PEPTIDE SEQUENCES BY MASS SPECTROMETRY

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"One significant result of this work is that it demonstrates that insulin, and probably other proteins, are homogeneous substances with unique structures and are not merely statistically random polymers of amino acids. This brings the science of proteins into the realms of classical organic chemistry and opens up the way to similar studies on the many other proteins that exist in nature and hence to a better understanding of the chemistry of life".

F. Sanger

1957

ABSTRACT.

The preparation and mass spectrometry of permethylated peptide derivatives was investigated. Procedures for the modification of free peptides prior to permethylation were examined. Acetylation with methanol+ acetic anhydride was found to result in partial esterification of the peptide. Specific cleavage of the C-terminal residue was also observed; a mechanism is proposed for this reaction. Esterification with HCl in methanol followed by acetylation of the peptide ester gave a mixture of products due to random methanolysis during the esterification. Methods of acetylating free peptides were examined, and it was found that the use of water + acetic anhydride at room temperature resulted in rapid quantitative acetylation, with no significant side reactions. Reaction of an ethereal solution of diazomethane with the acetyl-peptide gave quantitative esterification with negligible byproduct formation.

Use of dimethylsulfinyl sodium in dimethylsulfoxide, and methyl iodide for the permethylation of peptide derivatives was investigated. Suitable conditions were found for the preparation of the reagent and for its use in the permethylation reaction. Substitution at existing ester groups was found to occur during the permethylation, and the products were partially characterised. Use of the free acetyl-peptide rather than its methyl ester eliminated this side reaction. Introduction of more than the expected number of methyl groups was observed. This extra-methylation was found to occur mainly at specific residues, although some random methylation was observed. The conditions of permethylation were adjusted to minimise extra-methylation and limit it to specific sites in the molecule. Peptides containing aspartyl residues undergo chain cleavage; the products of this reaction were identified and a mechanism proposed for their formation. The permethylation reaction is discussed in relation to the formation of these artefacts; it is thought to

involve deprotonation of the peptide to form a multiple anion. Reaction conditions are suggested to eliminate these side reactions.

The mass spectrometry of permethylated peptide derivatives is discussed and the mass spectra of peptides of known sequence reported. The mass spectra show the sequence-determining fragments as the principal ions. This observation is rationalised in terms of the negative-inductive effect of the N-methyl groups. The simple procedure for interpreting the mass spectra of permethylated peptide derivatives is outlined, together with the use of minor fragmentation modes in identifying the molecular ion and sequencing peaks. Deuteriated methyl iodide, high resolution mass spectrometry and the detection of metastable transitions can all be used to confirm the deduced sequence.

The techniques developed were applied to a mixture of free peptides isolated from cheese; the three peptides present were sequenced. The results were confirmed by high resolution mass measurement and permethylation with deuteriated methyl iodide.

The present state of peptide sequence determination by mass spectometry is evaluated and possible future developments discussed.

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HISTORICAL DEVELOPMENT.

Protein Structure:

Historically, the determination of peptide sequencesfits into the classical chemical approach to the study of the covalent structure of proteins.

The polypeptide structure of proteins was first proposed independently by Emil Fischer and F. Hofmeister in 1902 (ref. 1). They postulated that α-amino acid residues were covalently linked by amide bonds through their α-amino and carboxyl groups, to form a linear molecule made up of a sequence of amino acyl residues. This remained unsupported by significant chemical evidence until the determination of the complete covalent structure of insulin by Sanger and his co-workers between 1945 and 1955 (ref. 2). The approach used in this classic study is essentially that used up to the present day. It involves the fragmentation (by at least two methods) of the large protein molecule and the separation and isolation of the peptides produced. The sequence of amino acyl residues in these is then determined and the overall sequence of the original protein molecule is deduced from this information.

Thus, the key to protein structure is the determination of the sequences of a large number of small peptides, usually available only in micro-amounts in their pure state. As with protein structure, basic strategy in determining peptide sequences has not changed since the early work in the field. The several methods originally used (ref. 3) are still those normally used today, though techniques have been refined.

