and DNS-glycine at F3.

These data are compatible with the peptide originating from the same region as TN1 & TA3.

12.12. Peptide CN3.

The mobility of this peptide requires that four of the six acid residues be amidated. The peptide was degraded with trypsin and the fragments were separated at pH 6.5 and the neutral fragment was purified at pH 3.5.

Table 12.12.1.

Tryptic peptides obtained from peptide CN3.

Peptide	m	m'	N-t.	Lys	Arg	Asp	Thr	Ser	Glu	Gly	Ile	Leu	Tyr
CN3TB1	+0.46		Leu	0.97	-	-	-	-	2.12	-	-	0.90	-
CN3TN1	0	+0.60	Ile	-	-	-	1.04	-	-	-	1.02	-	0.94
CN3TA1	-0.30		Ser	0.11	0.97	1.99	-	0.94	2.12	0.16	0.94	1.03	-

Both residues of glutamic acid in CN3TB1 must be glutamines, or else the peptide would not be basic. The apparent sequence was confirmed by DNS-PTC degradation and is

Leu-Gln-Gln-Lys.

The sequence of the neutral fragment was determined by the same method and was Ile-Thr-Tyr. The properties of CN3TA1 are the same as those of peptide TA13.

These results show that the sequence of CN3 is

Leu-Gln-Gln-Lys- TA13 -Ile-Thr-Tyr.

12.13. Peptides CN9, CA11, CA20 & CA24.

Peptide CA20 has a mobility which suggests the presence of only one acidic residue, so two amides are present. CPA released alanine and glutamine, and the DNS-PTC degradation gives a partial sequence Ser-Asx-Asx-Ala-Ala- . Thus we may write the sequence

Ser-(Asp,Asn)-Ala-Ala-Gln.

CA11/

CA11 has an additional aspartic acid residue and a leucine. The mobility shows that the additional residue must be asparagine. Exactly the same partial sequence was obtained by sequential degradation, showing that the additional residues are at the C-terminus. CPA released leucine, alanine, glutamine and possibly asparagine.

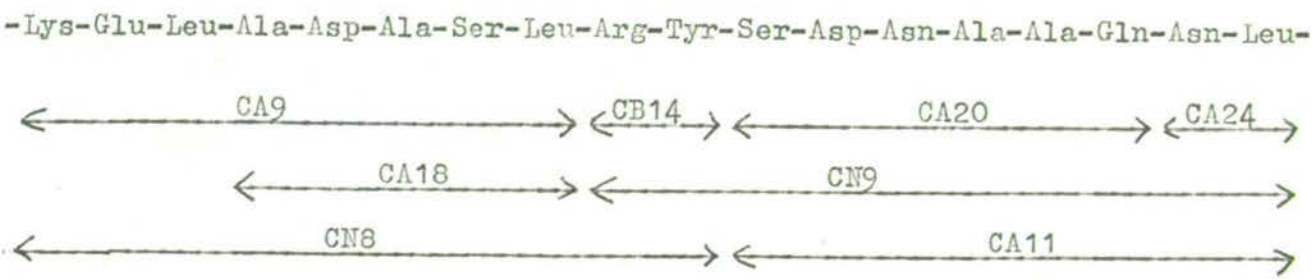
The order of the two additional residues may be settled by consideration of peptide CA24. The release of leucine and asparagine by CPA, show that the peptide is Asn-Leu. The slight acidic mobility is due to the weakening effect of the amide side chain on the α-amino group. There is no other likely origin for this peptide in the molecule. Thus CA11 is

Ser-(Asp,Asn)-Ala-Ala-Gln-Asn-Leu.

Peptide CN9 contains one residue each of arginine and tyrosine in addition to the composition of CA11. The N-terminus shows that these residues are at the N-terminus. Thus CN9 has the structure

CB14 - CA11.

These findings agree with those in Section 12.10. The structure of the region is



12.14./

12.14. Peptides CN10 & CA4.

Peptide CN10 did not contain a stoichiometric amount of methionine, but was too large for further fractionation to be certain of improving the analysis. The peptide was degraded with trypsin and the results are shown in Table 12.14.1.

Table 12.14.1.

Products of degradation of CN10 with trypsin

	CN10TB1	CN10TB2	CN10TN1[a]	CN10TN2	CN10TA1[b]
m	+1.12	+0.84	0	0	-0.38
m'			+0.81	+0.45	
N-t.	Lys	Arg	Asx	Thr	Ala
Lys	0.98	0.94	-	-	0.84
Arg	1.02	1.21	++	-	-
Asp	-	-	++	0.22	2.15
Thr	-	-	+	1.01	0.17
Ser	-	-	-	0.98	-
Glu	-	-	+	-	1.13
Pro	-	-	-	-	0.88
Gly	-	-	+	1.05	2.09
Ala	-	-	++	1.96	1.90
Val	-	-	-	-	2.00
Ile	-	-	+	0.10	-
Leu	-	-	+	0.10	-
Met	-	0.84	-	-	-
Tyr	-	-	-	0.52	-

[a]: Insufficient peptide isolated for quantitative analysis.

[b]: Qualitative analysis showed tryptophan present.

CN10TB1 is Lys-Arg, and the N-terminus of some of the CN10. peptides. It is difficult to see what else but Met-Lys-Arg CN10TB2 can be. The N-terminus is probably from contaminating free arginine. Thus these two peptides are each the N-terminus of part of CN10, and account for the 0.55 methionine found on amino acid analysis.

DNS-PTC examination of CN10TN2 showed Thr-Gly-Ala-Ala-Ser-, leaving the tyrosine at the N-terminus. CN10TA1 is peptide TA9.

The difference in composition between the sum of CN10TB2, CN10TN1 & CN10TA1 and whole CN10 is Arg, Asx_2, Thr_2, Gly, Ala, Ile, Leu. This agrees with the composition of TN7 and also with the qualitative analysis of CN10TN1, although threonine is somewhat weak and glutamic acid somewhat strong.

CA4 was isolated impure and in low yield. On tryptic degradation, two of the products were recognisable as TA9 & CN10TN1. The third fragment was a basic peptide which qualitative analysis showed to contain arginine and leucine/isoleucine. N-terminal determination showed DNS-Ile-Arg (Section 3.15.), suggesting that the peptide was Ile-Arg. These two residues are the C-terminal portion of TN7, so it seems reasonable to postulate that CA4 is Ile-Arg- TA9 - CN10TN1 and the amino acid analysis is approximately correct for this. This suggests that CN10 is

(Met)-Lys-Arg- TN7 - TA9 - Thr-Gly-Ala-Ala-Ser-Tyr.

12.15./

12.15. Peptides CN12, CN13 & CN17.

Peptides CN12 & CN13 differ by one residue each of alanine and phenylalanine. This suggests that the difference is due to the splitting out of peptide CN17, which is, from Table 12.4.2., slightly impure Ala-Phe.

A complete sequence for CN12 was determined by the DNS-PTC method. The results are shown in Table 12.17.1.

Table 12.16.1.

DNS-PTC degradation of peptide CN12.

Peptide	F1	F2	F3	F4	F5
N-t.	Pro	Asx	Glx	Arg	Phe
Substantial impurities.	Asx		Asx	Phe	
Slight impurities.		Glx			

The neutral mobility of the peptides shows that no amides are present. CPA released phenylalanine. Hence the sequence is

Arg-Pro-Asp-Glu-Arg-Phe.

A similar DNS-PTC degradation was done on CN13. The same sequence was obtained, but the impurity level was much lower. The material ran out before the seventh and eighth residues could be determined. Thus all the evidence on CN13 is consistent with a sequence/

sequence

Arg-Pro-Asp-Glu-Arg-Phe-Ala-Phe.

12.16. Peptides CN14, CA3 & CA8.

Peptide CA8 was examined chiefly through the fragments it gave on tryptic digestion. They are summarised in Table 12.17.1. In addition to these free arginine was released. There was also a faint basic peptide which could not be fully purified.

Table 12.16.1.

Products of tryptic digestion of CA8.

Peptide	m	m'	N-t.	Lys	Arg	Asp	Ser	Glu	Pro	Gly	Ala	Met	Ile	Leu
CA8TN1	0	+1.51	Ala	1.84	(1)[a]	0.97	1.00	2.22	1.14	-	1.06	-	-	1.7
CA8TN2	0	+1.36	Leu	1.00	-	-	1.11	1.21	0.91	-	-	-	-	0.7
CA8TA1	-0.20		Ala	1.75	-	1.13	1.00	2.23	0.95	0.17	0.90	-	-	2.0
CA8TA2	-0.34		Ala	0.83	-	1.07	0.18	1.16	-	-	0.73	-	-	1.2
CA8TA3[b]	-0.55		Glx	-	-	1.04	-	1.05	-	-	-	0.11	0.95	1.9

[a]: Defective analysis; found by qualitative analysis.

[b]: Peptide contains tryptophan detected by qualitative analysis.

The/

The sequence of CA8TA2 was determined by DNS-PTC degradation and proved to be Ala-Leu-Glu-Asp-Lys; the mobility ruled out the presence of amides. The same applied to CA8TA3 which gave the sequence Glu-Leu-Leu-Ile-Asp-. Elimination and the specificity of chymotrypsin then places tryptophan at the C-terminus suggesting Glu-Leu-Leu-Ile-Asp-Trp. It is possible that the methionine in the analysis is genuine and that it follows the tryptophan in a small proportion of molecules (see Section 12.14.).

Peptide CA8TA2 has the same properties as TN13 and is presumably Leu-Pro-Ser-Glu-Lys. Peptide CA8TA1 is then the sum of CA8TN2 & CA8TA2 and has the structure: CA8TA2 - TN13.

Peptide CA8TN1 differs from CA8TA1 only in containing the only arginine residue; the existence of free arginine suggests a -Lys-Arg- sequence, and this can only be accomodated after TN13. It is concluded that the structure of CA8 is

Ala-Leu-Glu-Asp-Lys-Leu-Pro-Ser-Glu-Lys-Arg-Glu-Leu-Leu-Ile-Asp-Trp.

The overall analysis of CA3 differs from CA8 by the absence of isoleucine and tryptophan and the loss of one residue of aspartic acid. CPA released leucine and glutamic acid, suggesting that the last three residues of CA8 are missing. Tryptic digestion gave free arginine and peptides corresponding to CA8TA1 & CA8TA2. The neutral peptides were not characterised. There were equal amounts of two acidic peptides, with mobilities of -0.50 & -0.63. The first contained Glu, 1.07; Leu, 1.93, and the second Glu, 0.99; Leu 1.01./

1.01. Both had N-terminal glutamic acid and were contaminated with 0.10 - 0.15 residues of aspartic acid and serine. The data suggest that these peptides are Glu-Leu-Leu and Glu-Leu, and it seems that they come from the C-terminal region of CA3. The likeliest origin for Glu-Leu is from a peptide with a C-terminal sequence -Arg-Glu-Leu, but if the peptide CA3 is a mixture of this with a form with -Arg-Glu-Leu-Leu, it is surprising that the analysis shows 4.07 residues of leucine. A less likely alternative is that the second leucine was removed by some chymotryptic or exo-peptidase activity originating from the trypsin.

Peptide CN14 is Ala-Leu. Although there are five -Ala-Leu- sequences in the molecule, there is no obvious origin for this peptide. Three of the alanines are preceded by lysine or arginine, and the other two sequences are Ala-Leu-Asp-(CA10) and Ala-Leu-Glu-(CA3 & CA8). The acidic residues would not be expected to encourage cleavage. No evidence of peptides starting with these aspartic acid and glutamic acid residues has been found. The origin of peptide CN14 remains an open question.

12.17. Peptides CA1, CA5 & CA6.

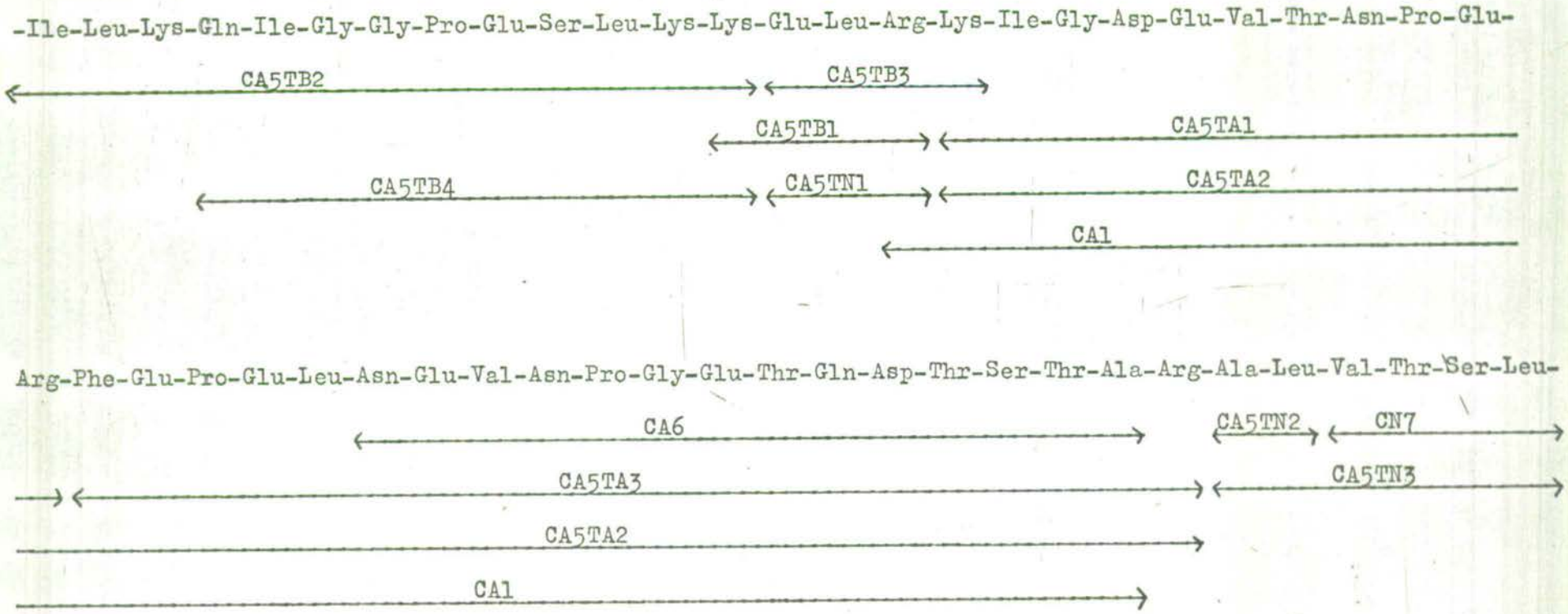
Peptide CA5 was very large and the analysis not particularly stoichiometric. It was not usefully purified by chromatography in BAWP, so the peptide was degraded with trypsin directly. The fragments are in Table 12.17.1.

Table 12.17.1.

Products of degradation of CA5 with trypsin

Peptide	CA5TB1,	CA5TB2	CA5TB3	CA5TB4	CA5TN1	CA5TN2	CA5TN3	CA5TA1	CA5TA2	CA5TA3
N-t.	Lys	Ile	Glx	Glx	Glx	Ala	Ala	Lys	Lys	Phe
m	+0.70	+0.50	+0.43	+0.24	0	0	0	-0.19	-0.42	-0.54
m'					+1.37	+1.00	+0.57			
Lys	1.17	3.52	0.93	1.94	0.11	0.17	0.22	0.86	0.80	0.15
Arg	0.78	-	0.89	-	0.94	-	-	0.85	1.78	1.03
Asp	-	-	-	-	-	-	0.23	2.08	5.16	3.16
Thr	-	-	-	-	-	-	0.93	0.95	3.73	2.75
Ser	-	0.88	0.30	0.93	-	-	0.96	0.20	1.03	0.86
Glu	1.03	1.90	0.93	2.10	1.04	0.31	0.15	2.30	7.40	5.27
Pro	-	0.83	-	0.99	-	-	-	1.02	2.93	2.04
Gly	-	1.78	0.33	1.97	-	0.27	-	1.06	2.13	1.18
Ala	-	-	-	-	0.10	0.88	1.02	0.26	1.03	0.90
Val	-	-	-	-	-	-	0.95	0.96	2.10	1.15
Ile	-	1.96	-	1.01	-	-	0.21	0.94	1.07	0.27
Leu	1.01	2.11	1.25	1.06	1.02	1.12	2.14	0.36	1.05	0.89
Phe	-	-	-	-	-	-	-	-	0.83	0.76

Figure 12.17.1. Peptides CA1, CA5 and CA6.



The fragments lacking basic residues presumably come from the C-terminus. CA5TN2 is evidently Ala-Leu, while the DNS-PTC method gave a partial sequence for CA5TN3 of Ala-Leu-Val-Thr-Ser-. So part of the heterogeneity of CA5 is due to the partial presence of CN7, Val-Thr-Ser-Leu, (Section 12.8.) at the C-terminus. The analytical figures for these four residues support this.

Peptides CA5TB1 and CA5TB3 are evidently related to CA5TN1, which is the tryptic peptide TN11. The N-terminal data suggest that the peptides are Lys-Glu-Leu-Arg and Glu-Leu-Arg-Lys respectively. The separation at pH 6.5 is due to the weakening of the ω-carboxyl of glutamic acid by a proximal lysine, but the large separation is surprising. Peptides CA5TA1, CA5TA2 & CA5TA3 correspond to TA5, TA1 and TA2 respectively.

The analysis of CA5TB4 is the same as TN1, but the peptide has an N-terminus and is basic. DNS-PTC degradation gave Glx-Ile-Gly-Gly-, and this peptide is discussed in Section 11.4. CA5TB2 has additionally second residues of leucine and isoleucine, and an extra lysine. This corresponds to the N-terminus of CA5, but insufficient material was available to allow the probable structure Ile-Leu-Lys-CA5TB4 - etc. to be verified directly.

The arrangement of these peptides is shown in Figure 12.17.1. The overall amino acid analysis of CA5 agrees with this, except that the value for glutamic acid is 16% high. It is possible the analyser was ill-calibrated for glutamic acid (Chapter 18.).

The/

Table 12.18.1.

Tryptic degradation of CA2 & CA7

	CA7TA1	CA7TA2	CA2TA1
m	-0.19	-0.23	-0.33
m'	+0.77	+0.77	
N-t.	Thr	His	Asx
Lys	0.93	-	0.95
His	-	0.61	-
Arg	0.95	-	-
Asp	4.10	1.22	0.86
Thr	1.94	2.06	1.28
Glu	1.11	-	1.05
Pro	0.94	-	0.95
Gly	0.14	1.11	0.38
Val	1.06	1.00	-
Met	-	0.95	0.19[a]
Ile	0.98	-	0.81
Leu	1.06	1.07	0.14
Tyr	0.71	-	-

[a]: as methionine sulphone.

The analysis of CA1 is identical with that of CA5TA2. The arginine residue is at the N-terminus instead of the C-terminus, and CPA released alanine, threonine and serine. The analysis of CA6 shows that it originates from the C-terminus of CA1.

12.18. Peptides CA2, CA7 & CA23.

Peptides CA2 & CA7 are the peptides from the two chymotryptic digests which contain the single histidine residue in the molecule. CA7 became divided into two fractions in the course of purification; one fraction gave the amino acid analysis shown in Table 12.4.2., the other was less pure judging from its amino acid analysis, but was present in much larger quantities. It was therefore degraded with trypsin, and two principal peptides were isolated (Table 12.18.1.) together with two other peptides which evidently originated from the contaminants. It was considered that, since the sum of the analyses of the two peptides equals the composition of CA7, it was justifiable to regard them as the tryptic degradation products of CA7.

Peptide CA2 was also degraded with trypsin; two acidic peptides were found; one was identical to CA7TA2, the other is shown in the Table as CA2TA1. CPA released threonine and leucine/isoleucine from CA2 and a similar result was obtained from CA7.

It was observed that the difference between CA2 & CA7 was the same as that between CA2TA1 and CA7TA1. This difference agrees with the composition of CA23 which also has the threonine N-terminus of CA7TA1 (it is not clear why an N-terminus was not demonstrable for CA7). DNS-PTC degradation of CA23 gave the partial sequence Thr-Arg-Asx-Asx-. After this multiple N-termini were obtained. It gave valine, leucine, asparagine and tyrosine with CPA.

The acidic mobility of CA7TA2 shows that the acid residue is not asparagine. The DNS-PTC method gave the partial sequence His-Val-Asx-Thr-Gly-.

These/

These data show that CA7 is

Thr-Arg-Asx-Asx-(Asx,Val,Leu,Tyr)-Asx-(Thr,Glx,Pro,Ile)-Lys-His-Val-Asp-Thr-Gly-(Met,Thr,Leu).

If this sequence is compared with the sequences of the tryptic peptides (Table 16.1.1.) it is evident that CA7TA2 differs from TN3 only in the absence of the C-terminal lysine, and that CA7TA1 chiefly consists of TA8. Peptide CA2TA1 is identical to TA8CN1 in composition (Section 11.25.). The peptide derived from CA2 is however acidic; its mobility is consistent with the N-terminal asparagine residue having become deamidated. Neutral material was present after tryptic degradation of CA2, but no peptide was isolated from it.

12.19. Peptides CA13 & CA19.

The large acidic mobility of CA13 shows that none of the three acid residues are amidated. CPA released threonine, alanine and glutamic acid; partial sequence determination by the DNS-PTC method showed Asp-Asp-Lys-.

CA19 cannot have any amides either. The DNS-PTC method gave a complete sequence Asp-Asp-Lys-Leu, confirmed by the release of leucine with CPA.

It seems very possible that these two peptides are related, but it was not possible to prove this directly. The remainder of peptide CA13 contained one residue each of leucine, isoleucine, threonine and/

and glutamic acid, together with two of alanine. The only tryptic peptide with which this is reconcilable is TN4, Leu-Ile-Ala-Glu-Ala-Thr-Lys. This suggests that chymotrypsin has cleaved a -Thr-Lys- bond, in agreement with the results obtained with CPA on CA13.

12.20. Peptide CA15.

The N-terminus and slight acid mobility of this peptide immediately suggest an asparaginyl N-terminus, in which case the other residue must be aspartic acid to leave the peptide approximately neutral in spite of the lysine residue. The peptide was resistant to the action of CPA. One cycle of DNS-PTC degradation gave some methionine sulphone as well as aspartic acid. After this no N-termini could be found.

This is part of peptide TB3, since the compositions of the other methionyl peptides are much less like CA15. Furthermore a partial sequence Asn-Met-(Asx,Gly)-Lys has been already deduced as the C-terminal portion of TB3 (Section 11.9.).

12.21. Peptide CA21.

CPA releases phenylalanine from this peptide, and the DNS-PTC method gave an N-terminal sequence Thr-Glu-Mes-. The mobility shows that none of the residues are amidated. So the peptide is

$$\text{Thr-Glu-Met-(Lys,Asp}_2\text{)-Phe.}$$

This shows the relation of the peptide to TA6 (Section 11.21.), and/

and since the material for the first chymotryptic digest, from which this peptide came, was trypsin-released enzyme, CA21 is the N-terminal peptide.

12.22. Peptides CA22 & CA25.

The compositions of these peptides suggest that CA25 is part of CA22, and this was confirmed by finding glutamine and phenylalanine released from both with CPA. CA25 gives Glx-Glx-Glx-Phe with the DNS-PTC method. The residual peptides after CPA have m of -0.94 & -1.11, and the former was distinguishable from free glutamic acid (-0.92). The mobility of CA25 shows that one residue of glutamine must be present. All these findings are consistent with a sequence

Glu-Glu-Gln-Phe

which was verified with an identical peptide found in a peptic digest (Section 13.11.).

CA22 gave lysine as its second residue with the DNS-PTC method. This suggests its sequence is

Ala-Lys-Leu-Glu-Glu-Gln-Phe.

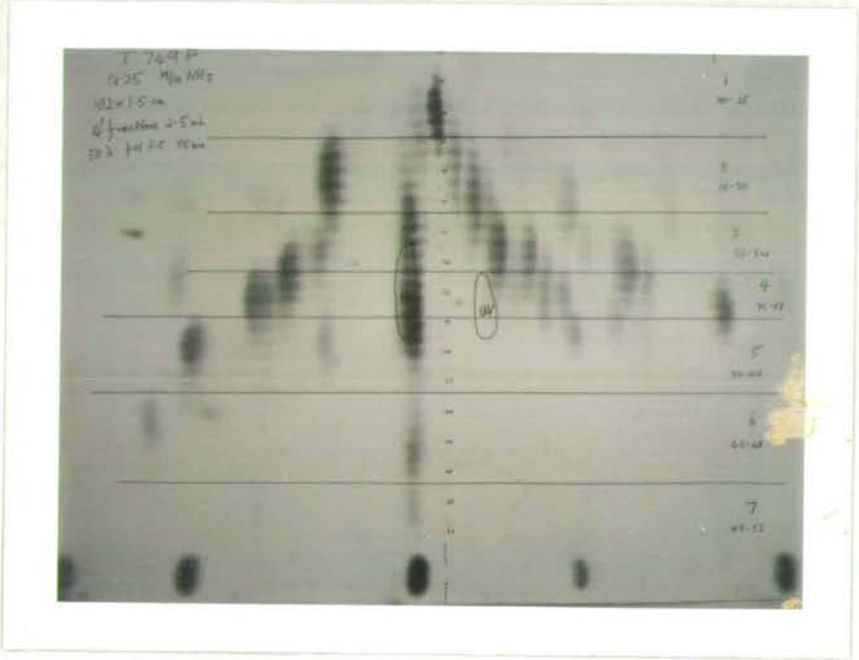


Figure 13.1.1.