Chemical Methods:

One general approach is to chemically modify a terminal residue, completely hydrolyse the peptide and identify the "labelled" amino acid. There are procedures for both N-terminal (ref. 4) and C-terminal (ref. 3) amino

acids. Similar information can be gained by using proteolytic enzymes such as the exopeptidases, carboxypeptidase A which liberates C-terminal amino acids and leucine aminopeptidase which is specific for N-terminal amino acids.

The most useful approach to sequencing peptides is due to Edman (ref. 7) and involves the removal, one at a time, of the N-terminal residues from the peptide and identification of the amino acid removed at each step. This is the only chemical method yet devised for such sequential degradation, although its usefulness has been extended by modifications since its original introduction in 1950. Briefly, the peptide is treated with phenyl isothiocyanate to give a phenylthiocarbamyl derivative at the N-terminal residue; treatment with anhydrous acid specifically cleaves this modified residue, finally giving a phenylthiohydantoin of the amino acid and the remaining peptide chain, which may then be subjected to another cycle of reactions. After each cycle, the amino acid split off is identified. Decreasing yields for each cycle generally limits to between six and ten the number of residues whose sequence can be determined in this way.

Edman (ref. 8) has described an automated modification of his procedure, for application to intact protein molecules. Sixty N-terminal residues were sequenced using only 0.25 μmole of myoglobin. Each cycle of reactions was performed with a yield of 98%. The "Edman sequenator", though commercially available, has not yet made a great impact on methods of sequence determination. It cannot be used for small peptides, as it depends for its successful operation on the insolubility of the protein in all solvents used.

Use of modifications of the Edman method is the best procedure available at present for determining peptide structure. Combined with the general approach to protein structure already outlined, it has enabled the determination of over four hundred protein sequences with a length of thirty or more residues, from a wide variety of sources (ref. 9). The complete structure of a protein molecule containing 1320 amino acid residues has been reported (ref. 69). Despite

this, the determination of the amino acid sequence of a protein cannot be considered a routine matter and the methods described have definite and sometimes serious limitations, outlined in the following section.

Mass Spectrometry and Peptide Sequencing:

Although the principles of mass spectrometry were demonstrated in the first years of this century, mass spectrometers adequate for organic chemical use have been available only since the early 1950's. In fact, it in only in the last ten years that organic chemists have made extensive use of this technique.

The advantages of mass spectrometry over more classical techniques are usually given as the requirement of only a small sample size (for even a complete unknown) and the provision of a large amount of information about the molecule in a short time. Samples in the order of 10 to 100 μg are routinely used and a few hours' work provides data such as molecular weight, elemental composition and structural information.

However, potential advantages of mass spectrometry in the determination of peptide sequences do not lie in small sample size or in shorter analytical time, despite assertions to the contrary (e.g. ref. 59). Mass spectrometry can, at best, only match the 20 nanomoles to 1 micromole routinely needed for the successful sequencing of penta- or hexapeptides by modifications of the Edman technique (ref. 10), while concurrent handling of many peptides considerably reduces the "per peptide" analytical time by normal methods, so that mass spectrometry is not considered to offer significant advantages in this area.

There are, however, several limitations of classical sequencing methods. One restriction is peptide size: normally only six to ten residues can be determined (ref. 1, p. 144). A much more serious limitation is the lack of universal applicability of the Edman method. It cannot be used on peptides with blocked N-terminal residues, such as acetyl- or formylpeptides or those

peptides with pyrrolidone carboxylic acid formed from an N-terminal glutaminyl residue during isolation.

Neither is the Edman method suited to the detection of new or unusual amino acid residues, making one large area of peptide chemistry particularly unsuited to classical sequencing methods. That is, the determination of the structures of naturally occurring small peptides, such as some antibiotics and fungal metabolites. These compounds frequently contain unusual or entirely new constituents and even common residues are often linked by other than the normal peptide bonds.

In these areas mass spectrometry potentially has something to offer the protein chemist. It is uniquely applicable in the verification of the sequence and purity of synthetic peptides where methods involving degradations must be avoided if unusual artefacts are to be found with any degree of reliability (refs. 34, 43).