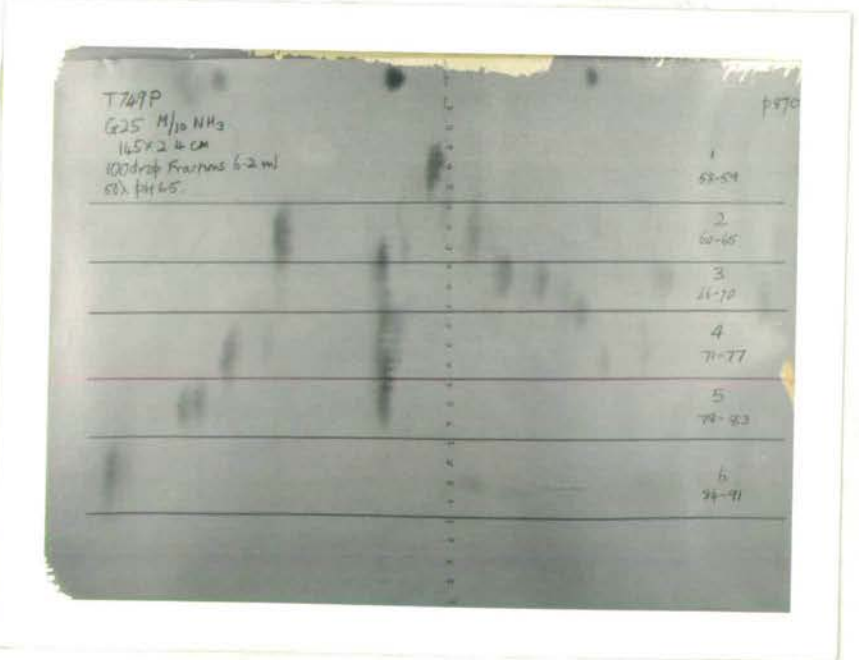


Figure 13.2.1.

Electrophoresis at pH 6.5 of peptic peptides separated on Sephadex G25.

CHAPTER 13

PEPTIC DIGESTION

13.1. Digest 1.

A total of three peptic digests have been done, but only the first was fractionated completely. The first digest was performed about 5 micromoles of PP11 (trypsin-released enzyme). The protein was dissolved in 70 ml. 8% acetic acid containing 2% formic acid (pH 2.0 electrophoresis buffer) 2.8 mg. pepsin was added, and after 2 hr. at 37° the digest was freeze-dried.

The digest was dissolved in 0.1 M-ammonia. A small precipitate was removed by centrifugation. The soluble fraction (about 3.5 ml.) was applied to a column of Sephadex G25 in 0.1 M-ammonia (102 cm. x 1.2 cm. diam.). It was eluted at approximately 60 ml./hr. and fractions of about 2.5 ml. were collected. 0.05 ml. of each was applied to 3MM paper for electrophoresis at pH 6.5 as described in Section 11.4. The result is shown in Figure 13.1.1. The seven fractions indicated in the Figure were pooled and freeze-dried.

Each was separated by electrophoresis at pH 6.5 on 30 cm. of 3MM paper. Marker strips were developed with ninhydrin and also with stains for tryptophan, tyrosine and methionine. Those fractions which/

which contained methionine but not tryptophan were oxidised with performic acid after elution (Section 3.5.). Fractions were then purified by chromatography in BAWP and electrophoresis at pH 3.5 as usual. Any additional purification is noted in Table 13.3.1.

13.2. Digests 2 & 3.

These two digests were performed to obtain peptides which represented regions of the molecule particularly accessible in peptic digests.

Digest 2 was performed on 5.5 micromoles of trypsin-released enzyme (PP15). Unless otherwise noted the experiment was performed as digest 1 had been. 4 mg. pepsin was used. The digest was removed by centrifugation, dissolved in 1 ml. 2 M-ammonia and added to the rest of the digest without re-precipitation. The gel-filtration was performed on a column of Sephadex G25 in 0.1 M-ammonia (145 cm. x 2.5 cm. diam.) at 40 ml./hr. and 6.2 ml. fractions were collected. Electrophoresis of 0.05 ml. portions gave the result shown in Figure 13.2.1. All fractions except those shown to contain tryptophan were oxidised with performic acid vapour prior to elution. Only the fractions considered to contain peptides of particular interest were further purified.

Digest 3 was performed on about 3 micromoles of PP18 (exo-enzyme) with 2.5 mg. pepsin. The digestion was performed in 10 ml. 5%/

5% formic acid; the pH was 2.0. Only the tryptophan containing peptides were purified.

13.3. Peptic peptides - summary.

Table 13.3.1. shows the amino acid analyses and N-termini of peptic peptides. The Sephadex fraction in which they eluted (Figure 13.1.1.), together with their mobilities and R_x values will be found in Table 13.3.2.

Table 13.3.1.

Amino acid analyses of peptic peptides

Peptide	PB1	PB2	PB3	PB4	PB5	PB6	PB7	PB8	PB9	PB10	PB11
Lys	3.86	3.80	0.87	0.21	2.00	-	0.82	1.15	1.00	-	0.76
His	-	-	-	-	-	-	-	-	-	-	-
Arg	0.87	0.88	1.08	2.38	-	1.15	-	-	-	1.04	-
Asp	4.23	4.07	2.04	1.86	2.08	-	-	-	-	-	-
Mes	-	-	-	-	-	-	-	-	-	-	-
Thr	-	-	1.97	1.95	-	0.95	0.90	1.02	-	-	-
Ser	2.10	1.85	-	-	-	1.09	-	1.04	-	-	-
Glu	-	-	-	1.07	-	-	-	-	-	-	-
Pro	-	-	-	-	-	-	-	-	-	-	-
Gly	-	-	1.18	0.42	1.02	-	-	-	-	-	-
Ala	1.88	1.17	1.07	0.31	1.00	1.07	1.02	1.83	0.99	0.96	1.13
Val	1.17	-	-	-	-	0.97	1.87	-	-	-	-
Met	-	-	0.81	-	0.87	-	0.96[d]	-	-	-	-
Ile	-	-	-	0.88	-	-	-	0.98	-	-	1.12
Leu	1.98	1.03	0.99	-	1.01	1.79	-	-	1.01	-	-
Tyr	0.88	0.40	-	0.88	-	-	-	-	-	-	-
Phe	-	-	-	-	-	-	-	-	-	-	-
Trp	-	-	-	-	-	-	-	-	-	-	-
N-t.	Ala	Ser	a	none	Lys	Ala & Leu	Ala	Ala	Ala	Arg	Ile[e]
Section	13.5	13.5	13.6	13.7	13.4	13.8 b	13.9 c	13.10	13.11	13.12	13.10 b

Notes [a]: Faint spots in DNS-Gly & DNS-Ser/Ala regions.

[b]: Finally purified at pH 9.5.

[c]: 96 hr. hydrolysis.

[d]: From 24 hr. hydrolysis of duplicate sample.

[e]: Together with material behaving like bis-DNS-lysine; see text.

Table 13.13.1

Peptide	PN1	PN2	PN3	PN4	PN5	PN6	PN7	PN8	PN9	PN10
Lys	-	-	0.87	0.93	-	1.75	-	0.93	-	1.00
His	-	-	-	-	-	-	-	-	-	-
Arg	3.03	2.78	(1)[f]	-	0.84	0.88	1.10	-	0.94	-
Asp	3.03	3.04	3.02	0.94	3.09	0.96	3.07	-	3.12	0.95
Mes	-	-	-	-	-	-	-	-	-	-
Thr	3.02	2.87	2.09	-	-	-	-	1.04	-	-
Ser	-	-	-	-	1.05	0.96	0.96	-	0.96	-
Glu	1.12	1.44	0.15	-	1.17	2.35	1.09	1.33	1.11	-
Pro	0.97	0.96	0.12	3.14	-	0.97	-	-	-	-
Gly	1.05	1.19	1.16	1.07	-	-	-	-	-	1.08
Ala	2.68	1.69	1.22	-	2.10	-	1.94	2.13	2.02	1.11
Val	1.16	1.03	0.12	2.05[g]	-	-	-	1.90	-	-
Met	-	-	0.76	-	-	-	-	0.96	-	-
Ile	-	-	0.88	1.81[g]	0.99	-	-	0.96	-	0.95
Leu	1.07	1.12	1.00	1.08	1.96	3.13	1.00	-	-	1.06
Tyr	0.97	0.94	0.11	-	0.81	-	0.83	-	0.85	-
Phe	1.88	0.96	0.14	-	-	-	-	-	-	0.85
Trp	-	-	f	f	-	-	-	-	-	-
N-t.	Ala	Ala	Ile	Ile	Arg	Asx	Arg	Ile	Arg	Asx
Notes				c						
Section	13.13.	13.13.	13.6.	13.14.	13.15.	13.12.	13.15,	13.9.	13.15.	13.11.

[f]: Detected by qualitative analysis.

[g]: 24 hr. values approx. 1.0 to 1.5 residues.

Table 13.3.1.

Peptide	PN11	PN12	PN13	PN14	PN15	PN16	PN17	PN18	PN19
Lys	-	-	-	-	0.97	-	-	-	-
His	-	-	-	-	-	-	-	-	-
Arg	0.90	-	-	-	-	-	-	-	-
Asp	-	-	0.53	-	1.11	-	0.15	0.17	-
Mes	-	-	-	-	-	-	-	-	-
Thr	-	1.02	1.23	0.90	-	-	0.90	0.99	-
Ser	0.18	-	1.06	0.95	-	0.11	1.21	-	-
Glu	1.16	-	-	-	-	-	-	-	-
Pro	-	-	-	-	-	-	-	-	-
Gly	-	0.99	-	0.22	0.19	0.94	-	0.20	-
Ala	1.95	-	1.78	-	1.00	-	0.89	0.93	-
Val	-	1.99	-	1.27	-	-	-	0.20	-
Met	-	-	-	-	-	-	-	-	-
Ile	-	-	-	-	-	1.02	-	0.20	0.99
Leu	1.00	2.00	-	1.87	0.02	-	-	1.08	2.01
Tyr	-	-	-	-	-	-	-	-	-
Phe	1.00	-	0.93	-	-	1.04	-	-	-
Trp	-	-	-	-	-	-	-	-	-
N-t.	Arg	Leu	Ala	Leu	Asx	Gly	Ala	Ala	Leu
Section	13.12.	13.14.	13.10.	13.8.	13.11.	13.11.	13.10.	13.16.	13.15.

Table 13.3.1.

Peptide	PA1	PA2	PA3	PA4	PA5	PA6	PA7	PA8	PA9	PA10
Lys	3.98	0.78	1.20	-	-	1.65	-	0.91	1.03	0.93
His	-	-	-	-	-	-	-	-	-	-
Arg	1.89	1.84	2.42	h	0.82	0.90	-	-	-	-
Asp	1.81	4.16	5.02	3.10	2.93	1.04	2.79	2.10	0.98	1.11
Mes	-	-	0.73	-	-	-	-	-	-	-
Thr	0.88	1.80	4.60	2.73	2.96	-	2.78	-	-	-
Ser	1.09	1.24	-	-	1.09	1.05	1.03	0.94	0.83	-
Glu	7.15	1.21	2.42	1.17	3.04	3.30	3.15	4.42	3.01	2.91
Pro	2.71	0.85	0.95	0.92	0.95	1.21	0.87	-	-	-
Gly	3.17	4.07	1.13	1.20	1.07	0.20	1.31	-	-	0.24
Ala	-	5.03	0.32	1.97	2.01	1.08	-	-	-	1.07
Val	0.78	2.07	2.13	1.04	1.02	-	1.08	-	-	-
Met	-	-	-	-	-	-	-	-	-	-
Ile	2.20	1.95	1.72	-	-	-	-	0.90	1.10	-
Leu	3.30	-	1.53	1.00	-	4.07	-	1.73	2.04	0.95
Tyr	-	1.03	0.98	0.86	-	-	-	-	-	-
Phe	1.12	-	-	-	-	0.74	-	-	-	1.03
Trp	-	f	-	-	-	-	-	-	-	-
N-t.	Lys	Ile	None	Ala	Asx	Phe	Asx	Leu	Leu	Glx
Section	13.17.	13.18.	13.7.	13.13.	13.19.	13.12.	13.19.	13.20.	13.20.	13.11.

Note

h: Not determined accurately.

Table 13.3.1.

Peptide	PA11	PA12	PA13	PA14	PA15	PA16	PA17	PA18	PA19
Lys	0.76	0.79	0.94	-	-	-	-	-	-
His	-	-	-	-	-	-	-	-	-
Arg	-	-	-	-	1.06	-	-	-	-
Asp	1.11	2.00	-	1.03	1.03	-	-	1.07	-
Mes	-	1.15	-	-	-	-	-	-	-
Thr	-	0.94	-	-	-	-	-	-	-
Ser	0.95	-	-	1.00	-	-	-	-	-
Glu	1.07	1.10	3.15	-	0.90	3.09	1.12	-	1.04
Pro	-	-	-	-	0.95	-	-	-	-
Gly	2.00	-	-	-	1.88	-	-	-	-
Ala	-	-	0.85	2.02	1.11	-	1.00	-	0.99
Val	-	-	-	-	1.03	-	-	-	-
Met	-	-	-	-	-	-	-	-	-
Ile	-	-	-	-	1.03	-	-	0.93	0.97
Lue	2.06	-	1.01	1.94	-	-	1.06	-	-
Tyr	-	-	-	-	-	-	-	-	-
Phe	-	-	1.06	-	-	0.91	0.82	-	-
Trp	-	-	-	-	f	-	-	f	-
N-t.	Lys	Thr	Ala	Leu	Ile	Glx	Phe	Ile	Ile
Section	13.16.	13.4.	13.11.	13.16.	13.18.	13.11.	13.12.	13.6.	13.9.

Note

[i]: Only found in digest 3.

Table 13.3.2.

Mobilities and R_x values for peptic peptides

Peptide	Sephadex fraction	m	R_x	m'	Peptide	Sephadex fraction	m	R_x	m'
PB1	2	+0.20	0.17	+1.60	PN15	5	0	0.49	+0.95
PB2	2	+0.20	0.08	+1.60	PN16	6	0	1.06	+0.66
PB3	5	+0.22	0.55	+1.31	PN17	5	0	0.41	+0.67
PB4	3	+0.19	0.41	+1.5[a]	PN18	5	0	0.90	+0.80
PB5	4	+0.27	0.27	+1.42	PN19	4	0	1.39	+0.92
PB6	4	+0.27	1.04	+1.33	PA1	1	-0.17	0.52	+1.31
PB7	4	+0.34	0.84	+1.45	PA2	1	-0.11	0.55	+0.83
PB8	4	+0.34	0.61	+1.54	PA3	2	-0.04	smear 0.2-0.4	smear ca.+1.00
PB9	5	+0.55	0.64	+2.45	PA4	3	-0.12	0.41	+0.83
PB10	6	+0.68	0.42	+2.40	PA5	2	-0.40	0.13	+0.46
PB11	5	+0.55	0.54	+2.19	PA6	2	-0.16	0.71	+1.23
PN1	3	0	0.78	+0.96	PA7	2	-0.58	0.17	-0.14
PN2	3	0	0.66	+1.06	PA8	3	-0.19	0.47	+0.74
PN3	4	0	0.72	+1.00	PA9	3	-0.19	0.68	+0.92
PN4	4	0	1.22	+0.85	PA10	3	-0.54	0.54	+0.65
PN5	4	0	0.80	+0.72	PA11	3	-0.28	0.71	+0.84
PN6	3	0	0.53	+1.34	PA12	3	-0.54	0.35	+0.62
PN7	4	0	0.47	+0.82	PA13	4	-0.30	0.61	+1.00
PN8	3	0	0.92	+1.00	PA14	4	-0.38	0.92	+0.33
PN9	4	0	0.20	+0.83	PA15	4[b]	-0.19	-	+0.79
PN10	4	0	0.89	+0.52	PA16	4	-0.78	0.47	+0.10
PN11	5	0	0.90	+1.34	PA17	5	-0.40	1.10	+0.49
PN12	4	0	1.39	+0.68	PA18	7	-0.42	0.93	+0.23
PN13	4	0	0.72	+0.52	PA19	4	-0.50	0.75	+0.55
PN14	4	0	1.22	+0.58					

[a]: Approximately. [b]: Found in corresponding fraction of digest 3.

13.4. Peptides PB5, PN12 & PA12.

PB5. The mobility of this peptide shows it contains one residue each of aspartic acid and asparagine. The peptide was resistant to the action of CPA. The sequence Lys-Ala-Leu-Asx-Mes-Asx-Gly- was determined by the DNS-PTC method, and the remaining lysine residue must presumably be at the C-terminus. The inhibitory effect of penultimate glycine on the action of CPA has been noted by Ambler (1967b). The findings are consistent with a sequence of

Lys-Ala-Leu-Asx-Mes-Asx-Gly-Lys.

PN12. This peptide released leucine and valine with CPA. The sequence

Leu-Thr-Val-Gly-Val-Leu

was determined by DNS-PTC degradation.

PA12. The sequence of this peptide was determined directly by the DNS-PTC method. The result was

Thr-Glu-Mes-Lys-Asp-Asp-Phe.

CPA released phenylalanine. The mobility of the peptide rules out amides.

13.5. Peptides PB1 & PB2.

These peptides apparently differ in analysis by one residue each of alanine, valine and leucine. The tyrosine recoveries were found/

found to be very variable in all peptic peptides from this region; in some analyses the tyrosine was almost completely destroyed. The difference in N-terminus suggests that the extra residues are at the N-terminus. Each peptide released leucine with CPA.

Peptide PB1 was degraded with trypsin. The fragments were separated by electrophoresis at pH 6.5 and the details are shown in Table 13.5.1.

Table 13.5.1.

Products of tryptic digestion of PB1.

Peptide	m	N-t.	Lys	Arg	Asp	Ser	Ala	Val	Leu	Tyr
PB1TB1	+0.58	Asx	1.91	-	1.08	0.21	-	-	-	-
PB1TB2	+0.49	Ala	0.13	0.87	-	2.03	1.04	1.11	0.95	-
PB1TN1	0	Asx	1.08	0.11	1.01	0.29	0.91	-	-	-
PB1TA1	-0.33	Tyr	0.93	-	2.25	-	-	-	1.08	0.75

The mobilities of the original peptides and its degradation products show that amides are absent. PB1TB1 and PB1TN1 are evidently the tryptic peptides TB1, Asp-Lys-Lys and TN10, Asp-Ala-Lys respectively. The analysis and properties of PB1TN2 are similar to the tryptic peptide TB11, and the sequence Ala-Val-Leu-Ser-Ser-Arg was confirmed by DNS-PTC degradation. Peptide PB1TA1 also gave/

gave leucine with CPA, and the DNS-PTC method gave the sequence Tyr-Asp-Asp-Lys-Leu.

The sum of the analyses of the four fragments agrees with PB1. It is evident that PB1TB2 is N-terminal and PB1TA1 C-terminal, but we cannot order the other two peptides. This problem was solved by DNS-PTC degradation of PB2. The sequence

Ser-Ser-Arg-Asx-Lys-Lys-Asx-Ala-

was obtained, sufficient to prove the hypothesis of the relation of PB1 and PB2 and to order TB1 and TN10. Thus the sequence of PB1 is

Ala-Val-Leu-Ser-Ser-Arg-Asp-Lys-Lys-Asp-Ala-Lys-Tyr-Asp-Asp-Lys-Leu.

13.6. Peptides PB3, PN3 & PA18.

Peptides PB3 and PN3 differ in analysis by one residue each of isoleucine, aspartic acid and tryptophan, which is the composition of PA18. The N-terminus of PB3 & PA18 were isoleucine, while the latter gave DNS-Asx after one cycle of DNS-PTC degradation. This suggests Ile-Asp-Trp as a likely sequence for PA18.

Peptide PN3 was degraded with trypsin, and gave free arginine, together with two peptides, shown in Table 13.6.1., which were purified by electrophoresis at pH 6.5.

Table 13.6.1.

Products of tryptic degradation of PN3.

Peptide	m	N-t.	Lys	Arg	Asp	Thr	Gly	Ala	Met	Ile	Leu	Trp
PN3TB1	+0.45	Ile	1.04	0.96	1.16	-	-	-	0.80	1.04	-	a
PN3TA1	-0.42	Asx	-	-	2.05	1.88	1.03	1.03	-	-	1.00	-

[a]: Detected by qualitative analysis.

Insufficient material was obtained to allow further characterisation.

The finding of free arginine suggests a sequence -Lys-Arg as the C-terminus of PN3TB1, and consideration of the data obtained with PA18 suggests that the likeliest sequence is Ile-Asp-Trp-Met-Lys-Arg.

From this it appears that the N-terminus of PB3 should be methionine. This was not satisfactorily demonstrated, but the finding of faint spots in front of and behind DNS-OH is not inconsistent with N-terminal methionine.

Peptide PB3 released leucine faintly with CPA.

The analyses of the two peptides suggest they are related to TA7 and TN7.

13.7. Peptides PB4 and PA3.

These peptides gave unsatisfactory amino acid analyses. PA3 contains the only histidine residue in the molecule. The analysis can best be approximately reconciled with the sequence of this region, derived/

derived from tryptic and chymotryptic peptides, if it is considered to be

Gln-Arg- TB8 - TA8 -His-Val-Asp-Thr-Gly-Met-Thr-Leu.

On this basis the worst discrepancy is 1.53 leucines for 2. The tyrosine is 0.98 for 2 but tyrosine recoveries were frequently bad in this digest. Pyrrolidonisation of glutamine might explain the absent N-terminus.

The analysis of PB4, which also failed to give an N-terminus, can also be explained in this region of the molecule. A sequence

Gln-Arg-Ile-Thr-Tyr-Thr-Arg-Asp-Asp

would agree with the amino acid analysis and also explain the absence of N-terminus. The peptide was isolated in small quantity. However, this is incompatible with the mobility and the material is better regarded as a mixture.

13.8. Peptides PB6 & PN14.

Peptide PN14 was degraded by the DNS-PTC method and gave the sequence

Leu-Val-Thr-Ser-Leu.

Peptide PB6 has in addition one residue each of alanine and arginine. Electrophoresis at pH 9.5 did not alter its two N-termini, alanine and leucine. Insufficient material remained after this for further experiment. If the sequence around peptide TB2

-Arg-Ala-Leu-Val-Thr-Ser-Leu-Arg-Ala-Phe-

is considered, it is evident that PB6 behaves as a mixture of two peptides, one with the alanine residue at the C-terminus and the other/

other with it at the N-terminus. The latter would be identical with TB2 and would require pepsin to split the two -Arg-Ala- bonds.

13.9. Peptides PB7, PN8 & PA19.

Peptide PN8 released lysine, valine and methionine sulphone with CPA. The mobility shows that the acid residue is not amidated. A partial sequence Ile-Ala-Glu-Ala- was determined by the DNS-PTC method.

The sum of the other two peptides is equal to PN8. PA19 gave the sequence Ile-Ala-Glu by the DNS-PTC method, confirming that it is the N-terminal part of PN8. Peptide PB7 also gave valine and methionine sulphone with CPA. 24 hr. hydrolysis gave about 1.4 residues of valine suggesting the presence of a -Val-Val- sequence. The data proves the partial sequence

$$\text{Ile-Ala-Glu-Ala-(Thr,Lys,Val}_2\text{,Met)}.$$

So the peptides are related to TB4 and TN4.

13.10. Peptides PB8, PB11, PN13 & PN17.

Peptide PN17 has the sequence Ala-Ser-Thr, determined by the DNS-PTC method.

Even after electrophoresis at pH 9.5, peptide PB11 apparently gave bis-DNS-lysine as well as DNS-Ile on N-terminal determination. It was, however, observed that the "bis-DNS-lysine" disappeared after

72/

72 hr. hydrolysis, and when the sequence Ile-Lys-Ala was determined by the DNS-PTC method, it was realised that the material was DNS-Ile-(ε-DNS)-Lys.

The analysis of PB8 was the sum of the above two peptides. It had N-terminal alanine, and also released alanine with CPA. The complete sequence was determined by the DNS-PTC method and was Ala-Ser-Thr-Ile-Lys-Ala.

Peptide PN13 was still impure. The composition shows the peptide must originate from peptide TB7, from which the three peptides above also come, and this suggests that the principal component may be Ala-Phe-Ala-Thr-Ser.

13.11. Peptides PB9, PN10, PN15, PN16, PA10, PA13 & PA16.

These seven peptides form a mutually overlapping set covering fourteen residues.

The sequences Ala-Lys-Leu and Gly-Ile-Phe for PB9 & PN16 were determined by the DNS-PTC method.

The aspartic acid residue in PN15 cannot be amidated. A partial sequence Asp-Ala-Lys- was determined by the DNS-PTC method, while leucine was released by CPA. So the peptide is Asp-Ala-Lys-Leu.

The mobility of PA16 shows that one of the residues is glutamine. DNS-PTC degradation was performed and the peptides were electrophoresed at each step. The data are shown in Table 13.11.1.

Table 13.11.1.

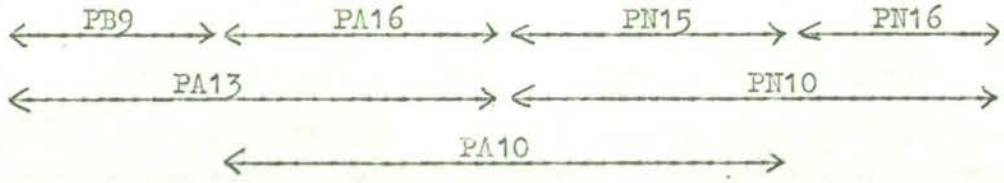
DNS-PTC degradation of PA16.

Peptide	E0	F1	F2	F3
N-terminus	Glx	Glx	Glx	Phe
m	-0.78	-0.38	-0.05	0

The slight acid mobility of PA16F2 is due to its glutamine N-terminus. Thus the sequence is Glu-Glu-Gln-Phe.

The compositions and N-termini of the other three peptides establish the relations:

Ala-Lys-Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys-Leu-Gly-Ile-Phe



In confirmation PA13 gave phenylalanine with CPA and the partial sequence Ala-Lys-Leu-Glx-Glx- with the DNS-PTC method, PA10 gave leucine with CPA and PN10 gave a partial sequence Asp-Ala-Lys-, as well as liberating isoleucine and phenylalanine with CPA.

13.12. Peptides PB10, PN6, PN11, PA6 & PA17.

The largest peptide (PA6) in this group differs from CA3 only in a phenylalanine residue, which is at the N-terminus of PA6.

Peptide PB10 is apparently Arg-Ala.

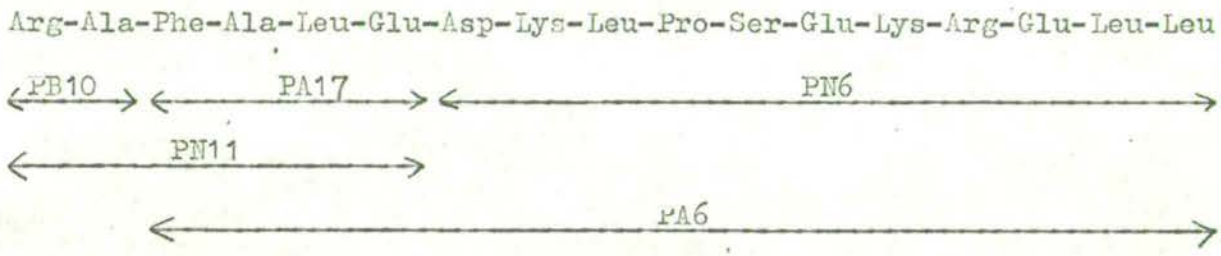
Peptide/

Peptide PN11 gave the partial sequence Arg-Ala-Phe-Ala- by the DNS-PTC method and released alanine, leucine and glutamic acid with CPA.

Peptide PA17 showed alanine as its second residue by the DNS-PTC method and was resistant to the action of CPA.

Peptides PA6 & PN6 both released leucine and glutamic acid with CPA, and the latter gave Asx-Lys-Leu- by the DNS-PTC method.

If these data are considered together with the sequence established for peptide CA3 in Section 12.17. they may be formulated



13.13. Peptides PN1, PN2 & PA4.

The two neutral peptides differ in analysis by one residue each of alanine and phenylalanine. Phenylalanine is released from PN2 by CPA. The sequence of these peptides was investigated by degrading PN1 with trypsin. Electrophoresis at pH 6.5 showed that all the fragments were neutral, and they were separated at pH 3.5. (Table 13.13.1.).

Table 13.13.1.

Products of tryptic degradation of PN1.

Peptide	N-t.	m	m'	Arg	Asp	Thr	Glu	Pro	Gly	Ala	Val	Leu	Tyr	Phe
PN1TN1	Thr	0	+1.23	1.79	0.98	0.90	1.15	1.05	-	1.05	1.07	-	0.31	-
PN1TN2	Ala	0	+0.85	0.91	2.14	1.97	-	-	1.05	0.95	-	0.99	-	-
PN1TN3	Phe	0	+0.62	-	-	-	-	-	0.15	1.11	-	-	-	1.89

Peptide PN1TN1 was identical in N-terminus and analysis to peptide TN8, and the mobility at pH 3.5 was substantially the same.

Similarly peptide PN1TN2 has the same amino acid analysis as the minor tryptic peptide TN12. The mobility at pH 3.5 was also the same. Partial sequence determination by the DNS-PTC method gave Ala-Leu-Asx.

The sequence of PN1TN3 was determined by the DNS-PTC method, and was Phe-Ala-Phe. This is evidently the C-terminus of PN1. The N-termini show that PN1TN2 is the N-terminal region, so PN1 is

TN12 - TN8 - Phe-Ala-Phe.

The properties of PN2 are fully consistent with its lacking the last two residues.

The amino acid analysis of PA4 suggests that it is the same as PN2, but lacking the phenylalanine residue. But the mobility of the peptide is acid, which is not consistent with this. However the analysis was defective and arginine was not determined. Tryptic digestion/

digestion gave an acidic peptide (m -0.21) which stained with the 1-nitroso-2-naphthol reagent. This is consistent with a peptide the size of TN8 with one charge, suggesting a sequence

TN12 - Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu

for PA4, the peptide lacking one residue each of arginine and phenylalanine. Nothing significant was liberated from PA4 with CPA.

13.14. Peptide PN4.

In view of the overall tryptophan analysis there was no reason to suppose that this peptide contained more than one tryptophan residue.

DNS-PTC degradation gave the result Ile-Ile- x-Pro-Pro- where no DNS-amino acid could be detected at the second cycle.

The peptide was degraded with trypsin. The fragments were separated at pH 6.5 and are shown in Table 13.14.1.

Table 13.14.1.

Products of tryptic degradation of PN4.

Peptide	m	N-t.	Lys	Asp	Pro	Gly	Val	Ile	Leu	Trp
PN4TB1	+0.36	Ile	1.03	-	2.09	-	-	1.88	-	a
PN4TA1	-0.38	Gly	-	1.03	1.00	0.97	1.99	-	1.01	-

Both these analyses were done after 96 hr. hydrolysis.

a: Detected after electrophoresis at pH 2.0 and in peptide by Ehrlich stain.

Peptide PN4TA1 was degraded by CPA and the amino acids analysed; valine and leucine had been released.

The sequence by the DNS-PTC method was Gly-Asp-Pro-Val-Val-Leu.

The mobilities of the peptides rule out asparagine.

These data show that the sequence of PN4 is

Ile-Ile-Trp-Pro-Pro-Lys-Gly-Asp-Pro-Val-Val-Leu.

13.15. Peptides PN5, PN7, PN9 & PN19.

The first three of these peptides had the same N-terminus and analyses differing only in a few residues. They contain one free acid residue each. PN9 gave alanine, glutamine and asparagine with CPA; PN7 gave the same amino acids and also leucine.

PN9 was degraded by the DNS-PTC method, and the mobilities of the peptides were determined at various stages.

Table 13.15.1.

DNS-PTC degradation of peptide PN9.

Peptide	F1	F2	F3	F4	F5
N-terminus	Tyr	Ser	Asx	Asx	Ala
m			-0.40	-0.08	0

These data show the partial sequence

Arg-Tyr-Ser-Asp-Asn-Ala-(Ala,Asn,Gln).

The/

The slight acid mobility of PN9F4 is due to its asparaginyl N-terminus.

Degradation by the same method of PN7 showed that the sequence was

Arg-Tyr-Ser-Asx-Asx-Ala-Ala-(Asn,Gln)-Leu.

The placing of the leucine residue is because it is the only difference from PN9.

The analysis of PN5 shows that it has in addition one residue each of leucine and isoleucine, and is

Arg-Tyr-Ser-Asp-Asn-Ala-Ala-(Asn,Gln)-Leu-(Ile,Leu).

Peptide PN19 must originate from these last three residues. DNS-PTC degradation showed the second residue to be isoleucine, so the peptide is

Leu-Ile-Leu.

13.16. Peptides PN18, PA11 & PA14.

The mobilities of these peptides show that amides are absent. A complete sequence determination by the DNS-PTC method was done on the two acid peptides and the sequences were:

PA11: Lys-Glu-Leu-Ala-Asp-Ala-Ser-Leu

PA14: Leu-Ala-Asp-Ala-Ser-Leu.

Peptide PN18 gives serine after one cycle of DNS-PTC degradation, and releases leucine with CPA, so it is Ala-Ser-Leu.

Thus/

Thus it is evident that these three peptides all have a common C-terminal sequence.

13.17. Peptide PA1.

This peptide was degraded with trypsin. The products formed are listed in Table 13.17.1. Free lysine was also observed.

Table 13.17.1.

Products of tryptic digestion of peptide PA1.

Peptide	PA1TB1	PA1TB2	PA1TN1	PA1TN2	PA1TA1	PA1TA2	PA1TA3
N-t.	Lys	Lys	Glu	none	Lys	Ile	Phe
m	+0.58	+0.35	0	0	-0.23	-0.47	-0.66
m'			+1.33	+1.05			
Lys	2.85	1.85	-	1.71	1.03	0.11	-
Arg	-	-	0.93	-	0.78	0.72	-
Asp	-	-	-	0.27	1.80	1.94	-
Thr	-	-	-	-	0.81	0.92	-
Ser	0.98	0.97	-	1.08	-	-	-
Glu	2.12	2.15	1.16	2.21	2.23	2.21	2.21
Pro	0.92	0.93	-	0.73	0.93	1.06	0.94
Gly	1.98	2.04	0.12	1.87	1.15	1.14	0.13
Val	-	-	-	-	1.32	1.09	-
Ile	1.00	0.93	-	0.88	0.95	0.91	-
Leu	1.08	1.01	0.90	1.23	-	0.16	0.97
Phe	-	-	-	-	-	-	0.89

This peptide evidently corresponds in part to peptide CA5, and peptides to which these correspond are: PA1TN1 to CA5TN1 and TN11; PA1TN2 to TN1; PA1TA1 to CA5TA1 and TA5; PA1TA2 to TA10; PA1TA3 to TA2PA2.

Three/

Table 13.18.1.

Products of tryptic degradation of PA2.

Peptide	PA2TB1	PA2TB2	PA2TA1	PA2TA2
N-t.	Ile[a]	Thr	Ala	Asx
m	+0.65	+0.30	-0.38	-0.52
Lys	-	-	0.92	-
Arg	1.01	0.96	-	-
Asp	-	-	2.01	1.99
Thr	-	1.94	-	-
Ser	-	1.15	0.12	-
Glu	-	-	1.10	-
Pro	-	-	1.03	-
Gly	0.13	2.09	2.03	0.22
Ala	-	2.02	1.98	1.06
Val	-	-	2.02	-
Ile	0.99	-	-	0.95
Tyr	-	0.84	-	-
Trp	-	-	b	-

[a]: Together with DNS-Ile-Arg.

[b]: Detected by qualitative analysis.

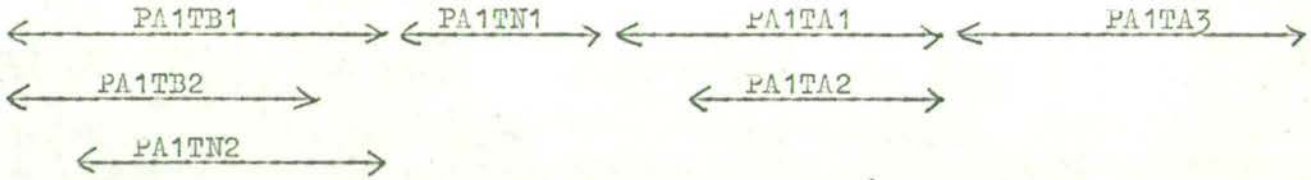
Three of these peptides evidently form a group, PA1TB1, PA1TB2 & PA1TN2 were all isolated in low yield and have the unique composition of TN1. Peptide PA1TB2 showed glutamic acid after one cycle of DNS-PTC degradation. The first two have lysine attached to the front of TN1, and differ according to the number of lysines at the C-terminus. The N-t glutamine of PA1TN2 is pyrrolidonised.

Direct DNS-PTC degradation of PA1 gave the partial sequence:

Lys-Glx-Ile-Gly-Gly-Pro-Glx-Ser-.

Hence the peptides are:

Lys-Gln- TA1 -Lys-Lys-Glu-Leu-Arg-Lys-Ile- TA10 -Arg-Phe-Glu-Pro-Glu-Leu



13.18. Peptides PA2 & PA15.

These two peptides, from digests 1 & 3 respectively, each contain the tryptophan residue which in the tryptic digest is found in peptide TA9.

Peptide TA2 was degraded with trypsin. The peptides were separated by electrophoresis at pH 6.5 and details are given in Table 13.18.1.

Peptides PA2TB2 & PA2TA1 correspond in analysis and mobility to tryptic peptides TB5 & TA9 respectively. After one cycle of DNS-PTC degradation PA2TB1 was converted to free arginine, identified by electrophoresis at pH 6.5, so the peptide is Ile-Arg.

Peptide PA2TA2 was degraded by the DNS-PTC method, showing the sequence Asx-Asx-Ile-Ala. The mobility of the peptide shows that one residue of asparagine is present. PA2TA2F1 was acidic, m -0.60, while PA2TA2F2 was neutral, so the sequence was

Asn-Asp-Ile-Ala.

The existence and analysis of PA15 shows that the Ile-Arg peptide is linked directly to TA9 and not to TB5. The sequence of PA2 is therefore

Ile-Arg- TA9 - TB5 -Asn-Asp-Ile-Ala.

It is evident from the composition of PA15 that it contains the N-terminal part only of TA9. It was degraded with subtilisin B and the fragments were separated by electrophoresis at pH 6.5 & pH 3.5. Table 13.18.2. shows those fragments which were purified in large amount. The other fragments were impure, but were not inconsistent with the structure proposed.

Table 13.18.2.

Subtilisin B digestion of peptide PA15.

			Qualitative analysis								
Peptide	m	m'	N-t.	Arg	Gly	Ala	Val	Ile	Pro	Glu	Asp	Trp
PA15SB1	+0.61	+2.02	Ile	+++	-	+++	-	+++	-	-	-	-
PA15SA1	-0.40	+0.52	Gly	-	++	-	-	-	-	++	-	++
PA15SA2	-0.43	+0.39	Gly	-	++	-	++	-	++	-	++	-
PA15SA3	-0.53	+0.25	Gly	-	++	-	++	-	++	++	++	++

These fragments were examined by the DNS-PTC method. PA15SA1 gave a little glutamic acid after one cycle and much after two cycles, suggesting the sequence Gly-Trp-Glu. PA15SB1 was Ile-Arg-Ala, while PA15SA2 gave the result Gly-Val-Pro-Asp, and PA15SA3 gave a partial sequence Gly-Val-Pro-Asp-. All these findings are consistent with the sequence

Ile-Arg-Ala-Gly-Val-Pro-Asp-Gly-Trp-Glu

PA15SB1 PA15SA2 PA15SA1

PA15SA3

13.19. Peptides PA5 & PA7.

The analyses of these peptides differed by two residues of alanine and one of arginine. Peptide PA7 released threonine and leucine or serine with CPA. Peptide PA5 was digested with trypsin

and/

and free alanine was released. The residual peptide was re-isolated and differed only in having 1.09 residues of alanine. These results suggest that the C-terminal region of PA5 is -Ala-Arg-Ala.

It is clear from the amino acid analyses that these peptides must come from the C-terminus of peptide TA2.

13.20. Peptides PA8 & PA9.

The mobilities of these peptides show that each must contain two acid groups to leave one net negative charge. With CPA, PA9 gave leucine, while PA8 gave asparagine or glutamine or both as well.

A complete sequence determination on PA9 by the DNS-PTC method gave

Leu-Glx-Glx-Lys-Ser-Ile-Glx-Asx-Leu.

Less of the other was available, and only Leu-Glx-Glx- could be demonstrated.

It is evident that the C-terminal portions of these peptides correspond to TA13, while the same four N-terminal residues were cleaved from CN3 to give a basic fragment. Thus PA8 is

Leu-Gln-Gln-Lys-Ser-Ile-Glu-Asp-Leu-Asn-Gln

while PA9 lacks the two amides at the C-terminus.

CHAPTER 14

EXPERIMENTS WITH MALEYL-PENICILLINASE.

14.1. Maleic anhydride.

If the basic character of either lysine or arginine can be reversibly altered, trypsin will only split at the other. It is easier to devise reagents which will react with the ε-amino groups of lysine residues. After reaction, the protein is digested with trypsin which only cleaves at arginine residues. Fractionation of this mixture of peptides should be easier than fractionation of a total tryptic digest. The blocking group is then removed and trypsin digestion then forms the normal tryptic peptides, and the larger peptides from which they originate show the groups they form between the arginine residues in the sequence.

Using the trifluoroacetyl group to block lysine, this method was applied to the sequence determination of glyceraldehyde 3-phosphate dehydrogenase (Davidson et al., 1967). The reagent maleic anhydride is more suitable for this method than S-ethyltrifluorothioacetate (Butler et al., 1969). It is much more pleasant to handle, and the ε-maleyl lysine is stable at the pH used for reaction. The hydrolysis of the anhydride at pH 9.0 is rapid, so the blocking reaction is a competition between amino groups and water for the reagent./

reagent. Peptides containing the group are easily soluble, and the group is indefinitely stable above pH 6.0, while at pH 3.5 and 37° about 20% remains after 30 hr.

The chief difficulty with this method is quantitative removal of blocking groups; Dixon & Perham (1968) suggest the use of 2-methylmaleic (citraconic) anhydride instead. 2-methylmaleyl lysine decomposes quantitatively overnight at pH 3.5 and 20°. The experiments reported were done before this latest modification was proposed, and maleic anhydride was used. The tryptic digest of maleyl-penicillinase was examined to see whether the method might help the amino acid sequence determination. Harris & Perham (1968) separated the twelve tryptic peptides formed from maleyl glyceraldehyde 3-phosphate dehydrogenase by gel-filtration, chromatography on DEAE-cellulose and electrophoresis.

14.2. Tryptic digestion of maleyl-penicillinase.

About 100 mg. exo-penicillinase (PP16) was dissolved in 6 ml. 0.33 M-tris base, in a small beaker with a pH electrode. About 160 mg. redistilled maleic anhydride was gradually added, and the pH was kept above 8 with 10 M-NaOH. The modified protein was desalted over Sephadex G25 in 0.1 M-ammonia, detected by its extinction at 254 nm. and freeze-dried. It was then dissolved in 4 ml. 0.2 M-ammonium acetate, pH 8.5, and treated with 2 mg. DPCC-trypsin for 3 hr. at 37°. 4 mg. SBTI was added and the digest was dried by rotary/

rotary evaporation after an hour, and then dissolved in 5 ml. 0.5 M-ammonia.

The digest was separated on a column of Sephadex G50 in 0.1 M-ammonia (89 cm. x 1.5 cm. diam.), pumped at 20 ml./hr. 3 ml. fractions were collected and 0.05 ml. of each was applied (Section 11.4.) to 3MM paper for electrophoresis with the origin near the cathode. After 100 min electrophoresis at 22 v./cm. the paper was dried and incubated overnight at 60° in the vapour of formic acid. The acid was dried off and the paper developed in the usual way. The larger material contained at least six smeary, faint acidic peptides, with mobilities between -0.40 and -0.95. The smaller material contained neutral and basic peptides which ran and stained normally. Four fractions, two early and two late, were freeze dried.

These last fractions were separated preparatively at pH 6.5 (30 cm. 3MM paper). The peptides, detected with chlorination and 1-nitroso-2-naphthol, were eluted, dried, and dissolved in 0.5 ml. 1% pyridine and an equal volume of 10% acetic acid was added to give a pH near 3. After 24 hr. at 60°, the fractions were dried and then purified at pH 3.5. The fractions were identified by N-terminal determination and qualitative amino acid analysis. Some were quantitatively analysed. The peptides considered to be present are shown in Table 14.2.1.

Table 14.2.1.

Small peptides formed by tryptic digestion of maleyl-penicillinase.

Certain.	Possible.
Ile-Thr-Tyr.	Ala-Leu-Val-Thr-Ser-Leu-Arg.
Ala-Leu.	Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg.
Phe-Ala-Phe-Ala-Ser.	Asn-Thr-Thr-Gly-Asp-Ala-Leu-Ile-Arg.
Ile-Thr-Tyr-Thr-Arg.	

The larger peptides were found not to run on paper electrophoresis so they were separated on columns of DEAE-cellulose (DE52; 8 cm. x 1 cm. diam.) with a gradient between 200 ml. of 0.1 M-ammonium acetate, pH 8.5 and the same volume of 1.0 M buffer. The record of extinction at 254 nm. of the effluent showed at least eight ill-resolved components, present in different proportions in the two fractions. Peaks eluting at the same position from the two separations were pooled and freeze-dried. Each pooled fraction was then desalted on a small column of Sephadex G25 in 0.1 M-ammonia and freeze-dried again.

Some fractions were re-separated on similar columns with shallower gradients calculated to separate the peptides further. Thus an early fraction was separated on a gradient between 0.1 and 0.4 M. There were some small, well resolved peaks. When small amounts of fractions were analysed qualitatively for amino acids, there was good correspondence between u.v. absorption and the presence of/

of amino acids. The different peaks had clearly different compositions.

14.3. Discussion.

The use of tris buffer for the original blocking was a mistake. The tris was present in about equivalent quantities to the anhydride, and in twenty fold excess over amino groups from protein. It nevertheless appears that substantially complete blocking occurred; the smaller fractions, which might have been expected to contain normal tryptic peptides, contained no trace of lysine at all.

Several of the small peptides isolated are those which would be expected. Three of the smaller peptides evidently originated from splitting at residues other than arginine. However the number is not greater than has been found in other tryptic digests. The apparent splitting of Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys at -Ser-Thr- has not been observed in any other digest. The other two anomalous splits are chymotrypsin-like. A similar effect was seen by Harris & Perham (1968).

Column chromatography on DEAE-cellulose clearly has considerable potential for separating the larger peptides produced in this experiment. It is unlikely on its own, however, to give pure peptides. The pH stability of the maleyl group and the failure of paper electrophoresis rule out many possible techniques. The method most likely to succeed is unblocking of fractions separated by DEAE-cellulose/

cellulose followed by further ion-exchange chromatography of the peptides. Their insolubility is likely to make the use of 8 M-urea necessary.

Such techniques are an alternative to fractionation of the large peptides produced by cyanogen bromide to complete the sequence of B. licheniformis penicillinase independently of its homology with the S. aureus enzyme.

CHAPTER 15

EXPERIMENTS WITH HNB-BROMIDE

15.1. HNB-bromide.

The reagent 2-hydroxy-5-nitrobenzyl (HNB) bromide has been introduced by Koshland (Horton & Koshland, 1965) as a selective reagent for tryptophan. The chemistry of the reaction of HNB bromide with tryptophan is not fully clear, but conditions have been described (Barman & Koshland, 1967) under which one HNB group reacts per residue of tryptophan. Since the group is yellow, spectrophotometric determination of HNB groups is easy. This procedure was tried with penicillinase and the results were compared with those obtained by other methods.

The coloured label has also been used to follow tryptophan peptides in the course of peptide fractionation, and to determine the sequences around them (Dopheide & Jones, 1968). In that work the removal of the yellow label indicated the removal of the tryptophan residue in the course of sequential degradation of peptides by the PTC method. Sequences on the carboxyl side of the tryptophan residue could also be determined. An attempt was made to apply these techniques to penicillinase.

15.2./

15.2. Determination of tryptophan in penicillinase.

The procedure follows that of Barman & Koshland (1967). The details of two experiments using different quantities of HNB-bromide are in Table 15.2.1.

About 5 mg. purified penicillinase was dissolved in 1 ml. 8 M-urea brought to pH 2.7 with conc. HCl. After 18 hr. at 37° the solution was cooled and the quantity of HNB-bromide shown was added in aectone with mixing. After 10 min. the solution was applied to a column of Sephadex G25 in 5% formic acid (40 cm. x 1 cm. diam.; 150 ml./hr) and fractions of a few ml. were collected. The protein was detected and precipitated with 0.1 vol. of 50% (w/v) trichloracetic acid in each fraction. After 30 min. the protein was centrifuged and washed twice with 95% ethanol containing 2% (v/v) conc. HCl. Residual ethanol was blown off with nitrogen and the protein dissolved in 1 ml. conc. HCl.

Protein was determined by analysing for lysine in a sample diluted with an equal volume of water and hydrolysed in the normal way. HNB groups were measured on an 0.15 ml. portion. 0.35 ml. 10 M-NaOH and 2 ml. water were added and the extinction at 410 nm. (1 cm. path length) was determined. The molar extinction coefficient of the HNB group at this wavelength is 18,000 M^{-1} $cm.^{-1}$ (Horton & Koshland, 1965).