Modification of Peptides:

Before peptides can be examined by mass spectrometry, the interrelated problems of their low volatility and lack of thermal stability must be overcome. Although free dipeptides (ref. 11) and tripeptides (ref. 12) give mass spectra containing molecular ions, extensive artefact formation, such as cyclisation to dioxopiperazines (ref. 11) occurs, often dominating the spectra.

The low volatility of free peptides has been ascribed to their zwitterion character (ref. 13) and to hydrogen-bonding involving the amide groups (ref. 14). Early methods of increasing volatility relied on the elimination of the zwitterion nature of the molecule by acylation of the N-terminal residue (ref. 15), usually followed by esterification of the terminal carboxyl group (ref. 13); or on reduction of the peptide to give the polyamino alcohol (ref. 16) or polyamine (ref. 17). More recently, methylation of the amide nitrogens of acyl peptides was introduced to eliminate intermolecular hydrogen-

-bonding (ref. 18).

Early Studies:

The development of mass spectrometry of peptides has occurred in several distinct steps. Starting in 1958, pioneering studies were carried out (see Table I). As organic mass spectrometry was in its infancy, these involved little more than cursory examinations of a few derivatives of simple synthetic peptides to see if mass spectra could be obtained and what type of information they could be expected to give.

The studies of Biemann and his co-workers (refs. 16, 17) on the mass spectrometry and gas liquid chromatography (GLC) of reduced peptides appeared very promising. The very volatile derivatives fragmented in such a way as to give information on the sequence of the original peptide. However, with increasing use of the direct insertion probe, there was no longer a need for derivatives of such volatility. Modification of the peptide by reduction was probably considered too drastic a procedure and this approach has not been pursued.

In the work of Heyns and Grutzmacher (ref. 15) concepts were introduced that had a great influence on later developments. They realised that the acyl group, originally used to eliminate the zwitterion nature of the peptide, acted as a marker for ions containing the N-terminal residue of the peptide. Secondly, they noted that characteristic ions are formed due to cleavage on both sides of the carbonyl group of the peptide linkage and that identification of these ions in the mass spectrum of a N-trifluoroacetyl--peptide methyl ester was sufficient to establish the amino acid sequence. Work in the field has since relied heavily on these two observations.

At about the same time, Weygand and Prox examined the combined GLC-mass spectrometry of mixtures of N-trifluoroacetyl-peptide methyl esters (ref. 67). The peptides were derived from oligopeptides by partial acid hydrolysis. These two workers also stressed that the sequence of a peptide

TABLE I.

EARLY MASS SPECTROMETRY OF PEPTIDES.

Anderson:	1958	ref. 13	an N-trifluoroacetyl-tripeptide methyl ester.
Biemann:	1959	ref. 16	polyaminoalcohols and polyamines from di-,
	1960	ref. 17	tri- and tetrapeptides; combined GLC-MS.
Stenhagen:	1961	ref. 19	N-trifluoroacetyl-peptide ethyl esters, several di- and one tripeptide.
Manusadzhyan:	1964	ref. 20	N-acetyl-peptide ethyl esters and polyamino-alcohols from di- and tripeptides.
Heyns and Grutzmacher:	1963	ref. 15 ref. 21	N-acetyl-peptides, N-trifluoroacetyl-peptides, up to a pentapeptide.
Weygand and Prox:	1963	ref. 67	N-trifluoroacetyl-peptide methyl esters, up to a pentapeptide; combined GLC-MS.

was defined by the ions formed due to cleavage on both sides of the carbonyl groups of the peptide bonds.

Naturally Occurring Peptides:

The next development was the use of mass spectrometry to examine a number of naturally occurring peptide derivatives, needing little or no prior modification. For most of these compounds, normal chemical studies had led to partial or complete structures (see Table II). The series of papers by Lederer's group at the Institute of Natural Product Chemistry in France, working in conjunction with A.E.I. Laboratories in England, represented a fundamental breakthrough as the first use of mass spectrometry in determining the sequence of oligopeptides of unknown structure. A particularly exciting discovery was that "Fortuitine", a peptidolipid of molecular weight 1359 containing nine amino acids, gave a mass spectrum in which the sequencing peaks were the principal ions (ref. 27).