Table 15.2.1.

Expt.	HNB-bromide ml.	HNB-bromide mg./ml.	μmoles	Protein μmoles	Molar excess HNB/Trp[a]	HNB groups μmoles	HNB/protein
1	0.1	50	21.6	0.091	80	0.426	4.68
2	0.08	20	6.9	0.107	21	0.13	1.21

[a]: Assuming 3 tryptophan residues/mole penicillinase.

15.3. Preparation of HNB-tryptophan peptides.

About 100 mg. trypsin-released penicillinase (PP15) was dissolved in 10 ml. 8 M-urea adjusted to pH 2.7 with HCl. 140 mg. HNB-bromide in 1 ml. acetone was added. After a few minutes, a small precipitate was spun off in a bench centrifuge and the solution was applied to a column of Sephadex G25 in 5% formic acid (70 cm. x 1.0 cm. diam.). The column was eluted at 150 ml./hr. and 5 ml. fractions were collected. The protein was detected and precipitated by making each fraction 5% (w/v) in trichloracetic acid. The precipitated protein was washed to remove trichloracetic acid and freeze-dried.

The protein was dissolved in 1 ml. 98% formic acid and diluted to 20 ml. One small portion was used to determine HNB groups spectrophotometrically as described in Section 15.2. Another portion was hydrolysed and analysed for amino acids. The results showed 8.5 HNB/

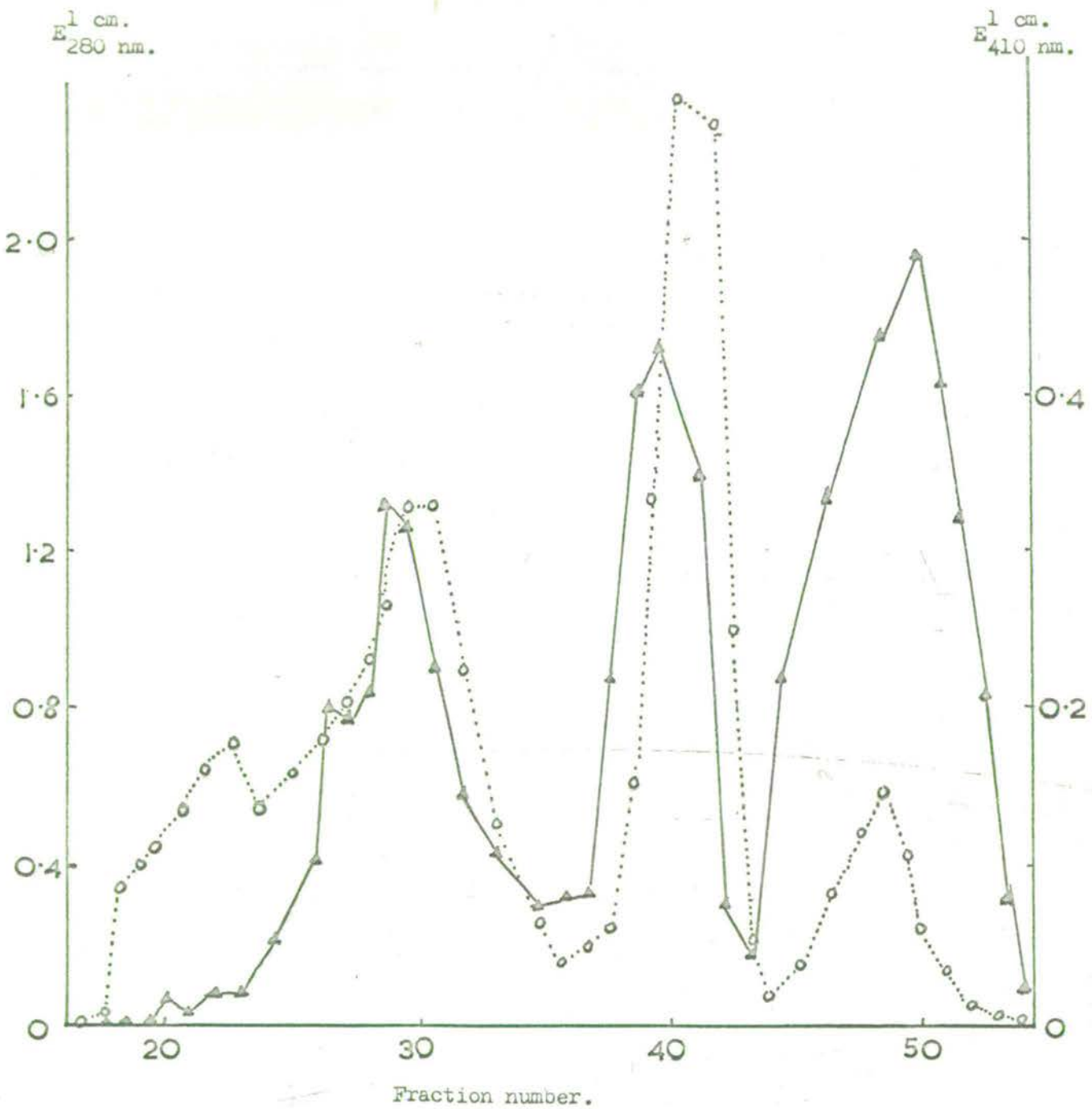


Figure 15.3.1. Chromatography of peptic digest of HNB-penicillinase on Sephadex G25. $E_{280\ nm.}$ ·····o····· . $E_{410\ nm.}$ —△— .

HNB groups per mole of protein. 2 mg. pepsin was added to the rest of the solution and after 7 hr. at 37° the digest was freeze-dried.

The digest was dissolved in 3.5 ml. 0.1 M-ammonia, and applied to a column of Sephadex G25 in the same solvent (130 cm. x 1.5 cm. diam.). The column was eluted at 80 ml./hr. 70 drop fractions were collected; each was 4.5 ml. The extinction of each fraction at 280 nm. and 410 nm. was determined and the results are shown in Figure 15.3.1. 0.05 ml. of each fraction was also electrophoresed at pH 6.5 (Section 11.4.). Before development with ninhydrin the paper was dipped in 10% aq. ammonia (sp. gr. .880) in acetone and the yellow spots were marked. Figure 15.3.2. shows the pattern that was obtained. Fractions 1, 2 & 3 were pooled and freeze-dried. No peptides were found in fraction 3, which, to judge from its elution volume, was free HNB-OH or HNB-tryptophan. It was not investigated further.

Fractions 1 & 2 were each dissolved in a little 0.1 M-ammonia and separated by electrophoresis at pH 6.5 on 13 cm. bands of 3MM paper. The peptide bands were located by exposure of the paper to ammonia vapour, but marker strips were also stained with ninhydrin to help assess the purity of the peptides.

4 yellow bands, numbered as in Figure 15.3.2. were eluted with 0.1 M-ammonia. 0.25 vol. 0.2 M-ammonium acetate, pH 8.5 was added directly to the eluate with 0.1 mg. thermolysin. The fractions were incubated/

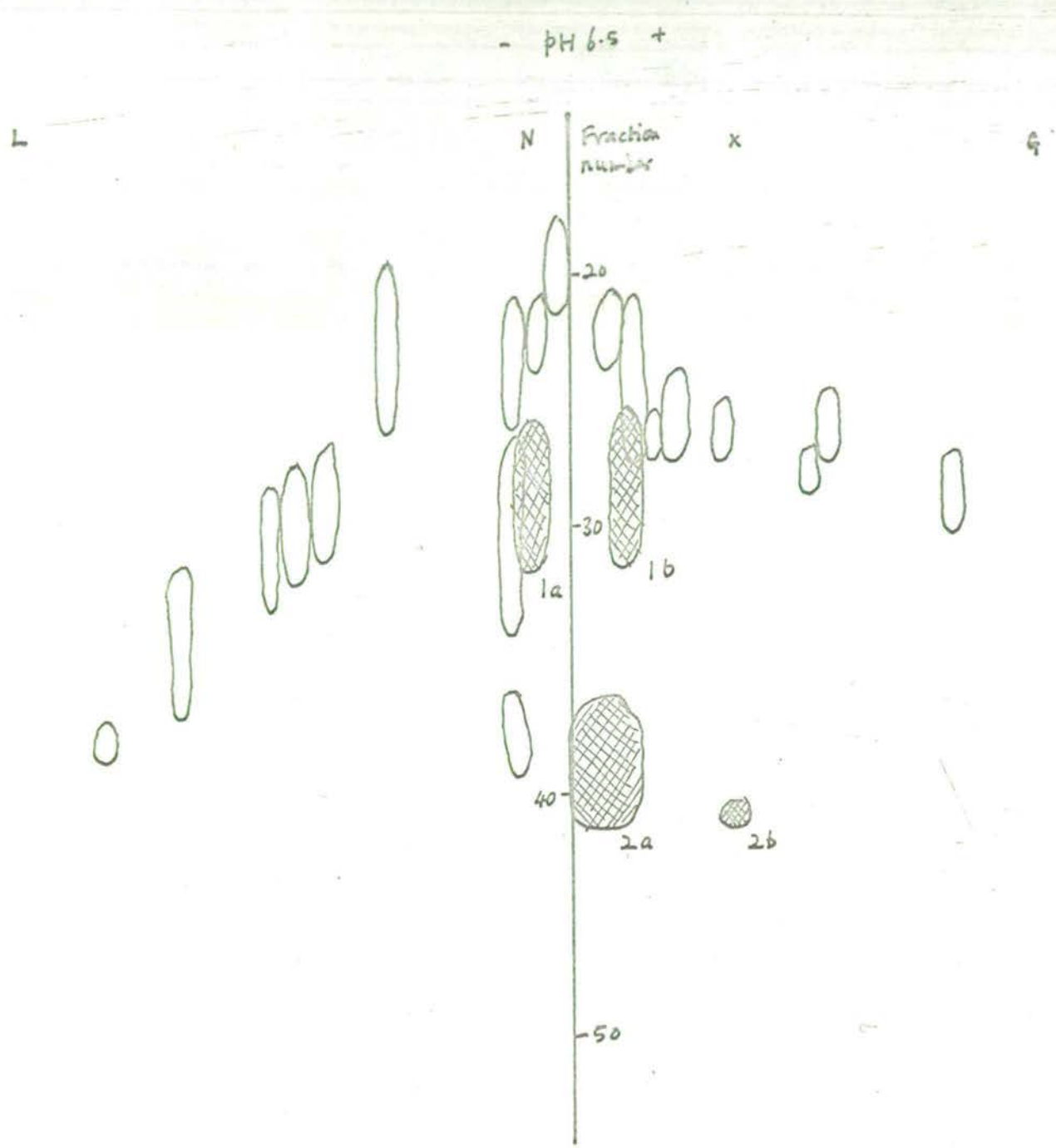


Figure 15.3.2. Electrophoresis at pH 6.5 of peptic digest of HNB-penicillinase separated on Sephadex G25 (Figure 15.3.1.).

○ : Peptide detected with ninhydrin. ⊗ : Peptide yellow with ammonia.

Standard substances:

L, lysine; N, monoaminomonocarboxylic acids; X, XCFF; G, glutamic acid.

incubated at 37° overnight and each was then applied directly to a column of Sephadex G15 (67 cm. x 1.0 cm. diam.) in 0.1 M-ammonia eluted at 80 ml./hr. 4 ml. fractions were collected, and fractions containing yellow material were pooled and freeze-dried. Fractions 1a & 1b gave two fractions with yellow material, labelled 1 & 2 in order of elution; fractions 2a & 2b did not split.

Each fraction was then separated by electrophoresis at pH 6.5, followed by pH 3.5 for neutral peptides. After this the ninhydrin-positive material seen on markers coincided with the yellow bands found after exposure to ammonia. Table 15.4.1. shows details of the peptides found. The samples used for amino acid were hydrolysed and dried. Each was then dissolved in 0.4 ml. 0.25 NaOH and the extinction at 410 nm. was determined in a micro-cell. After this 0.1 ml. M-HCl was added, followed by a suitable volume of amino analyser internal standard solution. These samples were found satisfactory for amino acid analysis. No agreement could be found between HNB-groups determined in this way and the amount of peptide; the figures gave 0.15 - 0.45 moles HNB/mole peptide.

Table 15.4.1.

Amino acid analyses of HNB-peptides.

Peptide	HNB1	HNB2	HNB3[b]	HNB4	HNB5
Fraction	1a2	2a	2a	1b	2b
m	+0.20	+0.2[a]	-0.13	-0.51	-0.40
N-t.	Ile	Ile	Ile	Gly	Ile
Lys	1.04	1.09	1.05	-	-
Arg	0.97	0.97	-	-	-
Asp	1.98	2.05	1.21	++	1.06
Glu	-	-	-	++	-
Pro	-	-	2.06	++	-
Gly	-	-	1.02	++	-
Ala	-	-	-	++	-
Val	-	-	0.49	++	-
Met	0.90	0.78	-	-	-
Ile	1.10	1.00	1.70	-	0.94
Leu	0.33	-	0.29	-	-

[a]: Approximately.

[b]: 96 hr. hydrolysis.

It was not possible to obtain a satisfactory amino acid analysis on HNB4. Fractions 1a1 and 1b2 contained peptides which, to judge from their N-termini and qualitative compositions, were closely related to HNB3 & HNB4 respectively.

15.4. DNS-PTC degradation of HNB peptides.

At each stage of the degradation, the peptide was dissolved in 0.4 ml. 0.1 M-ammonia, and the extinction at 410 nm. was determined. It was hoped that the extinction would drop when the tryptophan residue was removed. It was found, however, that the extinctions, when corrected for the amount of material removed for N-terminal determination, remained constant or rose, even when the peptide ceased to be yellow or, from other evidence, the tryptophan was removed. The data are therefore not quoted in detail.

Peptide HNB5 gave the partial sequence Ile-Asp-, as also did the larger peptide HNB2. Peptide HNB1, which is evidently related, was not examined.

Peptide HNB3 gave the partial sequence Ile-Ile-x-Pro-Pro-, where x indicates that no DNS-amino acids were observed at that cycle.

Peptide HNB4 gave the data which may be interpreted as Gly-Val-Pro-Asx-Gly-, after which no further amino acids were observed, although the material was by no means exhausted. See Section 15.5. for discussion of this peptide.

15.5. Discussion.

There is strong evidence that the penicillinase molecule contains three tryptophan residues. Spectrophotometric and chemical methods (Section 8.6.) give 2.82 and 2.86 residues respectively. The results/

results of the peptic digest (Chapter 13) and of experiments in this Chapter show that there are three tryptophan sequences.

Horton & Koshland (1965) say that although gel-filtration fails to remove non-covalently linked HNB groups, the use of urea and acid precipitation remove such groups. Section 15.3. shows that tyrosine residues have not reacted. Thus it seems most likely that the HNB is covalently linked to tryptophan.

The experiments of Barman & Koshland (1967) with carboxymethyl chymotrypsinogen show that 20 moles HNB/tryptophan incorporates 1 mole HNB/residue. At 15 & 30 moles HNB/tryptophan the incorporation is substantially less and more respectively. Thus the level of labelling of the tryptophan residues is very sensitive to the excess of reagent. In view of this it is perhaps not surprising that it has not been possible to find the precise conditions required to incorporate 1 HNB/tryptophan residue.

It does not seem that conditions have been adequately defined for reliable measurement of tryptophan in an unknown protein. The method does not seem likely to displace those of Goodwin & Morton (1946) and Spies & Chambers (1948).

Peptides HNB1, HNB2 & HNB5 come from the sequence

-Glu-Leu-Leu-Ile-Asp-Trp-Met-Lys-Arg-Asn-Thr-.

Peptide HNB5 is Ile-Asp-Trp, corresponding to PA18. The analysis of HNB2 shows that it is Ile-Asp-Trp-Met-Lys-Arg-Asn, a sequence for which it provides useful confirmation. HNB1 is chiefly the same peptide/

peptide, together with a little of the peptide with leucine added on the front, an effect which thermolysin might well be expected to produce with such a sequence.

Peptide HNB3 corresponds to the N-terminal part of PN4, and the DNS-PTC findings are identical. The sequence is

Ile-Ile-Trp-Pro-Pro-Lys-Gly-Asp-Pro-Val-Val-Leu.

Probably the impurities in the analysis arise from a small proportion of the molecules containing some of the last four residues.

HNB4 evidently originates wholly within TA9. The data are suggestive of a sequence

Gly-Val-Pro-Asp-Gly-Trp-.

The data for the third residue were interpreted at the time as an alanine residue. This sequence is discussed in Section 11.26.

It is interesting that although HNB3 gave data by the DNS-PTC method on the C-terminal side of the tryptophan residue, HNB4 would not do this, although the five residues had been determined with only 3/5 of the total material.

The failure of the HNB analysis of the HNB peptides cannot be explained at present. The procedure differs from that of Dopheide & Jones (1968) only in that they performed the analysis before hydrolysis, whereas here it was performed after. However Barman & Koshland (1967) state quite definitely that HNB groups are stable to acid hydrolysis. They were determined in 0.1 M ammonia which has a pH/

pH of 10.7, which according to Horton & Koshland (1965) is sufficient to convert the HNB group to the yellow form.

The complete agreement of the HNB-tryptophan peptides with tryptophan peptides completely rules out the possibility of reaction of HNB-bromide with other residues than tryptophan (e.g. tyrosine, which reacts under other conditions; Horton & Koshland, 1965). Since tryptophan has been quantitated in other ways, and HNB-peptides are not significantly superior to plain tryptophan peptides for sequence determination, further effort has not been devoted to trying to explain these anomalies.

CHAPTER 16

DEDUCTION OF AMINO ACID SEQUENCE OF PENICILLINASE

16.1. Sequences of tryptic peptides.

The sequences of the tryptic peptides are listed in Table 16.1.1. Most of these were established with the tryptic peptides themselves, and here the section containing the evidence is noted. Others require the consideration of data from other peptides, and these are considered individually.

Peptide PB5 (Section 13.4.) differs from TB3 only in an additional lysine at the N-terminus, and the sequence can clearly correspond to no other peptide.

The analysis of TB7 shows it to be the only tryptic peptide with two phenylalanine residues. Thus the C-terminal portion of PN1 (Section 13.13.), Phe-Ala-Phe must be the N-terminal portion of TB7. The only peptic peptide which has the composition of the rest of TB7 is PB8 (Section 13.10.) so the rest of the sequence is Ala-Ser-Thr-Ile-Lys.

The doubtful area in the sequence of TN1 is covered by the direct sequence determination on peptide PA1 (Section 13.17.).

The sequence determination on peptides CA11 and CA20 (Section 12.13.) covers that part of TN6 which was left doubtful by direct experiment./

experiment.

Peptide CA10 covers all of TN12 but the C-terminal arginine (Section 12.9.) and its complete sequence was determined.

The doubtful region of peptide TA7 is covered by peptides PA18 and PN3 (Section 13.6.).

The location of the amides in TA16 was determined with peptide PA16 (Section 13.11.).

The sequence of the N-terminal region of TN3 was determined with peptide CA7TA2 (Section 12.18.).

The sequence of peptide TA9 was determined partly by subtilisin B digestion of peptide PA15 (Section 13.18.).

Table 16.1.1. also includes all the regions of sequence which have not been found in tryptic peptides, together with a note of which peptides they were found in.

Table 16.1.1.

TB1 Asp-Lys-Lys. (11.7.)

TB2 Ala-Leu-Val-Thr-Ser-Leu-Arg. (11.8.)

TB3 Ala-Leu-Asn-Met-Asn-Gly-Lys. (11.9. & 13.4.)

TB4 Val-Val-Met-Lys. (11.7.)

TB5 Thr-Gly-Ala-Ala-Ser-Tyr-Gly-Thr-Arg. (11.10.)

TB7 Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys. (11.11., 13.10. & 13.13.)

TB8/

Table 16.1.1. continued.

TB8 Ile-Thr-Tyr-Thr-Arg. (11.12.)

TB11 Ala-Val-Leu-Ser-Ser-Arg. (11.13.)

TN1 Gln-Ile-Gly-Gly-Pro-Glu-Ser-Leu-Lys-Lys. (11.4. & 13.17.)

TN3 His-Val-Asp-Thr-Gly-Met-Thr-Leu-Lys. (11.15. & 12.18.)

TN4 Leu-Ile-Ala-Glu-Ala-Thr-Lys. (11.16.)

TN6 Tyr-Ser-Asp-Asn-Ala-Ala-Gln-Asn-Leu-Ile-Leu-Lys. (11.17. & 12.13.)

TN7 Asn-Thr-Thr-Gly-Asp-Ala-Leu-Ile-Arg. (11.18.)

TN8 Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg. (11.19.)

TN10 Asp-Ala-Lys. (11.7.)

TN11 Glu-Leu-Arg. (11.7.)

TN12 Ala-Leu-Asp-Thr-Gly-Thr-Asn-Arg. (11.20. & 12.9.)

TN13 Leu-Pro-Ser-Glu-Lys. (11.7.)

TN14 Lys-Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys. (11.21.)

TA2 Phe-Glu-Pro-Glu-Leu-Asn-Glu-Val-Asn-Pro-Gly-Glu-Thr-Gln-Asp-Thr-Ser-Thr-Ala-Arg. (11.22.)

TA5 Lys-Ile-Gly-Asp-Glu-Val-Thr-Asn-Pro-Glu-Arg. (11.23.)

TA7 Glu-Leu-Leu-Ile-Asp-Trp-Met-Lys. (11.23. & 13.6.)

TA8 Asp-Asp-Leu-Val-Asn-Tyr-Asn-Pro-Ile-Thr-Glu-Lys. (11.25.)

TA9 Ala-Gly-Val-Pro-Asp-Gly-Trp-Glu-Val-Ala-Asp-Lys. (11.25. & 13.18.)

TA11 Ala-Phe-Ala-Leu-Glu-Asp-Lys. (11.27.)

TA12 Glu-Leu-Ala-Asp-Ala-Ser-Leu-Arg. (11.28.)

TA13 Ser-Ile-Glu-Asp-Leu-Asn-Gln-Arg. (11.29.)

TA14 Tyr-Asp-Asp-Lys. (11.7.)

TA16 Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys. (11.30. & 13.11.)

Fragments/

Table 16.1.1. continued.

Fragments not represented in tryptic peptides

Leu-Gly-Ile-Phe (attached to C-terminus of TA16; PN10, CN11 & CN18, 12.6. & 13.11.)

Ala-Leu-Thr-Val-Gly-Val-Leu (attached to C-terminus of TB7; PB8, PN12, CB5 & CN6, 12.5., 12.6., 13.4. & 13.10.)

Leu-Gln-Gln-Lys (attached to N-terminus of TA13; CN3 & PA8, 12.12. & 13.20.)

Asn-Asp-Ile-Ala (attached to C-terminus of TB5; PA2, 13.18.)

Ile-Ile-Trp-Pro-Pro-Lys-Gly-Asp-Pro-Val-Val-Leu. (PN4, 13.14.)

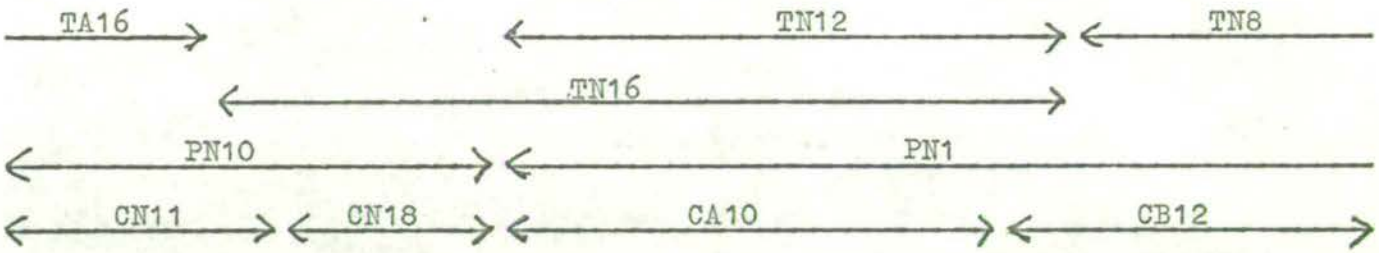
16.2. Deduction of the sequence.

The agreement with the X1 peptides (Section 10.3.) and the N-terminal results (Section 9.1.) show that peptides TN14 & TA6 (Section 11.21.) represent the N-terminus of the molecule. The corresponding peptides CA21 (12.21.) and PA12 (13.4.) terminate at the phenylalanine residue, leaving Ala-Lys as a two residue overlap. The only peptic peptides which contain this region in a form compatible with the other data are PB9 & PA13, as shown in Section 13.11. PA13 shows that the next tryptic digest is TA16. Peptide CA22 (12.22.) corresponds in the chymotryptic digest. The peptide PN10 (13.11.) shows that we now run into the first region where the tryptic peptides are ill-defined. The only tryptic fraction in which the phenyl-

alanine/

phenylalanine from the sequence Leu-Gly-Ile-Phe is found is the ill-purified peptide TN16 (11.20.). If these four residues are subtracted from the apparent composition of TN16, the residue agrees with the composition of TN12, which is a low yield peptide whose alanine N-terminus is found as a free N-terminus of peptic (13.13.) and chymotryptic (12.13.) peptides. This suggests that the phenylalanine is subject to chymotrypsin-like splitting in tryptic digests. The postulated structure is

Asp-Ala-Lys-Leu-Gly-Ile-Phe-Ala-Leu-Asp-Thr-Gly-Thr-Asn-Arg-Thr-Val-Ala-Tyr-



This same peptide TN12 is found as the N-terminus of the peptic peptide PN1. This peptide released TN8 as its central portion on tryptic digestion, and for its C-terminus the unique peptide Phe-Ala-Phe, which is the N-terminus of TB7. Thus PN1 shows the structure TN12 - TN8 - TB7.

The C-terminal portion of TB7 is found in peptides PB8 and CB5. These peptides run into a region of the protein which has never been found in tryptic digests. Section 12.17. shows the Ala-Leu of TB2 cannot be attached here. The extra leucine on CB5 suggests that we look for peptic and chymotryptic peptides, not reconcileable with any tryptic peptides, differing by a leucine residue. The peptides CN6 and/

and PN12 conform to this description, but they both end at the same leucine residue, so we can deduce directly no further.

If we then start from the C-terminus, the X3 peptides (Section 10.5.) represent the C-terminus, and all the peptides containing the structure -Met-Asn-Gly-Lys end at this point (peptides TB3, PB5 and CA15). Peptide CB7 added to CA15 makes PB5. The peptide X2 evidently originates from peptide TB3, so to find the next tryptic peptide we must consider tryptic peptides ending in -Met-Lys. Three of the five methionines in the molecule are followed by lysines, but we may immediately eliminate the one at the N-terminus, in peptide TN14. The -Met-Lys sequence in peptide TA7 is followed by an arginine residue, as is seen in Section 13.6. Thus the next tryptic peptide must be TB4. Peptide CB3 shows that this is preceded by another lysine residue. This -Val-Val- sequence is also found in peptide PB7, and the data in Section 13.9. clearly shows that the next tryptic peptide is peptide TN4.

The composition of peptide CA13 shows that it contains all but the lysine residue of TN4, while its N-terminal sequence is Asp-Asp-Lys-, showing that the next tryptic peptide is TA14. These residues provide a large overlap into PB1, which was thoroughly investigated. The N-terminal portion of PB1 is another peptide found in low yield in the tryptic digest, corresponding to TB11. Thus we have now run into another region intractable in both tryptic and chymotryptic digests, and can only show directly the C-terminal sequence

TB11 - TB1 - TN10 - TA14 - TN4 - TB4 - TB3.

The/

The rest of the molecule can be formed into two groups. There is peptide PN4, which does not overlap with the other regions at all. The only sign of a peptide with its very characteristic composition was an impure fraction referred to in Section 12.2.

The rest of the peptides can be formed into one large piece as follows. We start with the fragment Leu-Gln-Gln-Lys because it is the only N-terminal fragments not found in tryptic peptides (Table 16.1.1.).

Our starting point is attached to peptide TA13, as peptides CN3 & PA8 show. The C-terminal portion of CN3 is Ile-Thr-Tyr, showing that TA13 is attached to TB8. Here we are in a rather unsatisfactory region of peptic digest so we proceed to the peptide CA23, whose N-terminus comprises the C-terminal portion of TB8. This peptide is evidently related to CA7, and the C-terminal portion of CA23 corresponds to the first half of TA8, in agreement with the findings in Section 12.19 which show that the next peptide is TN3. This tryptic peptide is longer than the chymotryptic peptide only by the lysine residue. There are seven chymotryptic peptides with lysine N-termini, and CB3 & CB7 have been covered already, while peptides CB4 & CN2 are shown in Section 12.19. to have other regions at their N-termini. The data on CN10, Section 12.14., strongly suggests that it is preceded by a methionine residue. This leaves peptides CN8 & CA9 as the possible overlap peptides, and the finding that this permits all other peptides to be linked satisfactorily reinforces/

reinforces the evidence.

If we accept this it is immediately apparent that the next tryptic peptide is TA12 (Section 12.10.), and the data in that section shows that the next tryptic peptide starts with a tyrosine residue. Since we have already accounted for TA14, this must be TN6, and this region that in Section 12.14. The peptic peptides from this region were also well characterised (Sections 13.15. & 13.16.).

The N-terminal sequence of CA5TB2 is clearly the product of adding a residue each of lysine, isoleucine and leucine to the N-terminus of TN1. This cannot be the C-terminus of other than the tryptic peptide TN6, so we now come to the substantial region of sequence discussed in Sections 12.17. & 13.17., the structure of which is TN1 - TN11 - TA5 - TA2 - Ala-Leu-Val-Thr-Ser-Leu. The next peptide is therefore TB2. The overlap to the next tryptic peptide is a free arginine residue. There are eight chymotryptic peptides with arginine N-termini, and CB9, CB12, CB14, CN9, CN12, CN13 & CA1 have already been accounted for, leaving only CB10. This requires an Ala-Phe- N-terminus for the next tryptic peptide, suggesting TA11. The peptic peptide PN11 (Section 13.12.) supports this overlap. The peptides in this section show that peptide TN13 follows, in agreement with the double tryptic peptide TA17. This region was also well characterised in the chymotryptic digest (Section 12.16.) where a very substantial overlap into TA7 was demonstrated. Peptide PN3 has a large/

large overlap with TA7 from the C-terminus, and shows that TA7 has an arginine residue after it, followed by peptide TN7. Peptide CN10 (Section 12.14.) also covers this region, and shows that peptide TA9 is next, followed by the N-terminal portion of TB5. PA2 (Section 3.18.) has the last two characteristic residues of TN7, followed by the whole of TA9 & TB5 and then the fragment -Asn-Asp-Ile-Ala, which has not been found in any tryptic peptide.

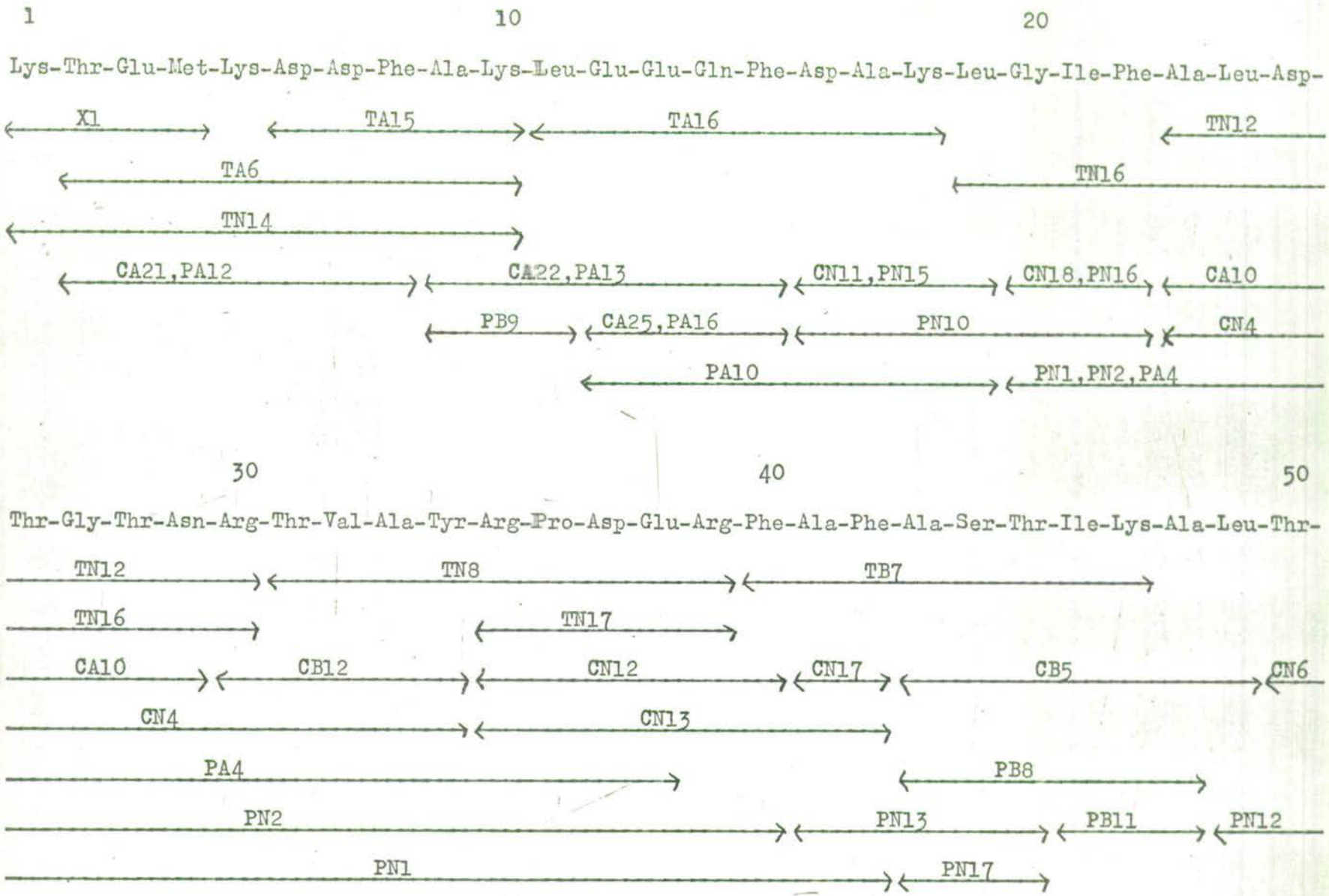
All the peptides found may thus be reconciled with four fragments whose structures are shown in Table 16.2.1.

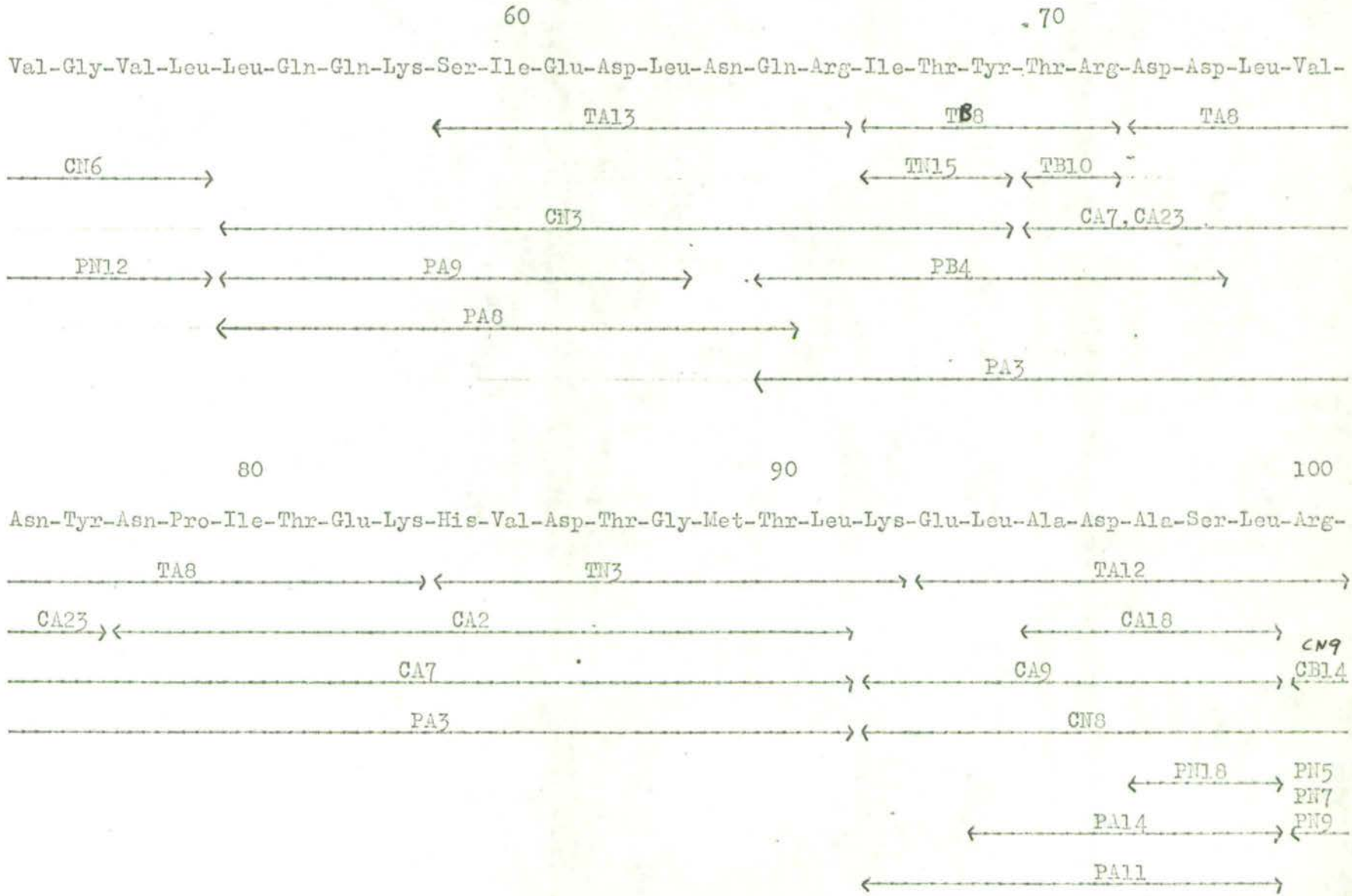
Table 16.2.1.

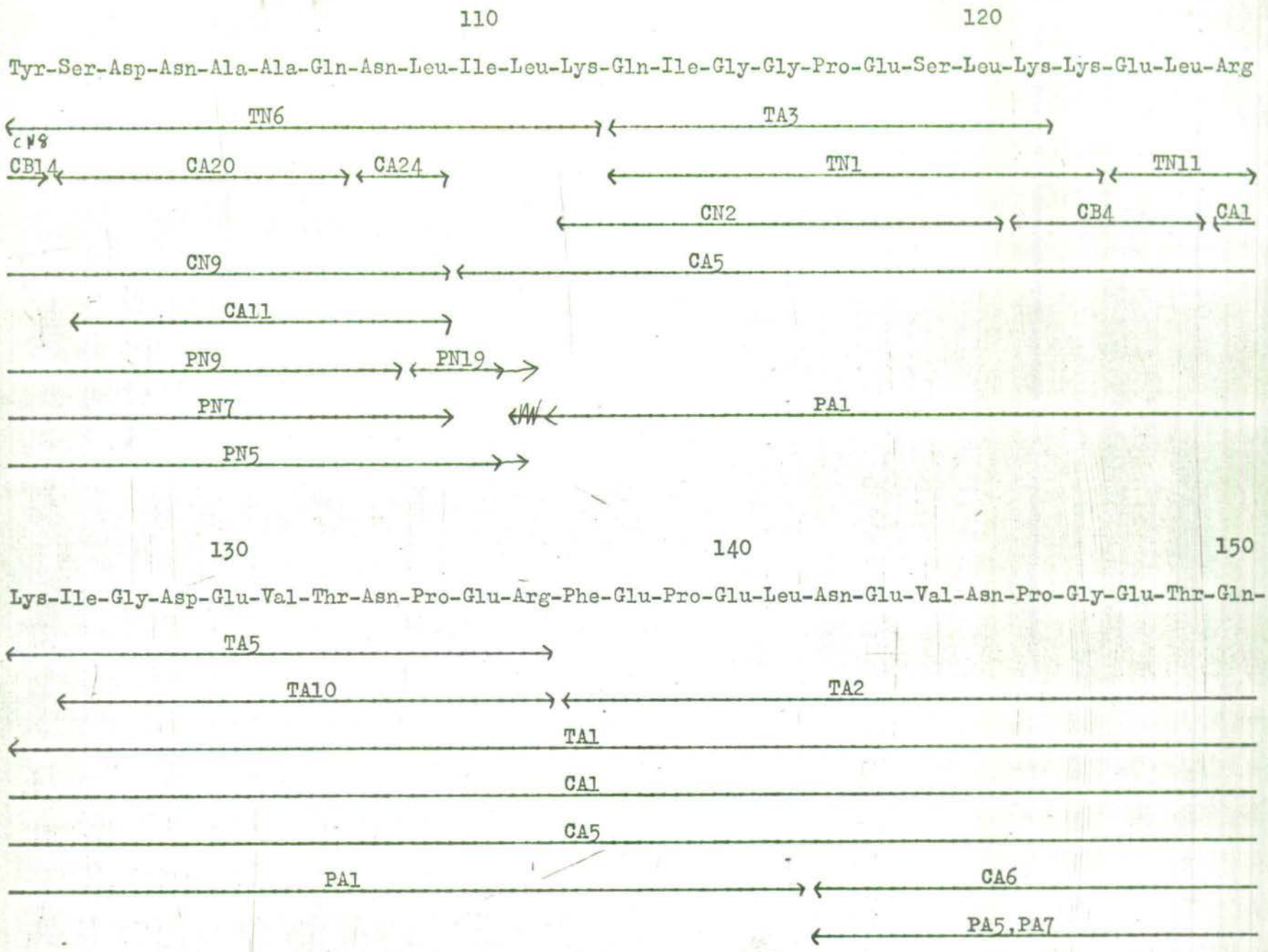
N-terminus	TN14 - TA16 - TN16 - TN8 - TB7 - Ala-Leu-Thr-Val-Gly-Val-Leu
C-terminus	TB11 - TB1 - TN10 - TA14 - TN4 - TB4 - TB3
PN4	Ile-Ile-Trp-Pro-Pro-Lys-Gly-Asp-Pro-Val-Val-Leu
Central	Leu-Gln-Gln-Lys- TA13 - TB8 - TA8 - TN3 - TA12 - TN6 - TN1 -
	- TN11 - TA5 - TA2 - TB2 - TA11 - TN13 - TA7 - Arg - TN7 - TA9 -
	- TB5 - Asn-Asp-Ile-Ala

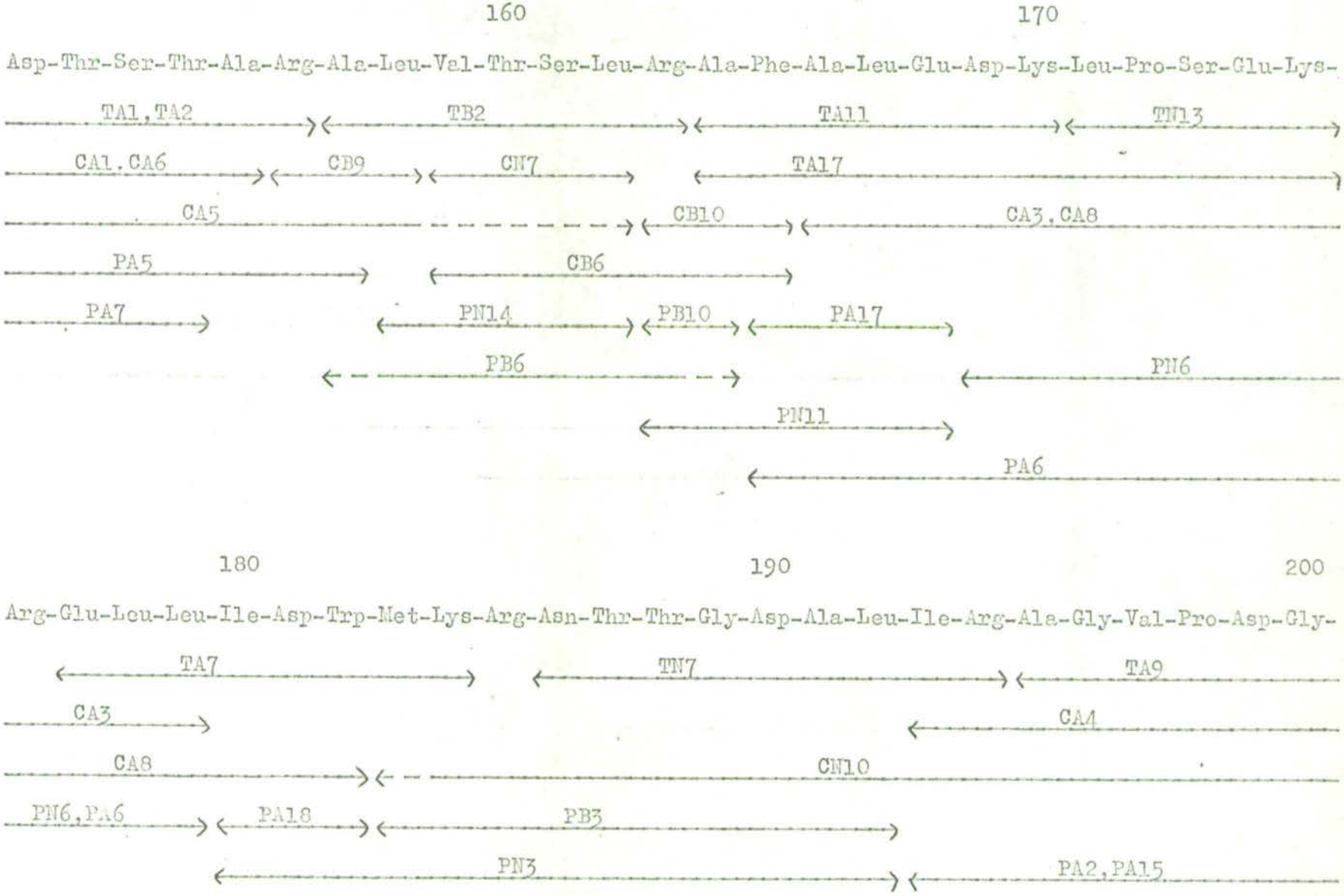
Thus there are three places in the molecule where no overlaps have been forthcoming at all, and we cannot satisfactorily show whether PN4 precedes or follows the large central fragment. What little evidence there is arises from the large cyanogen bromide peptide XC. This is not by any means pure, but the fragments obtained by digesting this with trypsin are listed in Section 10.7. and it/

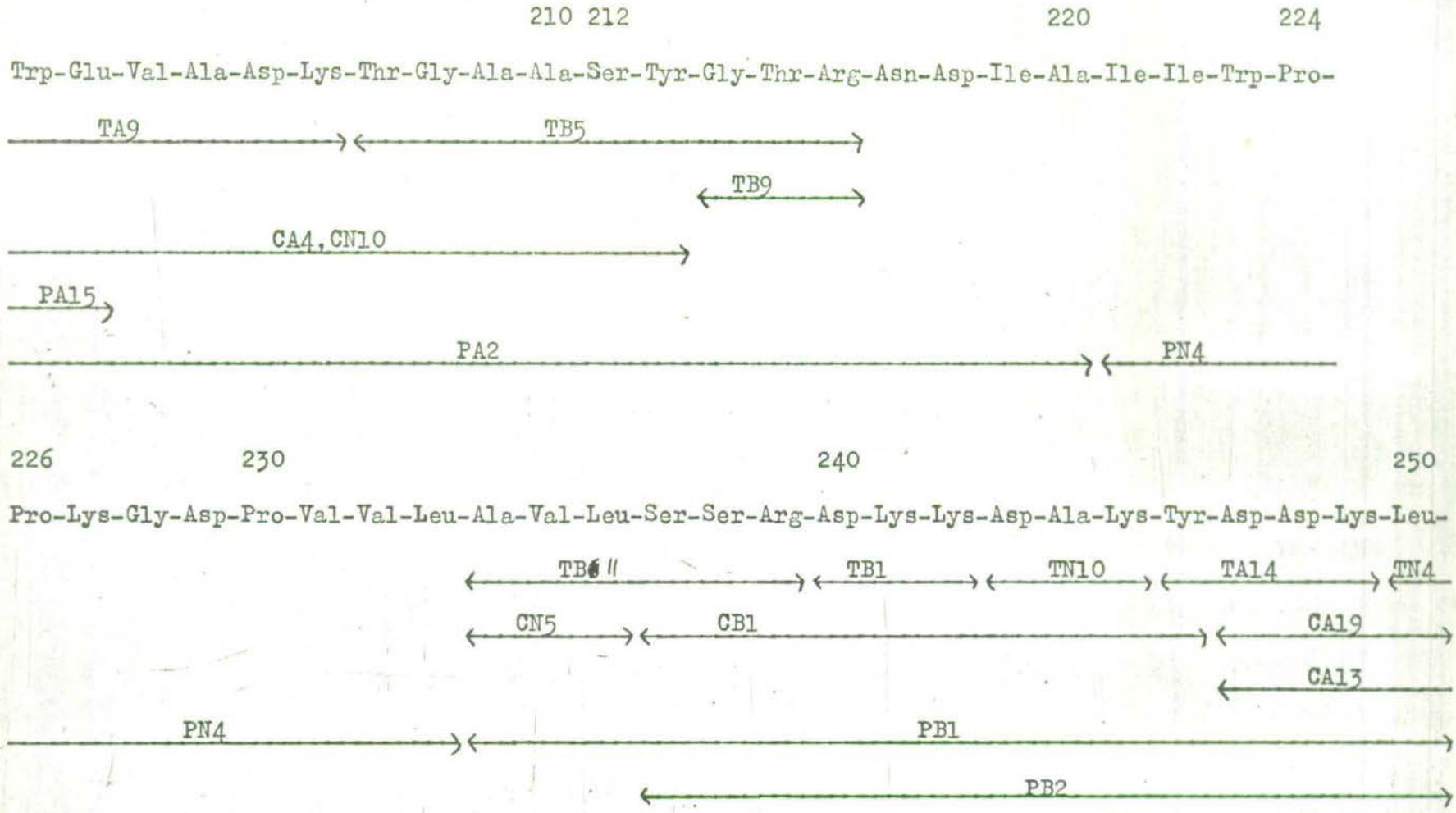
Figure 16.2.1.

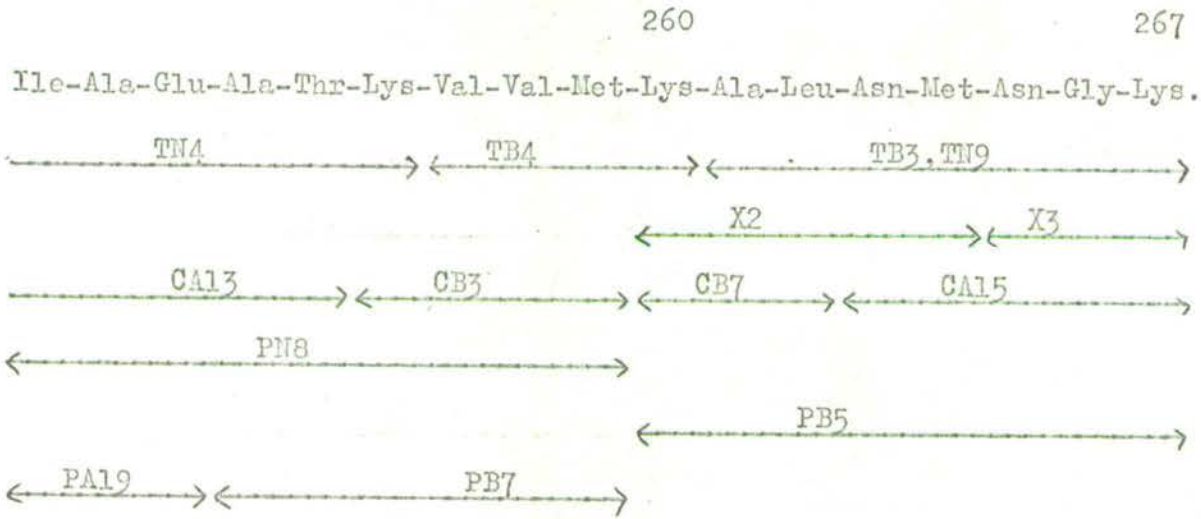

1
10
20
Lys-Thr-Glu-Met-Lys-Asp-Asp-Phe-Ala-Lys-Leu-Glu-Glu-Gln-Phe-Asp-Ala-Lys-Leu-Gly-Ile-Phe-Ala-Leu-Asp-
X1
TA15
TA16
TN12
TA6
TN16
TN14
CA21,PA12
CA22,PA13
CN11,PN15
CN18,PN16
CA10
PB9
CA25,PA16
PN10
CN4
PA10
PN1,PN2,PA4
30
40
50
Thr-Gly-Thr-Asn-Arg-Thr-Val-Ala-Tyr-Arg-Pro-Asp-Glu-Arg-Phe-Ala-Phe-Ala-Ser-Thr-Ile-Lys-Ala-Leu-Thr-
TN12
TN8
TB7
TN16
TN17
CA10
CB12
CN12
CN17
CB5
CN6
CN4
CN13
PA4
PB8
PN2
PN13
PB11
PN12
PN1
PN17


60
70
Val-Gly-Val-Leu-Leu-Gln-Gln-Lys-Ser-Ile-Glu-Asp-Leu-Asn-Gln-Arg-Ile-Thr-Tyr-Thr-Arg-Asp-Asp-Leu-Val-
TA13
TB8
TA8
CN6
TN15
TB10
CN3
CA7, CA23
PN12
PA9
PB4
PA8
PA3
80
90
100
Asn-Tyr-Asn-Pro-Ile-Thr-Glu-Lys-His-Val-Asp-Thr-Gly-Met-Thr-Leu-Lys-Glu-Leu-Ala-Asp-Ala-Ser-Leu-Arg-
TA8
TN3
TA12
CA23
CA2
CA18
CN9
CA7
CA9
CB14
PA3
CN8
PN18
PN5
PN7
PA14
PN9
PA11


110
120
Tyr-Ser-Asp-Asn-Ala-Ala-Gln-Asn-Leu-Ile-Leu-Lys-Gln-Ile-Gly-Gly-Pro-Glu-Ser-Leu-Lys-Lys-Glu-Leu-Arg
TN6
TA3
CN8
CB14
CA20
CA24
TN1
TN11
CN2
CB4
CA1
CN9
CA5
CA11
PN9
PN19
PN7
PA1
PN5
130
140
150
Lys-Ile-Gly-Asp-Glu-Val-Thr-Asn-Pro-Glu-Arg-Phe-Glu-Pro-Glu-Leu-Asn-Glu-Val-Asn-Pro-Gly-Glu-Thr-Gln-
TA5
TA10
TA2
TA1
CA1
CA5
PA1
CA6
PA5,PA7


160
170
Asp-Thr-Ser-Thr-Ala-Arg-Ala-Leu-Val-Thr-Ser-Leu-Arg-Ala-Phe-Ala-Leu-Glu-Asp-Lys-Leu-Pro-Ser-Glu-Lys-
TA1, TA2
TB2
TA11
TN13
CA1. CA6
CB9
CN7
TA17
CA5
CB10
CA3, CA8
PA5
CB6
PA7
PN14
PB10
PA17
PB6
PN6
PN11
PA6
180
190
200
Arg-Glu-Leu-Leu-Ile-Asp-Trp-Met-Lys-Arg-Asn-Thr-Thr-Gly-Asp-Ala-Leu-Ile-Arg-Ala-Gly-Val-Pro-Asp-Gly-
TA7
TN7
TA9
CA3
CA4
CA8
CN10
PN6, PA6
PA18
PB3
PN3
PA2, PA15


210 212
220
224
Trp-Glu-Val-Ala-Asp-Lys-Thr-Gly-Ala-Ala-Ser-Tyr-Gly-Thr-Arg-Asn-Asp-Ile-Ala-Ile-Ile-Trp-Pro-
TA9
TB5
TB9
CA4, CN10
PA15
PA2
PN4
226
230
240
250
Pro-Lys-Gly-Asp-Pro-Val-Val-Leu-Ala-Val-Leu-Ser-Ser-Arg-Asp-Lys-Lys-Asp-Ala-Lys-Tyr-Asp-Asp-Lys-Leu-
TB6 II
TB1
TN10
TA14
TN4
CN5
CB1
CA19
CA13
PN4
PB1
PB2


260
267
Ile-Ala-Glu-Ala-Thr-Lys-Val-Val-Met-Lys-Ala-Leu-Asn-Met-Asn-Gly-Lys.
TN4
TB4
TB3, TN9
X2
X3
CA13
CB3
CB7
CA15
PN8
PB5
PA19
PB7

it apparently runs from the methionine in TA7 to that in TB4. The analysis of an insoluble fraction arising from this digest showed an impure fraction (XCTP3; Table 10.7.1.). This peptide showed an increase of 83% in valine and 67% in isoleucine on going from 24 hr. to 96 hr. hydrolysis, suggesting that the -Ile-Ile- & -Val-Val- sequences of PN4 were present. This supports an overall structure N-Terminus - Central - PN4 - C-terminus for the molecule.

Were it not for the existence of the staphylococcal penicillinase sequence, it would be necessary to fractionate thoroughly the cyanogen bromide peptides as suggested in Section 10.8. to confirm this. However as has been discussed in Appendix II, it has been possible to demonstrate beyond all reasonable doubt that the fragments of the B. licheniformis penicillinase are homologous with parts of the S. aureus penicillinase. On this basis, the N-terminal fragment ends at residue 54 (Fig. 1, Appendix II), the central fragment runs from 55 to 219 and the C-terminal fragment starts at 234. A better match for the region 210 to 219 of the S. aureus sequence is obtained if residue 209 of the S. aureus sequence is considered an insertion (or to be matched by a deletion in the B. licheniformis sequence) (Figure 16.2.2.) This then leaves the sequence shown in Table 16.2.2. unmatched, and this can be reasonably matched with peptide PN4 if another deletion is incorporated.

Table 16.2.2.

	221	222	223	224	225	226	227	228	229	230	231	232	233
S. aureus	Phe	Val	Tyr	Pro	Lys	Gly	Gln	Ser	Glu	Pro	Ile	Val	Leu
PN4	Ile	Ile	Trp	Pro	-	-	Pro	Lys	Gly	Asp	Pro	Val	Val	Leu

The homology fully confirms all the overlaps deduced for the B. licheniformis sequence. Figure 16.2.1. shows the complete sequence for the B. licheniformis penicillinase together with all the peptides, while Figure 16.2.2. shows the homology between the two penicillinases in one-letter code.

Figure 16.2.2.

Amino acid sequences of penicillinase

from

Staphylococcus aureus PC1 (top) and Bacillus licheniformis 749/C (bottom)

```
                                1                               2                               3                               4
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
          k e l n d L E k k y n A h i G v y A L D T k s g k e V k f n s D k R F
k t e m k d d f a k L E e q f d A k l G i f A L D T g t n r t V a y r p D e R F

4                               5                               6                               7                               8
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
A y A S T s K A i n s a i L L e Q v p y n k L N k k v h i n k D D i V a Y s P I
A f A S T i K A l t v g v L L q Q k s i e d L N q r i t y t r D D l V n Y n P I

                                                                1                               1                               1
8                               9                               0                               1                               2
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
l E K y V g k d i T L K a L i e A S m t Y S D N t A n N k I i K e I G G i k v k
t E K h V d t g m T L K e L a d A S l r Y S D N a A q N l I l K q I G G p e s l

1                               1                               1                               1                               1
2                               3                               4                               5                               6
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
K q r L k e l G D k V T N P v R y E i E L N y y s P k s k k D T S T p a A f g k
K k e L r k i G D e V T N P e R f E p E L N f v n P g e t q D T S T a r A l v t
                                                                e

1                               1                               1                               1                               2
6                               7                               8                               9                               0
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
t L n k l i a n g K L s k E n k k f L l D l M l n N k s G D t L I k d G V P k d
s L r a f a l e d K L p s E k r e l L i D w M k r N t t G D a L I r a G V P d g
```

Figure 16.2.2. continued.

```
2                 2                 2                 2                 2
0                 1                 2                 3                 4
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
y k V A D K s G q A i t Y a s R N D v A f v y P k g q s e P i V L v i f t n k D
w e V A D K t G a A - s Y g t R N D i A i i w P - p k g d P v V L a v l s s r D
```

```
2                 2                 2                 2
4                 5                 6                 6
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7
n K s d K p n D K L I s E t a K s V M K e f
k K d a K y d D K L I a E a t K v V M K a l n m n g k
```

Amino acids the same in both proteins at corresponding positions are in capitals.

CHAPTER 17

EXPERIMENTS WITH TETRANITROMETHANE

17.1. Tetranitromethane.

The lability of many penicillinases to dilute iodine solutions has long suggested that a tyrosine or histidine residue might be involved in the activity of the enzyme (Citri, 1958). The characterisation (Sokolovsky, Riordan & Vallee, 1966) of tetranitromethane (TNM) as a specific reagent for tyrosine residues in proteins suggested that it might be interesting to examine its reaction with penicillinase. The reaction can be conducted under very mild conditions, and the only other residue present in penicillinase with which it has been reported to react is tryptophan (Cuatrecasas, Fuchs & Anfinsen, 1968). The reagent is extremely explosive and toxic (Sievers et al., 1947), and was handled with suitable care.

17.2. Effect of TNM on penicillinase activity.

About 50 mg. exo-penicillinase (PP17) was dissolved in 0.25 M-tris chloride buffer, pH 8.5 at a concentration of 20 mg./ml. Some 0.15 ml. portions were diluted with 0.3 ml. buffer. 0.01 ml. TNM was diluted in 4.2 ml. 95% ethanol. This solution contained 20 micromoles/ml. 0.005/

0.005 & 0.015 ml. portions of TNM solution were added to portions of dilute protein solution. After 45 min. at room temperature a small portion of each solution was diluted 1000 fold with 0.05 M-ammonium acetate, pH 4.5 to prevent further action. The dilute solutions were assayed for penicillinase activity by the Citri method. The results are shown in Table 17.2.1.

Table 17.2.1.

Molar ratio TNM/penicillinase	Relative activity
0	100
1	63
3	60

Stronger conditions were not used because it was desired to restrict modification to one residue, if possible.

17.3. Properties of nitro-penicillinase.

About 1.5 ml. of the solution described in the last section was used. It was treated with 0.05 ml. of the TNM solution described and allowed to react for 1 hr. at room temperature (TNM equimolar with penicillinase). After this the nitro-penicillinase was separated from other compounds by gel-filtration on Sephadex G25

(41/

(41 cm. x 1.0 cm. diam.; in 0.1 M-ammonia) and amino acid analysis of a small portion showed about 0.2 residues nitrotyrosine, assuming 7 residues of phenylalanine. Since the peak was small and the analyser constant for tyrosine was used, this figure is not particularly accurate.

The solution was adjusted to pH 8.5 with acetic acid, and incubated at 37° with 1 mg. DPCC-trypsin for 2 hr. and freeze-dried. The digest was redissolved in 0.3 ml. 0.1 M-ammonia, and a small portion was examined by electrophoresis at pH 6.5. At pH 6.5. yellow material was detected running at -0.40, together with some which remained at the origin by dipping the paper in acetone containing 10% aq. ammonia (sp. gr. 0.880). The rest of the digest was partially purified by passing it through the same Sephadex column and collecting and re-freeze-drying all the yellow material.

The yellow fraction was dissolved in 0.5 ml. 0.2 M-ammonium acetate, pH 8.5 and digested with 1 mg. SBTI-chymotrypsin for 4 hr. at 37°. A small portion of the digest was electrophoresed at pH 6.5 and developed with ammonia. It showed one yellow band, running acidic. The peptide was therefore purified successively at pH 3.5 & pH 6.5 on 8 cm. No. 1 paper. It had mobilities of about -0.5 & -0.65 respectively. No other ninhydrin positive material was visible on the final electrophoresis. N-terminal Asx was determined by the DNS method, and the rest of the peptide was used for amino acid analysis. The result/

result was

Asx, 2.90; Val, 1.00; Leu, 1.10; Tyr, 0.33; nitro-Tyr, 0.24.

The peptide also contained 0.15 - 0.25 residues of serine, glutamic acid and glycine.

CHAPTER 18

DISCUSSION AND CONCLUSIONS

It will be evident from the experimental data that most of this discussion will deal with the amino acid sequence determination of penicillinase, but a few other aspects of the work first require mention.

Experiments with release by SBTI-chymotrypsin from lysed, nuclease treated cells (Section 6.1.) show that chymotrypsin will release penicillinase from cells, at about 10^{-3} of the rate found with trypsin. The different starch-gel pattern found with this material rules out release by contaminating trypsin. (Figure 6.1.2.). This finding is contrary to that of Lampen (1967a). The high control values found in the experiments on release are probably due to continuing natural release. That the material is not, for instance, a free intra-cellular fraction, is shown by the fact that low temperature reduces the value to 5% from about 20% (Table 6.2.1.).

The experiments aimed at releasing penicillinase from membrane without the use of protease failed as have those of other workers. What fragmentation of membrane was obtained gave penicillinase which smeared at the origin of starch gels just as described by Lampen (1967a). He/

He considered this material to be penicillinase attached to heterogeneous membrane fragments. Presumably the fragments here are small enough not to sediment under the conditions used.

The data in Chapter 9 enables us to propose structures for the terminal regions of the material responsible for each starch-gel band. The differences found at the ends are sufficient to explain the heterogeneity found, so there is no need to postulate heterogeneity at other sites in the molecule.

The proposed terminal structures for the material responsible for each band are shown in Table 18.1.; band E1 is a mixture of the two forms shown.

Table 18.1.

Terminal structures of penicillinase

Type of enzyme	Band	Structure
Trypsin-released	T1	Thr-Glu-Met-.....-Met-Asn-Gly-Lys.
	T2	Thr-Glu-Met-.....-Met-Asp-Gly-Lys.
Exo-enzyme	E0	Lys-Thr-Glu-Met-.....-Met-Asn-Gly-Lys.
	E1a	Lys-Thr-Glu-Met-.....-Met-Asp-Gly-Lys.
	E1b	Glu-Met-.....-Met-Asn-Gly-Lys.
	E2	Glu-Met-.....-Met-Asp-Gly-Lys.

The/

The other faint bands detected on starch gels (Figure 9.3.2.) were not found in the fractionation of enzyme on DEAE-cellulose and no structural information about them was obtained.

Both methods of N-terminal investigation agreed that threonine is the N-terminus of trypsin-released enzyme. The yield of DNP-Thr (27%; Section 9.1.) is not inconsistent with all the N-terminus being threonine; Fraenkel-Conrat, Harris & Levy (1955) say that correction factors of 2 to 4 are required to correct for destruction in the course of hydrolysis of DNP-amino acids from DNP-protein. Both methods also agree that lysine is one N-terminus of exo-enzyme, and Section 9.5. shows clearly that it is found, as would be expected, in the most basic band. The other N-terminus was found to be glutamic acid by the DNS method (Sections 9.1. & 9.5.) but no derivative of Glu-Met- has been isolated from the cyanogen bromide digests. The DNP experiment with unfractionated enzyme showed threonine and glycine but neither was supported by other evidence; in particular Thr-Glu-Hsl was not found in exo-enzyme, whether fractionated or not. The principal ground for preferring the DNS evidence is that the DNS method has been used extensively with peptides, whereas the DNP method has been used very little. Even if threonine were the correct N-terminus for these fractions it would not alter the explanation these data provide for the mobility differences of the bands.

A quantitative yield of C-terminal lysine has not been obtained (Section 9.2.). With CPB, it may be that the -Asp-Gly-Lys sequence slows/

slows the release by CPB considerably. A brief digression is necessary before considering the results obtained by hydrazinolysis. Current opinion (Braun & Schroeder, 1967; Fraenkel-Conrat & Tsung, 1967) differs on whether distillation of the hydrazine is necessary. It was previously shown (Bradbury, 1958a; Niu & Fraenkel-Conrat, 1955) that up to 10% of water in the hydrazine did not cause the appearance of non-terminal amino acids, and since the distillation of hydrazine is not without risk it was omitted. It is by no means certain that the non-terminal amino acids found are due to hydrolysis; Bradbury (1958b) using proteins much smaller than penicillinase found up to 5% of non-terminal amino acids due to some process independent of water. The recovery of lysine (46%, Section 9.2.) is about what might be expected since Braun & Schroeder (1967) recovered only 41% after 11 hr. at 80°. These findings are therefore fully consistent with the view that lysine is the C-terminal amino acid, although a small proportion of serine (18% found) cannot entirely be ruled out.

The similar properties of T1 & E1 and also T2 & E2 support the structures proposed. The properties of trypsin-released material and exo-enzyme are identical with regard to the C-terminus.

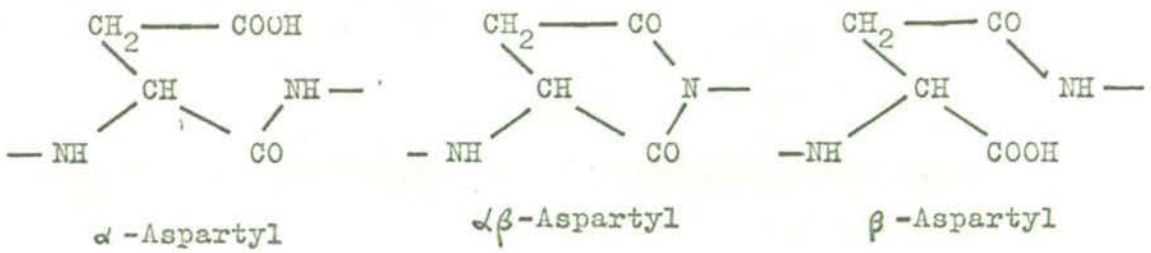
The sequence determination of tryptic peptides by the DNS-PTC method (TA6 & TN14; Section 11.21.) confirms that the N-terminal lysine residue is attached directly to the threonine.

The observations on cyanogen bromide peptides from the C-terminus show/

show the following points. The sequence is Asx-Gly-Lys. This peptide has been found in two neutral forms with different mobilities at pH 3.5, as well as in a basic form (X3c). Table 9.5.1. shows that the neutral forms are found in equal amounts after cyanogen bromide treatment, while in Section 10.5. it was observed that when the basic form was purified at pH 3.5, only one more acidic spot was seen. All these data are consistent with α-Asp-Gly-Lys (X3a) and β-Asp-Gly-Lys (X3b) being found in the cyanogen bromide experiment, while repurification of Asn-Gly-Lys gave rise to mostly α-Asp-Gly-Lys (X3d). (The conditions were such that a quarter of the amount of the isomer would have been observed.)

N-terminal β-aspartyl groups have been reported to arise in two different circumstances. Haley & Corcoran (1967) showed that an Asn-Gly- peptide N-terminus, released on enzymic digestion of ribonuclease, was converted increasingly to β-Asp-Gly- as digestion continued. They considered this to be a non-enzymic process, and did not report observing any α-Asp-Gly- peptide. Ambler (1963b) found N-terminal Asn-Gly- sequences released by digestion very prone to deamidation.

Naughton et al., (1960) showed that under the conditions used for partial acid hydrolysis α-Asp- was converted to β-Asp- through an αβ-Asp- form:



α-Aspartyl α/β-Aspartyl β-Aspartyl

Their experimental situation did not involve an asparagine residue.

The evidence here shows that β-Asp-Gly-Lys has been formed; the bluish tint the peptide gave with ninhydrin on heating vigorously is presumably a combination of the blue colour from N-terminal β-aspartyl (John & Young, 1954) with the normal purple colour from the lysine ε-amino group.

The existence of multiple bands in trypsin-released enzyme shows that deamidation occurs while the protein is still intact. Since β-aspartyl groups were only found in the cyanogen bromide experiment this suggests they arose by the mechanism of Naughton et al., (1960) in acid concurrently with cyanogen bromide cleavage. Although it is possible that the deamidation in whole protein went partly to the β-form, or that the β-form was formed subsequent to the acid treatment as reported by Haley & Corcoran (1967), it is not necessary to use these explanations to account for the observed facts.

Deamidation is also observed in the material separated by DEAE-chromatography. Thus when the second peak (P2) found on DEAE-cellulose chromatography of exo-enzyme was re-chromatographed after a time, the new P3 is five times larger than the P1 (Table 9.3.2.). Presumably the/

the former is mostly due to further deamidation, while the latter is a measure of the cross-contamination of the peaks in the original fractionation.

Deamidation arises in the course of purification. Thus band T2 is found to be much weaker in material treated gently with trypsin than in material treated in the normal way (Section 8.1.). Evidently deamidation increases in the course of purification of the enzyme. It is not possible to say whether any of it occurs in vivo, but even if it does there is not reason to postulate any reason other than the instability of -Asn-Gly- sequences.

The distribution of material between the peaks on chromatography enables us to estimate the fractions of exo-enzyme lacking the amide group and also lacking the N-terminal lysine. From Figure 9.3.1., peaks P1, P2 & P3 contain respectively 48, 52 & 11 x 10^6 units of penicillinase. Thus 0.432 of the enzyme is E0 and 0.099 is E2, while the sum of E1a & E1b makes up 0.469. If we assume that deamidation and the lysine N-terminus are independent, then

$$E0 / E1a = E1b / E2.$$

From this we may deduce a quadratic equation, both sets of roots of which are real, and correspond to possible situations. They give

	E0	E1a	E1b	E2
1	0.432	0.125	0.344	0.099
2	0.432	0.344	0.125	0.099

Physically, set 1 has 27% of P2 with lysine-1 present and aspartic/

aspartic acid-265 and the rest lacks lysine-1 & has asparagine-265, (both of which give the same overall charge), while set 2 has the proportions reversed. The extent to which P2 can still deamidate on rechromatography suggests that the first set is correct. Considering unfractionated exo-enzyme, the first set predicts 0.557 of the enzyme with a lysine residue, while the second gives 0.776. The relative strengths of the N-termini observed also supports the first set, though quantitative assessment of DNS-amino acid spots is not easy.

Thus we may tentatively conclude that 55% of exo-enzyme has a lysine N-terminus, and that by the time the enzyme is pure about 25% has lost the amide from asparagine-265.

The data in Section 9.4. show that all these forms have substantially identical substrate profiles, although it is possible they differ with regard to their behaviour with antiserum. Further experiment would be needed to be sure whether this effect is genuine.

These findings offer a simple explanation of the variability of the starch gel bands and the differences between exo-enzyme and trypsin-released enzyme. There are very few reports of amide loss being established as the cause of multiple bands on starch gel, although this is reported to be the case for Neurospora cytochrome c (E.L. Smith, unpublished, per Kaplan, 1968). The sequence around the amide involved is extremely fortunate in this work; were the asparagine involved anywhere/

anywhere else in the molecule it would have been much more difficult to establish the point.

It has not been possible to isolate any larger form of penicillinase than that starting with lysine-1. It may be that lysine-1 is removed by trypsin incidentally to the destruction of some protein structure in which the enzyme is non-covalently bound. The absence of the first two residues of part of exo-enzyme may be due to incidental proteolytic action. Certainly the pH curve for the release process is not normal for a protease. Hall, Kunkel & Prescott (1966) have described proteolytic enzymes found in culture supernatants of B. licheniformis, but their specificities are broad so it is not possible to be sure whether they are involved. It may be that release can be catalysed by any protease which can split one of the bonds in a limited length of polypeptide chain exposed to proteolytic action. Possibly chymotrypsin does not have such a specificity, which would explain its inefficiency.

Two other systems bear some similarity to this. Sasaki, Sugimoto & Chiba (1966) found that phosphoglyceric acid mutase existed in forms which differed in mobility on moving boundary electrophoresis. They were due to digestion of the enzyme by protease and had different specific activities. The alteration was stopped by DFP. The electrophoretic differences were considered to be due to differences in the number of lysine residues in the molecule.

Eaker, King & Craig (1965a, b) found two anomalous forms of ribonuclease/

ribonuclease by counter-current fractionation. Amino acid analysis showed each to lack one of the ten lysine residues of ribonuclease. The N-terminal sequence of ribonuclease is Lys-Glu-Thr- and studies of peptides showed that both were lacking the N-terminal lysine and were Glu-Thr- and Glp-Thr- (pyrrolidone carboxylic acid N-terminus). Since the Lys-Glu bond is resistant to the action of most proteases, they considered that the forms had arisen through esterification of Glu-2 in the course of purification (which involved removal of ethanol under vacuum) followed by proteolysis and hydrolysis of the ester.

The preparation and purification of penicillinase have been discussed in Section 7.8. and the conclusion was reached that the material obtained was sufficiently pure for sequence studies.

The separation of peptides into size fractions by gel filtration is a very valuable method of simplifying the subsequent fractionation. The use of a pH far from that at which the protease present is active prevents further digestion of large peptides during the chromatography. In general peptides are separated only on the basis of size, although aromatic, particularly tryptophan, peptides are anomalously retarded (Determann & Walter, 1968).

The specificity of the protease used has generally been in accord with expectation. In addition to lysine and arginine, trypsin has split at phenylalanine-22, tyrosine-68, leucine-233 and possibly serine-237, although the evidence for the last is not good. The bonds split/

split slowly were lysine-5 (-Met-Lys-Asp-), arginine-136 (-Glu-Arg-Phe-) and lysine-170 (-Asp-Lys-Leu-). No evidence of splitting at -Lys-Pro- and -Arg-Pro- bonds (Milstein, Clegg & Jarvis, 1968) has been observed. It is not possible to tell whether the anomalous splits are the result of chymotrypsin contamination or a minor specificity of trypsin. In view of the selective nature of the anomalous cleavage the latter may be the more likely explanation.

In addition to the normal cleavage at phenylalanine, tyrosine, tryptophan, methionine and its sulphone, leucine and asparagine, chymotrypsin also split well at alanine-155 and threonine-255 and also at glutamine-107. Cleavage at threonine and glutamine has frequently been recorded, but Hill (1965) lists alanine as one of six residues at which chymotrypsin has not been observed to split.

The specificity of pepsin is not as precise as of the other two enzymes (Tang, 1963). Most splits observed can be explained by cleavage on both sides of phenylalanine, leucine and tryptophan, and on the C-terminal side of tyrosine, threonine, glutamine, alanine, methionine and aspartic and glutamic acids.

Thermolysin (Matsubara et al., 1966) has a very useful specificity for determination of sequences of peptides (Ambler & Meadway, 1968; Appendix I). It generally cleaves on the N-terminal side of hydrophobic residues. Subtilisin B (Hill, 1965) and papain (Smyth, 1967) have been of value when more specific proteases have not been applicable.

Relative/

Relative yields of peptides have not been generally recorded. The great variability in yields from elution from paper makes the information of very limited value. Where the yields of peptides were noticeably much lower than comparable peptides this has been noted.

In the tryptic digest, residues 19-30 were only recovered with difficulty. (Section 11.20.). The peptides containing residues 48-58 and 217-233 have not been observed at all. In one experiment the fraction precipitated by trichloracetic acid and those eluted from Sephadex were analysed (Table 11.4.1.). The majority of the precipitated material could be accounted for by trypsin and no peptide had been precipitated. The comparison of recoveries of different amino acids shows no sign of unusual deficiencies of valine, isoleucine or proline and it appears that substantially all the peptides were recovered from the Sephadex column. It seems most likely that the missing peptides failed to elute from the paper. In view of the care taken, particularly in some experiments, to detect and elute all peptide material, using both ninhydrin and chlorination, at early purification stages these peptides were probably mixed with others subsequently purified, so that the loss was not observed.

In the chymotryptic digest the only region not detected was 214-233. All precipitated fractions were examined, but nothing related to this region was found. A fraction (Section 12.2.) was observed which had a very high proline content and probably contained part of this region,/

region, but it was little and impure and could not be further characterised.

The peptides isolated have generally been adequately pure, and few fractions containing a significant amount of material have had analyses uninterpretable as peptides.

All interpretable analyses have been included. Opinions differ on what level of impurity in peptides need be reported (0.01 moles/mole, Hirs, 1960; 0.02, Light & Greenberg, 1964; 0.05, Ambler & Brown, 1967; 0.15, Guest, Carlton & Yanofsky, 1967) and a level of 0.10 has been adopted here. Many of the peptides with residues differing by more than 20% from theoretical were minor, or contained tyrosine. Hirs (1960) reported that C-terminal tyrosine gave low values on analysis. In this work the mean recovery of C-terminal tyrosine was 0.74 compared with 0.83 for non-terminal tyrosine, but the difference was not statistically significant.

A few peptides had no N-terminus demonstrable by the DNS-method. In some cases N-terminal glutamine had apparently pyrrolidonised. In some cases the presumptive N-terminus reacted with DNS-Cl after enzymic degradation and fractionation of the peptide. No explanation is available for these phenomena.

The DNS-PTC method has not been used to the limit of its sensitivity. The larger amounts of peptide used may account for the occasional observation of impure N-termini. About .05 micromoles/cycle/

cycle has been considered very adequate, while quite extensive sequences have been determined with 0.02 micromoles/cycle. Peptides of eight or more residues have been usually degraded with an enzyme before DNS-PTC degradation.

Three asparagine residues have shown an inclination to deamidation. The case of residue 265 (-Asn-Gly-) has been discussed already. The observations on peptide X2 (Section 9.5.) suggest that asparagine-263 may also deamidate. It may be that this is provoked by the acid conditions or the presence of a homoserine residue next, since deamidation here was not observed in enzymic digests. Evidence in Section 12.18. suggests that asparagine-78 may have partially deamidated, but no other evidence for this has been found.

Deviations from neutrality have been occasionally observed for formally neutral peptides, but these cases can all be explained by the arguments used by Ambler & Brown (1967).

The experiments with maleyl-penicillinase and HNB-bromide have already been discussed. (Sections 14.3. & 15.5.).

The deduction of the sequence from the data forms the subject of Chapter 16, where it was shown that the data could scarcely establish by themselves a unique sequence for B. licheniformis penicillinase, but that taken in conjunction with the homology between this enzyme and the penicillinase from S. aureus the data very strongly suggest that the sequence deduced is correct.

The sequence agrees fairly well with the amino acid analysis of whole/

whole protein (Section 8.4.). The only discrepancy larger than one residue is glutamic acid, where the sequence is three residues short compared with the analysis. Anomalously high yields of glutamic acid have also been noted in peptide CA5 (Table 12.4.2.). This suggested that the analyser might have been miscalibrated for glutamic acid, and the error is in the direction compatible with loss of glutamic acid from the standard mixture. Glutamic acid was found to be one of the most variable in an examination of several batches of standard amino acids (R.P. Ambler, personal communication). To check this suggestion the average glutamic acid recovery was computed for each set of peptides from enzymic digests. The tryptic peptides gave 105% and the chymotryptic and peptic 108% each. However some of the excess may originate through contamination of the peptides with glutamic acid, and without calculating glutamic acid contents of non-glutamic acid containing peptides to a 1% level it is not possible to assess the contribution. It may also not be coincidental that the organisms were grown on a medium with a very high content of glutamic acid.

It is, however, clear that the insertion of a glutamic acid (or other) residue in the gaps where the fragments of penicillinase have not been decisively linked would make the homology with the staphylococcal enzyme very much worse.

It is also possible that the excess glutamic acid arises through slight contamination of penicillinase with another protein. The glutamic/

glutamic acid content of penicillinase is already high, so such a protein would require a very high content indeed. It seems more likely that the error is a combination of analytical errors and possible contamination with free glutamic acid.

The sequence (Figure 16.2.1.) differs in four places (residues 74-76, 117, 142 & 186) from the partial sequence data given in Ambler & Meadway (1969) - Appendix II. The first, the misplacement of an asparagine residue, arose from the misinterpretation of the N-terminus of CA23F4, combined with the asparagine being overlooked after release with CPA. The second arose by contaminating DNS-Glu being taken as the N-terminus of a proline peptide, when DNS-Pro had been extensively destroyed after 18 hr. hydrolysis. The third, glutamine for asparagine, was due to misinterpretation of the result of electrophoresis of DNS-amino acids at pH 4.38, while the fourth (aspartic acid for asparagine) arose through an error in transcription.

To what extent do all the molecules of penicillinase have identical sequences when synthesised? The possibility of "microheterogeneity" was recently raised again by Ehrenstein (1966), who observed more than one amino acid at several sites in rabbit haemoglobin. Among others, Kilmartin & Clegg(1967) reported similar phenomena. The difficulty in these cases is to distinguish the possibility of heterogeneous polypeptide chains being synthesised under the control of one stretch of DNA coding for it, and the protein examined being the mixed product of several genes. In several cases where the experimental situation permitted,/

permitted, such heterogeneity has been shown to be due to several genes (Walsh, Ericsson & Neurath, 1966; Hilse & Popp, 1968; Schroeder et al., 1968), and the other cases are proteins where multiple genes are likely. A reinvestigation of the rabbit haemoglobin (Braunitzer et al., 1968) did not find any evidence of heterogeneity.

No case of microheterogeneity in bacterial proteins has been reported. The absence of pure peptides not reconcilable with the sequence suggests there is no gross heterogeneity, although up to 25% of an anomalous amino acid might have been overlooked.

It will be recalled (Table 1.4.) that strain 6346/C enzyme has different properties from that of 749/C. The specific activity against benzylpenicillin is about one-sixth of the 749 value. Preliminary experiments have been performed on protein from this strain (R.P. Ambler, personal communication) and have shown that the differences are very few. Two which appear well established are that 6346 has glutamine for arginine at residue 163 and valine for methionine at residue 259. It would be very interesting to know which difference or differences is responsible for the large differences in activity.

In order to obtain the maximum homology between the two enzymes it has been necessary to postulate two deletions in the B. licheniformis sequence. In each case regions at either end of a peptide match well with the S. aureus sequence suggesting that an amino acid has not been overlooked. It must be admitted that the deletions lie in the region of/

of the sequence where a twelve residue peptide has not been directly linked to the rest of the molecule. However insertion of one or two amino acids in the gaps would make the homology in the region very much worse.

Using the current version of the genetic code for E. coli (Crick, 1966), it is possible to calculate for each amino acid how many bases require to have mutated to convert one penicillinase to the other. The sume of the results, which is

No bases, 110 amino acids; 1 base, 99; 2, 46; 3, 0,

is of course a minimum value for the amount of mutation which has taken place. The results are in accord with the supposition that the proteins are homologous.

Judging from experience with other systems (Fitch & Margoliash, 1967; Epstein, 1967) we may predict that there will be certain sites at which mutation is incompatible with function, and that those mutations which have occurred will have been selected to retain the conformation of the polypeptide chain.

It is interesting to observe the effect of this mutation on the tryptic peptides. At only 22 of the 61 sites where a basic residue occurs in one protein does one occur in both. Eight tryptic peptides cover the same residues, in most of which the charge distribution is preserved, but none are the same. Harris & Perham (1968) have remarked on the ease with which basic residues

are/

are altered by mutation. There are eleven mutations from lysine to glutamic acid or aspartic acid. It is clear that tryptic peptide maps are unlikely to reveal similarities except between closely related proteins.

The experiments with tetranitromethane have not been carried very far. Tetranitromethane reacts with penicillinase to lower the enzyme activity. It is not clear to what extent the introduction of 1 nitro group/mole would alter penicillinase activity. Any experiment would have to separate penicillinase from the nitrated derivative to show that residual activity was not due to unmodified penicillinase. It is interesting that one and three moles TNM/mole penicillinase both lower activity to 60% but it is not clear whether this is the maximum inhibition attainable.

The nitro-tyrosine has been found only in the peptide Asp-Asp-Leu-Val-Asn-Tyr and presumably the nitro group is on tyrosine-77. The analysis and mobility of the yellow peptide are in full agreement with this finding, which eliminates the possibility that the reagent has reacted with tryptophan. The peptide has a higher nitro-Tyr/Tyr ratio than the parent protein, presumably because of selective purification of the nitro-peptide. The total tyrosine found is not less than with some other tyrosine peptides (Hirs (1960) reported unexplained low yields of carboxyl-terminal tyrosine). No visible yellow material was discarded in the course of purification, so tyrosine-77 is much the

most/

most reactive residue in the molecule. In order to show the relation of this residue to the active site of the enzyme, further experiments will be needed. If a substrate or inhibitor could be made to protect the enzyme from TNM, this would be very useful evidence. The enzyme has such a high turnover number that it turns over 3,000 times its weight of methicillin, the best inhibitor known, in a minute, and assay of the modified enzyme would require the removal of the product.

What then are the implications of these results for the study of penicillinase? The two questions asked by Citri & Pollock (1966) may be answered to some extent. The penicillinases from S. aureus & B. licheniformis have evolved from a common ancestor, since the similarity throughout the molecules is far too great to be the result of convergent evolution. It is very likely that they share structure to a greater extent than is required by the needs of penicillinase activity.

The structural relation of the exo-enzyme from B. cereus to these is susceptible to direct investigation. We would expect on the basis of amino acid analyses that it should be more similar to the B. licheniformis enzyme than to the enzyme from S. aureus but to say anything else would be the purest speculation. The enzymes from Esch. coli TEM & Enterobacter cloacae have amino acid analyses more similar than would be expected through chance alone. The result of a computer search on the lines of those used in Ambler & Meadway (1969) - Appendix II has failed/

failed to show any evidence of internal reduplication or relation or homology. It therefore appears unlikely that the Gram positive penicillinases have evolved through recent reduplication.

Is the structure shown here invariably present in all penicillinases of the 749 type? This is a question which it is impossible to answer without considerable structural work on several different isolates. There are very many sites at which a change of one amino acid might have very little effect on activity and be difficult to detect without, at least, amino acid analysis of small peptides covering the whole molecule. This question of the amino acid sequence differences found in different strains in the same species is a general one and is likely to be investigated in small proteins first where less work is needed to examine several strains.

We can say little about the tertiary structure of the enzyme. The facile deamidation of asparagine-265 without alteration in the specific activity argues that it lies on the surface. The same argument applies to residues 1 & 2. We may therefore consider that both ends of the protein lie in exterior positions. There are comparatively few regions of any length lacking charged residues in both the staphylococcal and bacilliary sequences. The most notable are residues 40-46 and 48-55, fifteen residues with a lysine in the middle, and regions 207-215, 219-224 and 230-238. It would seem likely that these will prove to be internal rather than external residues. Presumably tyrosine-77 is also external.

We/

We cannot either say very much about taxonomic relationships between B. licheniformis and S. aureus. There is at present a complete lack of other protein sequences from which to judge whether the differences between these two sequences are more or less than we should expect. It is not possible at present even to hazard a guess at what is the usual evolutionary difference between two bacterial proteins. Thus 70% similarities in sequence have been found between azurins from different genera (Ambler, 1968) and subtilisins from closely related species, B. subtilis and B. amyloliquefaciens (Smith et al., 1968; Wright, Alden & Kraut, 1969).

The extent of homology rules out the possibility of completely independent origins for the two proteins, so three possibilities remain for the similarities (Ambler & Meadway, 1969).

1. Penicillinase first evolved in a common ancestor of staphylococci and bacilli. Though it has been lost from most of the descendants it survives in these two groups.

2. The enzyme evolved on two separate occasions from the same enzyme with another activity, e.g. peptidoglycan transpeptidase (Chapter 1.).

3. The penicillinase gene evolved in one or other genus after their separation, and was then transferred (e.g. by transformation or transduction) to the other. The best hope of proving this lies in showing that the other proteins of the organisms are much more different than the penicillinases, or showing that the penicillinase in the "recipient"/

"recipient" is much more closely related to a precursor enzyme in the "donor" than in the "recipient".

The fact that in staphylococci the penicillinase gene is located on an extra-chromosomal plasmid while in B. licheniformis it is very near the end of a linear chromosome (D.J. Sherratt & J.F. Collins, personal communication) might be interpreted as evidence for the third hypothesis, showing that in neither species is the gene as fully integrated into the chromosome as are others.

The conclusions of this work are as follows. The amino acid sequence of the penicillinase from Bacillus licheniformis 749/C has been determined and is as shown in Figure 16.2.1. and in the Summary. The enzyme released from cells by trypsin lacks the first residue (Lys-1). The double band found on starch gel electrophoresis is due to deamidation at asparagine-265. The three bands found with exo-enzyme are due to this combined with a form lacking lysine-1 and threonine-2. The protein is apparently homologous with the penicillinase from Staphylococcus aureus of which the sequence has been previously determined. 110 residues are identical in the two proteins in corresponding positions. Two deletions are necessary in the B. licheni-formis sequence to obtain good matching at the C-terminus. Tyrosine-77 is exceptionally reactive with the reagent tetranitromethane.

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The Use of Thermolysin in Amino Acid Sequence Determination

By R. P. AMBLER and R. J. MEADWAY
Department of Molecular Biology, University of Edinburgh

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Thermolysin is a protease from the thermophilic bacterium *Bacillus thermoproteolyticus* Rokko (Endo, 1962). The enzyme has a specificity that is quite different from that from any of the proteases previously available for sequence investigations, as has been shown by studies on synthetic peptide substrates (Ohta & Ogura, 1965) and on proteins of known structure (Matsubara, Singer, Sasaki & Jukes, 1965; Matsubara, Sasaki, Singer & Jukes, 1966). These studies have shown that normally thermolysin will hydrolyse the peptide bonds on the *N*-terminal side of hydrophobic residues.

The present investigation was undertaken to see how useful this enzyme would be for investigation of amino acid sequences, and to clarify points about its specificity. *Pseudomonas* azurin, a small protein of known structure (Ambler & Brown, 1967), was used as substrate, the products of digestion under different conditions were fractionated and an estimate of the thermolysin-sensitive bonds in the molecule was made. These results were compared with the known effects of trypsin, chymotrypsin, pepsin and subtilisin B on the molecule. The specificity of thermolysin was also examined with peptides of known sequence derived from *Pseudomonas* azurin.

Methods and materials. Thermolysin was obtained from Chugai Boyaki Co. Ltd., P.O. Box Higashi no. 106, Osaka, Japan. *Pseudomonas* apoazurin and azurin peptides were prepared as described by Ambler & Brown (1967). Except where otherwise mentioned, the methods used were those of Ambler (1963) and Ambler & Brown (1967).

The conditions used for digestion were very similar to those that have been used for digestions with trypsin, chymotrypsin and subtilisin B (Ambler, 1963). Thermolysin (0·5mg./ml.) was dissolved in ammonium acetate (0·2N with respect to acetic acid), pH 8·5, containing 5mM-$CaCl_2$. Protein (20mg./ml.) or peptide (1–0·1 μmole/ml.) was dissolved in the same buffer, and after addition of enzyme incubated at 37° or 57° for ¼–7hr. The enzyme/substrate ratios used were from 1:20 to 1:100 (by wt.) for protein, and from 0·02–0·5mg./μmole for peptides. After digestion the mixtures were evaporated to dryness in a vacuum desiccator.

The mixtures were fractionated by gel filtration (whole protein digests only), high-voltage paper electrophoresis and paper chromatography, and the isolated peptides were identified by qualitative analysis for amino acid composition and *N*-terminal group. In a few cases the identity of a peptide was confirmed by *C*-terminal analysis (with carboxypeptidases) or by quantitative amino acid analysis.

Action of thermolysin on azurin. Fig. 1 shows the peptides isolated from a thermolysin digest of apoazurin (5μmoles, 57°, 1½hr., enzyme/substrate ratio 1:50 by wt.). Also shown are those peptides isolated from a digest of performic-acid oxidized apoazurin (2·5μmoles, 37°, 3hr., enzyme/substrate ration 1:80 by wt.) that were not detected in the digest of unoxidized protein.

Small-scale experiments showed no qualitative difference between digests at 37° and 57°. Under milder conditions of digestion (time, temperature or enzyme/substrate ratio) two peptides (residues 1–14 and 111–119) were produced in greater quantity, whereas after more vigorous digestion two further peptides (residues 81–83 and 117–119) had increased in amount. In general the differences in peptide pattern between digestions at the extremes of the ranges of conditions were very small.

The effect of the proximity of free α-amino and carboxyl groups on the action of thermolysin was investigated with azurin peptides that contained bonds shown to be sensitive to the enzyme when in the intact protein. The tripeptides Asp-Val-Ser (98–100), Thr-Val-Asn (30–32), Mes*-Phe-Phe (109–111), Mes-Gln-Phe (13–15), Asn-Val-Mes (42–44) and Trp-Val-Leu (48–50) were not split, nor were the larger peptides Leu-Ile-Gly-Ser (86–89) and Asp-Val-Ser-Lys-Leu (98–102). Peptides were split by thermolysin when they contained bonds that were both susceptible in the whole protein and not adjacent to either end. Thus Val-Ile-Ala-His-Thr-Lys (80–85) was cloven at His-Thr, Ser-His-Pro-Gly-Asn-Leu-Pro-Lys-Asn-Val-Mes (34–44) at Asn-Val (but not at Asn-Leu), and peptides containing the sequence 113–121 were split at Gly-His and Ala-Leu, but not at Thr-Phe.

Specificity of thermolysin. The results described above confirm that thermolysin rapidly hydrolyses peptide bonds on the *N*-terminal side of valine, leucine, isoleucine and phenylalanine residues.

* Abbreviation (in amino acid sequences): Mes, methionine sulphone.

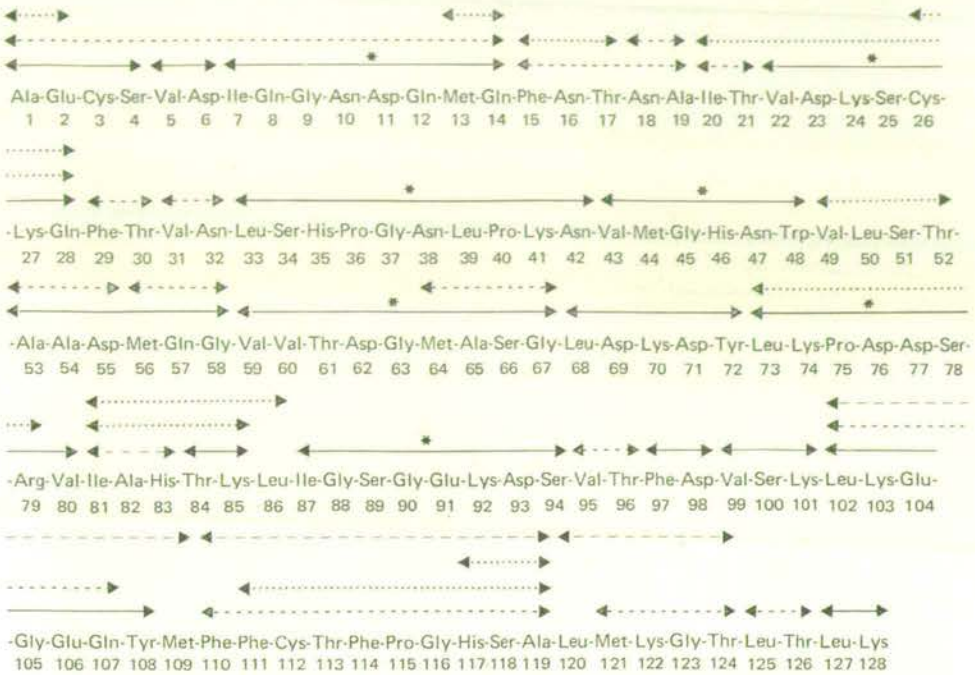

Fig. 1. Peptides formed by the action of thermolysin on *Pseudomonas fluorescens* azurin. ◀-▶, Peptides isolated in high yield; ◀---- ▶, peptides isolated in moderate yield; ◀·····▶, peptides isolated in low yield; ◀ – –▶, peptides isolated only from digest of performic acid-oxidized azurin. The peptides were isolated by gel filtration followed by high-voltage paper electrophoresis and paper chromatography, and identified by qualitative amino acid analysis and *N*-terminal group determination. The amino acid compositions of peptides labelled * were determined quantitatively.

Bonds on the *N*-terminal side of methionine residues are also cloven, as would be predicted from the hydrophobic nature of the side chain, but if the substrate is oxidized bonds on the *N*-terminal side of methionine sulphone residues are not affected. Most bonds on the *N*-terminal side of tyrosine residues, and some (but not all) on the *N*-terminal side of alanine residues are split. A small proportion of bonds on the *N*-terminal side of asparagine, threonine, histidine and glycine residues are susceptible, but not enough cases have yet been encountered for it to be apparent what distinguishes these sites. The bonds on the *N*-terminal side of the cystine and cysteine residues in azurin show a slight susceptibility to hydrolysis, but no such effect has been found in insulin.

Thermolysin does not appear to be able to hydrolyse peptide bonds on the *N*-terminal side of a hydrophobic residue if the next but one residue is proline (e.g. bonds 38–39 and 113–114). In azurin there is no case of a proline residue on the *N*-terminal side of a hydrophobic residue, but thermolysin splits Pro-Ile and Pro-Val bonds in staphylococcal penicillinase (R. P. Ambler, unpublished work).

Our results with natural tri-, tetra- and penta-peptides confirm the findings of Matsubara (1966) with synthetic peptides that adjacent α-amino or carboxyl groups prevent the hydrolysis of peptide bonds by thermolysin. ω-Amino and carboxyl groups do not have a marked effect on the susceptibility of adjacent bonds.

In our experiments temperature did not have a significant effect on the specificity of thermolysin.

Thermolysin in sequence determination. In most proteins more peptide bonds will be susceptible to thermolysin than to chymotrypsin or trypsin. In azurin, 39 of the 127 peptide bonds are hydrolysed at a significant rate, compared with 14 by trypsin and 26 by chymotrypsin. Pepsin hydrolyses 32 bonds, and subtilisin B at least 52 (Ambler & Brown, 1967). Of the bonds sensitive to thermolysin, 11 are not hydrolysed by any of the other enzymes. Thermolysin digests of large proteins will generally be too complex to be worth fractionating, but the enzyme is potentially very useful for the secondary degradation of large tryptic or chymotryptic peptides, particularly those that are insoluble because of their high content of hydrophobic residues.

The enzyme has already proved extremely useful

for the primary digestion of small proteins. For instance *Desulphovibrio* cytochrome c_3 contains only 12 bonds susceptible to chymotrypsin, and most of the chymotryptic peptides are large and difficult to purify, whereas thermolysin hydrolyses 18 bonds, and the peptides are easy to purify, and in conjunction with the tryptic peptides allow the sequence of the protein to be established (Ambler, 1968).

Thermolysin digests contain many peptides that have *N*-terminal valine or isoleucine, and so are likely to have low colour yields with ninhydrin. Great care must be taken to ensure that such peptides are not lost, particularly if paper methods are used for purification.

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Chemical Structure of Bacterial Penicillinases

by

R. P. AMBLER
R. J. MEADWAY

Department of Molecular Biology,
University of Edinburgh

Analysis of amino-acid sequence shows a genetic relationship between bacterial genera.

THE bacterial penicillinases[1] (penicillin-amido-β-lactam hydrolase, E.C.3.5.2.6) are enzymes that destroy penicillins by hydrolysis. Traces of enzyme activity have been detected in cultures of many different bacteria, but only rarely at high levels. Some Gram-positive bacteria, for example, strains of *Bacillus cereus*, *B. licheniformis* and *Staphylococcus aureus*, are able to produce very large amounts of the enzyme on induction, and some Gram-negative enterobacteria can also produce large amounts.

Evolution of penicillinases has been the subject of speculation[2]. The enzyme certainly confers penicillin resistance on populations of bacilli or staphylococci, but resistance has not been acquired recently; penicillinases have been detected in bacilli and staphylococci isolated before the days of penicillin chemotherapy[3]. Bacilli and some of the moulds that can produce penicillins inhabit the soil, but it has not yet been shown that these antibiotics act as selective agents in the microecology of the soil. Staphylococci are not soil bacteria, but may come into contact with penicillin when they are present on animal surfaces also inhabited by pathogenic fungi such as *Trichophyton*[4].

The enzyme and chemical properties of the penicillinases from *B. cereus*, *B. licheniformis* and *S. aureus* have been extensively investigated[1]. The enzymes have similar molecular weights, and similar enzyme properties, but the gross chemical properties such as isoelectric point and amino-acid composition (Table 1) are very different. We report here our studies of the amino-acid sequence of penicillinases from representative strains of *S. aureus* and *B. licheniformis*.

Richmond[5] purified the extracellular magnoconstitutive penicillinase from *S. aureus* PC1 and determined its amino-acid composition, molecular weight and other properties. We have purified this enzyme by a modification of the original method, involving adsorption on to cellulose phosphate, elution with 4 M ammonium sulphate, *p*H 7·5 and gel-filtration on 'Sephadex *G*-100', with overall yields of up to 20 mg/l. culture. We have determined the amino-acid sequence by the characterization of the peptides produced by digestion of the protein with trypsin, chymotrypsin or pepsin using methods described previously[6,7]. The protein is very rich in lysine (43 out of 257 residues), and about sixty different tryptic peptides are produced and have been isolated and analysed, as compared with only about forty-five peptides from chymotryptic or peptic digests. The block sequence of the molecule was first deduced from the overlapping of these enzyme peptides, but has been confirmed by fractionation of the products of cyanogen bromide cleavage[8] of the protein which yields three large fragments (of ninety-four, eighty-four and seventy-six residues) and a tripeptide. The large fragments were too similar in size to be adequately separated by gel-filtration, but were resolved by ion-exchange chromatography in 8 M urea solution on carboxymethyl-cellulose. The fragments were characterized by amino-acid and terminal group analysis and by isolation and identification of the peptides produced from each by tryptic digestion. Formal proof that the protein studied was the penicillinase was obtained by characterizing the amino-acid lesions in material isolated from cultures of artificially induced mutants in which the penicillinase activity is altered (M. H. Richmond and R. P. A., unpublished results).

Table 1. AMINO-ACID COMPOSITIONS OF PENICILLINASES

	Staphylococcus aureus PC1	*Bacillus licheniformis* 749/C
Glycine	12	16
Alanine	18	26
Valine	16	17
Leucine	22	27
Isoleucine	19	15
Serine	19	12
Threonine	13	21
Aspartic acid*	39	38
Glutamic acid*	18	30
Phenylalanine	7	7
Tyrosine	13	6
Tryptophan	—	3
Cyst(e)ine	—	—
Methionine	3	5
Proline	9	12
Lysine	43	24
Histidine	2	1
Arginine	4	15
Total	257	275

* Values include asparagine and glutamine.

The values for the *S. aureus* protein are taken from the sequence (Fig. 1). The values for the *B. licheniformis* enzyme are based on amino-acid analysis of the whole protein. The total number of residues in the *B. licheniformis* sequence would be 267 if all the gaps shown in Fig. 1 were just filled.

The sequence of the magnoconstitutive penicillinase from *B. licheniformis* 749/C is also being investigated. The organism was grown with high aeration in a medium based on monosodium glutamate (4 per cent w/v), and yields of up to 20 mg of enzyme/l. culture have been obtained (Collins, Hughes, Meadway, Shah and Thompson, unpublished). Using developments of the method of Pollock[9] we have purified both the penicillinase released into the culture medium (the extracellular enzyme) and that released from the cells by treatment with trypsin ("cell-bound" enzyme). Differences between these forms are extremely small, and can be completely explained by "ragged ends" at the N-terminus, presumably caused by the differences in method of release. The enzyme liberated from cells by trypsin has the threonine residue 2 (Fig. 1) as N-terminus, whereas the extracellular enzyme is a mixture of two forms, one with lysine (residue 1) and the other with glutamic acid (residue 3) as N-terminus. Peptides corresponding to about 90 per cent of the molecule have been characterized, and their sequences linked together to form five large fragments (Fig. 1).

No similarities between the *S. aureus* and the *B. licheniformis* proteins were detected on peptide maps, and so the proteins were treated as completely different amino-acid sequences. When the sequence of the staphylococcal enzyme was known, and the information about the *B. licheniformis* protein had nearly reached the stage shown in Fig. 1, we tried to match the two sequences. By inspection, positions of match were chosen for each of the five *B. licheniformis* fragments, and we have subsequently used a programmed computer to search for other matching positions and to assess the significance of the matches chosen.

For four of the fragments (LA, LB, LC, LE, Fig. 1) the positions chosen gave the best possible matches of any on the *S. aureus* sequence. The matches were also highly significantly better than the best expected if random sequences of the same composition were matched (Table 2). The distribution of the number of matches at each of the other possible positions followed the binomial distribution expected for the matching of random sequences. The fifth fragment (LD, Fig. 1), which forms the N-terminus of the *B. licheniformis* protein, does not match any better with any part of the staphylococcal sequence than the best match with a random sequence of the same composition. When the computer program was altered to consider "conservative substitutions"[10] as well as identical matches, the best matched position for fragment D was as shown in Fig. 1, but the match was not significantly better than the best expected from a random sequence. Table 2 shows the significance of the match of each fragment when the similarity was first determined, and also the very much greater significance of the matching at the present stage of the investigation. We recommend the use of the computer technique early in the investigation of the structure of a protein if any sequences which may be homologous are already known.

In the positions of best match, none of the fragments overlap, except fragments LE and LB which share a lysine residue. Fragments LD and LC abut directly, and the N and C-termini of the whole molecule are in the right places. The *B. licheniformis* protein is a few residues longer at each end.

The amino-acid analysis data on *B. licheniformis* penicillinase suggest that there are only about twenty residues not accounted for in the fragments shown in Fig. 1 and these would just fill the predicted gaps, with a need for very few insertions or deletions in the whole alignment. Both gaps coincide with runs of hydrophobic residues in the *S. aureus* sequence, which formed tryptic "core" peptides in the *S. aureus* protein and were particularly difficult to obtain. The nature of the missing residues in the *B. licheniformis* protein is consistent with the gaps being the result of the same technical difficulties.

Few convincing attempts have been made to interrelate bacterial genera, or to produce phylogenetic trees that include both *Bacillus* and *Staphylococcus*. The DNA base ratios of the organisms, a property that has sometimes revealed a clear difference between species that had been classed together, are fairly similar (*B. licheniformis* 42 per cent G + C, *S. aureus* 32 per cent G + C[11]).

The similarity in the amino-acid sequences of the two penicillinases has several possible explanations.

(1) Penicillinase first evolved in an ancestor common to both staphylococci and bacilli. The penicillinase gene has been lost from most of the descendants, but still survives in recognizably homologous form in *S. aureus* and *B. licheniformis*.

(2) An enzyme with penicillinase activity has evolved on at least two independent occasions from an enzyme with a different activity (say, cell wall mucopeptide synthesis[2]). The precursor enzyme would be present in all Gram-positive bacteria, and for the purpose of this hypothesis would need to have a highly conserved structure.

(3) The penicillinase gene evolved in one or other of the genera subsequent to their divergence, and has reached the other genus by some process such as inter-generic transduction or transformation.

(4) The two penicillinases have evolved quite independently, and the similarity in amino-acid sequence is a result of analogy rather than homology.

We believe that this fourth possibility is statistically so unlikely that it can be discounted. At present there is little evidence to help choose between the other three hypotheses. The penicillinase gene in *S. aureus* PC1 is in an extrachromosomal plasmid[12], and in *B. licheniformis* 749/C seems to be very near the end of an apparently linear chromosome (D. Sherratt and J. F. Collins, personal communication). These observations can be interpreted as evidence for the third hypothesis, indicating that in both organisms the gene has arrived so recently that it has not been totally incorporated into the chromosome. Further comparisons between the amino-acid sequences of other proteins common to both organisms are needed, however, before a firm choice can be made between the hypotheses.

Existing evidence (Fig. 1) suggests that when the complete amino-acid sequence of the *B. licheniformis* protein is known, about 40 per cent of the residues will be

Table 2. MATCHING OF SEQUENCES OF FRAGMENTS FROM *Bacillus licheniformis* PENICILLINASE WITH THE SEQUENCE OF *Staphylococcus aureus* PENICILLINASE

Fragment	Stage 1 Length of fragment	Stage 1 Maximum possible match	Stage 1 Matches at chosen position	Stage 1 P	Stage 2 Length of fragment	Stage 2 Maximum possible match	Stage 2 Matches at chosen position	Stage 2 P
LA	34	32	12	$1{\cdot}7\times10^{-3}$	34	32	12	$1{\cdot}7\times10^{-3}$
LB	130	78	29	$1{\cdot}5\times10^{-11}$	128	94	34	3×10^{-15}
LC	27	21	11	3×10^{-4}	27	27	14	2×10^{-4}
LD	22	22	4	1*	22	22	4	1*
LE	35	26	11	$1{\cdot}7\times10^{-4}$	35	30	11	$1{\cdot}2\times10^{-3}$

Matches and probabilities are shown for two stages in the investigation; at stage 1 the general sequence similarity was first noticed, and stage 2 represents the present state of work. For fragment LA and so on, see Fig. 1. The "maximum possible match" is the number of residues in each fragment that have been completely placed into sequence. The "matches at chosen position" are the number of residues in the fragment that are identical to the corresponding residue in the *S. aureus* protein when the sequences are arranged as shown in Fig. 1. For all fragments except LD, the position shown gives many more matches than any other. P is the probability that a match at least as good as that chosen would occur if the fragments had the same compositions but random sequences. Values of P were calculated from amino-acid compositions, and checked by trial matching of random sequences.

* When "conservative replacements" of amino-acids[10] were allowed to count towards matching, the probability of fragment LD matching at any place on the *S. aureus* sequence by chance as well as or better than at the chosen position was 0·12. Direct experiments have shown that this fragment contains the N-terminus of the *B. licheniformis* protein.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
					Lys-	Glu-	Leu-	Asn-	Asp-	LEU-LD	GLU-	Lys-	Lys-	Tyr-	Asn-	ALA-	His-	Ile-	GLY-	Val-	Tyr-	ALA-	LEU-	ASP-
Lys-	Thr-	Glu-	Met-	Lys-	Asp-	Asp-	Phe-	Ala-	Lys-	Leu-	Glu-	Glu-	Gln-	Phe-	Asp-	Ala-	Lys-	Leu-	Gly-	Ile-	Phe	Ala-	Leu-	Asp-
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
-THR-	Lys-	Ser-	Gly-	Lys-	Glu-	VAL-	Lys-	Phe-	Asn-	Ser-LC	ASP-	Lys-	ARG-	PHE-	ALA-	Tyr-	ALA-	SER-	THR-	Ser-	LYS-	ALA-	Ile-	Asn-
-Thr-	Gly-	Thr-	Asn-	Arg-	Thr-	Val-	Ala-	Tyr-	Arg-	Pro-	Asp-	Glu-	Arg-	Phe-	Ala-	Phe-	Ala-	Ser-	Thr-	Ile-	Lys-	Ala-	Leu	
51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
-Ser-	Ala-	Ile-	Leu-	Leu-	Glu-	Gln-	Val-	Pro-	Tyr-	Asn-	Lys-	Leu-	Asn-	Lys-	Lys-	Val-LE	His-	Ile-	Asn-	Lys-	ASP-	ASP-	Ile-	Val-
							Lys-	Ser-	Ile	(Glx,	Glx,	Asx,	Asx,	Leu)	Arg-	Ile-	Thr-	Tyr-	Thr-	Arg-	Asp-	Asp-	Asn-	Leu-
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
-Ala-	TYR-	Ser-	PRO-	ILE-	Leu-	GLU-LE	LYS-	Tyr-	VAL-	Gly-	Lys-	Asp-	Ile-	THR-	LEU-	LYS-	Ala-	LEU-	Ile-	Glu-	Ala-	Ser-	Met-	Thr-
-Val-	Tyr-	Asn-	Pro-	Ile-	Thr-	Glu-	Lys-	His-	Val-	Asp-	Thr-	Gly-	Met-	Thr-	Leu-	Lys								
																Lys-	Glu-	Leu-	Ala	(Asp,	Ala,	Ser)	Leu-	Arg-
101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
-TYR-	SER-	Asp-	Asn-	Thr-	ALA-	Asn-	ASN-	Lys-	ILE-	Ile-	LYS-	Glu-LB	ILE-	GLY-	GLY-	Ile-	Lys-	Val-	Lys-	LYS-	Gln-	Arg-	LEU-	Lys-
-Tyr-	Ser	(Asp,	Asn)	Ala-	Ala-	Gln-	Asn-	Leu-	Ile-	Leu-	Lys-	Gln-	Ile-	Gly-	Gly-	Glu	(Ser,	Pro)	Leu-	Lys-	Lys-	Glu-	Leu-	Arg-
126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
-Glu-	Leu-	GLY-	ASP-	Lys-	VAL-	Thr-	Asn-	Pro-	Val-	ARG-	Tyr-	GLU-LB	Ile-	GLU-	LEU-	Asn-	Tyr-	Tyr-	Ser-	Pro-	Lys-	Ser-	Lys-	Lys-
-Lys-	Ile-	Gly-	Asp-	Glu-	Val	(Thr,	Asx,	Pro,	Glx)	Arg-	Phe-	Glu-	Pro-	Glu-	Leu-	Gln-	Glu-	Val	(Gly,	Pro,	Thr,	Glx,	Asx,	Asx,
151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
-Asp-	THR-	Ser-	Thr-	Pro-	Ala-	ALA-	Phe-	Gly-	Lys-	Thr-	LEU-	Asn-LB	Lys-	Leu-	Ile-	Ala-	Asn-	Gly-	LYS-	LEU-	Ser-	Lys-	GLU-	Asn
Asx)	Thr	(Thr,	Ser)	Ala-	Arg-	Ala-	Leu-	Val-	Thr-	Ser-	Leu-	Arg-	Ala-	Phe-	Ala-	Leu-	Glu-	Asp-	Lys-	Leu-	Pro-	Ser-	Glu-	Lys-
176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
-Lys-	Lys-	Phe-	LEU-	Leu-	ASP-	Leu-	MET-	Leu-	Asn-	Asn-	Lys-	Ser-LB	Gly-	Asp-	Thr-	LEU-	ILE-	Lys-	Asp-	GLY-	Val-	Pro-	Lys-	Asp-
-Arg-	Glu-	Leu-	Leu-	Ile-	Asp-	Trp-	Met-	Lys-	Arg-	Asp	(Thr,	Thr,	Ala-	Gly,	Asn)	Leu-	Ile-	Arg-	Ala-	Gly	(Asp,	Glu,	Pro,	Trp,
201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225
-Tyr-	Lys-	Val-	Ala-	ASP-	LYS-	Ser-	GLY-	Gln-	ALA-LB	Ile-	Thr-	Tyr-	Ala-	Ser-	Arg-	Asn-	Asp-	Val-	Ala-	Phe-	Val-	Tyr-	Pro-	Lys-
Gly,	Ala,	Val,	Val)	Asp-	Lys-	Thr-	Gly-	Ala-	Ala-	Ser-	Tyr-	Gly-	Thr-	Arg-	Asn-	Asp-	Ile-	Ala						
226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
-Gly-	Gln-	Ser-	Glu-	Pro-	Ile-	Val-	Leu-	Val-	Ile-	Phe-	Thr-	Asn-	Lys-	ASP-	Asn-	Lys-	Ser-LA	Asp-	LYS-	Pro-	Asn-	ASP-	LYS-	LEU-
								Ala-	Val-	Leu-	Ser-	Ser-	Arg-	Asp	(Ala,	Lys,	Asp,	Lys)	Lys-	Tyr-	Asp-	Asp-	Lys-	Leu-
251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267								
-ILE-	Ser-	GLU-	Thr-	Ala-	LYS-	Ser-	VAL-LA	MET-	LYS-	Glu-	Phe													
-Ile-	Ala-	Glu-	Ala-	Thr-	Lys-	Val-	Val-	Met-	Lys-	Ala-	Leu-	Asn-	Met-	Asn-	Gly-	Lys								

Fig. 1. Amino-acid sequences from penicillinases. The top line of amino-acids shows the sequence of *Staphylococcus aureus* PC1 penicillinase, and below are arranged the sequences of five fragments from *Bacillus licheniformis* 749/C penicillinase (LA–LE). Residues in the *S. aureus* sequence that appear to be identical with corresponding residues in the *B. licheniformis* sequence are shown in capitals. If there were a single amino-acid deletion in the sequence of the *B. licheniformis* protein at about residue 211, the match in the region 213–218 would be much better. There is direct evidence that LD contains the N-terminus and LA the C-terminus of the *B. licheniformis* protein.

identical with those in the *S. aureus* protein, and that extremely few deletions or insertions will need to be postulated to get the best match. This is a greater difference than between the most divergent "higher organism" cytochrome *c* (54 per cent identity between vertebrates and the yeast *Candida krusei*), but less difference than between myoglobin and any haemoglobin chain[13]. There is little evidence yet available for amino-acid sequence differences in bacteria, but 70 per cent similarities are found between the azurins from different genera[14] and between the subtilisins from different strains of *B. subtilis*[15]. The scarcity of deletions or insertions between the penicillinases is also in accord with results with the other bacterial systems[14,15] and markedly different from the observations for proteins from metazoa[13].

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APPENDIX III

Proteases

Carboxypeptidase A, DFP-treated, Sigma, 87B-1630.

Carboxypeptidase B, DFP-treated, Worthington, COB-DFP 33.

Chymotrypsin, Worthington 3X crystallised, CD 16108-9.

Leucine amino peptidase, Sigma, Hog Kidney Type II, 125B-2560.

Papain, Sigma, 2x crystallised, 25B-1420.

Pepsin, Worthington, 2x crystallised PM 692.

Pronase, Sigma, (Protease, Type VI, from Streptomyces griseus) 17B-1720.

Subtilisin B, Novo, 56-2.

Thermolysin, Diawa Kasei K.K., T7F C72.

Trypsin, Worthington, TRSF-6188 (for sequence studies).

Trypsin, Seravac, 3x crystallised, 337BL (for penicillinase preparation).

Other materials

Alanyl-glycine, Koch-Light Laboratories Ltd.

Amino acid analyser resins, Beckman Instruments Ltd. and Locarte Co. Ltd.

Amino acids, Sigma Chemical Co. Ltd.

Amino acids, standard mixture, Evans Electroselenium Ltd.

Ampholine, LKB Instruments Ltd.

Casamino acids, Oxoid Ltd.

Collidine (Fraction 643, 45-50% 2,4,6-collidine, 32-37% 2,3,6-collidine, lutidines to 100%), Midland-Yorkshire Tar Distillers Ltd.

Cyanogen/

Cyanogen bromide (Eastman), Kodak Ltd.

Deoxyribonuclease, Seravac Laboratories Ltd.

Diphenylcarbamylchloride, Koch-Light Laboratories Ltd.

N-ethyl morpholine, Koch-Light Laboratories Ltd.

Gases, British Oxygen Co. Ltd.

Hydrazine (95%+, Eastman), Kodak Ltd.

Ion-exchange celluloses (Whatman), H. Reeve Angel & Co. Ltd.

Lysozyme, Seravac Laboratories Ltd.

Ninhydrin, Koch-Light Laboratories.

Paper (Whatman No. 1 & 3MM), H. Reeve Angel & Co. Ltd.

Permutit ion -exchange resins, BDH Chemicals Ltd.

N-phenyl-1-naphthylamine-8-O-carboxybenzene, BDH Chemicals Ltd.

PPG 2000, Shell Chemicals Ltd.

Ribonuclease, Seravac Laboratories Ltd.

Sephadex, Pharmacia Ltd.

Soya bean trypsin inhibitor, Seravac Laboratories Ltd.

Starch (Connaught), BDH Chemicals Ltd.

Tetranitromethane (Aldrich), Ralph N. Emanuel Ltd.

White spirit 100, Esso Petroleum Co. Ltd.

Addresses

BDH Chemicals Ltd., Poole, Dorset.

Beckman Instruments Ltd., Glenrothes, Fife.

British Oxygen Co. Ltd., Hammersmith, London, W.6.

Diawa Kasei, K.K., Osaka, Japan.

Ralph/

Ralph N. Emanuel, Ltd., 264 Water Road, Alperton, Middlesex.

Esso Petroleum Co. Ltd., London, S.W.1.

Evans Electroselenium Ltd., Braintree, Essex.

Glaxo Laboratories Ltd., Greenford, Middlesex.

Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Kodak Ltd., Kirkby, Liverpool.

LKB Instruments Ltd., LKB House, 137 Anerley Road, London, S.E.20.

Locarte Co. Ltd., 24 Emperor's Gate, London, S.W.7.

Midland-Yorkshire Tar Distillers Ltd., Four Ashes, Staffs.

Novo Terapeutisk Laboratorium, Copenhagen, Denmark.

Oxoid Ltd., London, S.E.1.

Pharmacia (G.B.) Ltd., Paramount House, 75 Uxbridge Road, London, W.5.

H. Reeve Angel & Co. Ltd., 14 New Bridge Street, London, E.C.4.

Seravac Laboratories Ltd., Maidenhead, Berks.

Shell Chemicals (U.K.) Ltd., Shell Centre, London, S.E.1.

Sigma London Chemical Co. Ltd., 12 Lettice Street, London, S.W.6.

Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.

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