In the naturally occurring long-chain N-acyl-oligopeptide methyl esters examined by Lederer's group, the principal mode of fragmentation observed was rupture of the peptide bond. It was soon shown that there was no basic difference in the fragmentation modes of short and long-chain N-acyl derivatives of synthetic peptide esters. However, with the long-chain N-acyl group, fragment-ions containing the intact N-terminal residue and part of the peptide chain were shifted to higher mass and were thus more easily recognised (ref. 36).

Acyl Marking Groups:

The determination of the structure of fortuitine and subsequent study of acyl-peptide derivatives led to a rash of papers stressing the importance of identifying by mass spectrometry those fragments containing the intact N-terminal residue. The climate of thought at that time is exemplified by McLafferty's generalisation that "the structure of a linear molecule is determined unequivocally by using only the possible fragments which contain one end of

TABLE II.

MASS SPECTROMETRY OF NATURAL PEPTIDES

Shannon:	1964a,b	ref. 22	"sporidesmolides", cyclodepsipeptides.
	1964c	ref. 23	"angolide", a cyclodepsipeptide.
Pais:	1964	ref. 24	a partially hydrolysed tripeptide alkaloid.
Lederer, et al.	1964 1965a	ref. 25 ref. 26	"peptidolipin NA_1", a cyclic hepta-peptidolipid.
	1965b	ref. 27	"fortuitine", a nonapeptidolipid.
	1965c	ref. 28	a pentapeptidolipid.
	1966a	ref. 29	"peptidolipin NA_2".
	1966b	ref. 31	"mycoside C_b", a tripeptidoglycolipid.
Vining:	1966	ref. 30	"isariin", a cyclic pentapeptidolipid.
Kiryushkin:	1966	ref. 32	"isariin".
	1967	ref. 33	"staphlomycin S", a cyclodepsi-hexapeptide.
Biemann:	1966	ref. 34	"isariin".
Kingston:	1966	ref. 35	"ostreogrycin A", a cyclodepsipeptide.

the chain" (ref. 38).

Special methods of marking the N-terminal residue were proposed for easy recognition of sequencing peaks in low resolution mass spectra, usually involving N-acyl substituents of characteristic composition. Other authors proposed recognising sequence peaks by routine high resolution measurement of the entire spectrum, followed by computerised interpretation (see Table III). Peptides used as examples in computer-aided interpretation of mass spectra were generally synthetically derived, though two groups (refs. 34,41) used derivatives of "isariin", a naturally occurring cyclic pentapeptidolipid. Their results confirmed the structure proposed by Wolstenholme and Vining (ref. 30) rather than that suggested by Kiryushkin (ref. 32), both of which had been inferred from mass spectrometry.

Although very promising, the computer-aided interpretation of the mass spectra of peptide derivatives has fallen into disuse because of the lack of universal and reliable methods of preparing peptides for mass spectrometry.

N-methylated Peptides:

At the beginning of 1967, mass spectrometry was normally limited to small peptides of six or seven residues made up of simple, non-polar amino acids; even then it was difficult to interpret the spectra in terms of sequence. The single exception to this generalization was the aptly named "Fortuitine".

This was a peptidolipid isolated from Mycobacterium fortuitum and was known to be an N-acyl-oligopeptide methyl ester. The preliminary structure (I) had been proposed:** (over page).

** (All abbreviations for structural formulae are in accord with the IUPAC-IUB rules: see Biochem. 5, 2485 (1966)).

$CH_3(CH_2)_nCO$-Val-Val-Val-Thr(Ac)-Thr-Ala-Pro-OMe

(I) n=18, 20.

Mass spectrometry of fortuitine gave a mass spectrum containing two molecular ions at m/e1331 and m/e1359, due to the homologous fatty acyl substituents. The spectrum consisted predominantly of the sequencing peaks corresponding to cleavage of the peptide bonds and allowed the complete structure to be determined as:

$CH_3(CH_2)_nCO$-Val(Me)-Leu-Val-Val(Me)-Leu-Thr(Ac)-Thr(Ac)-Ala-Pro-OMe

(II) n=18, 20.

In retrospect, the exceptional volatility of this compound can be attributed to the presence of three amino acid residues with substituted amide nitrogens: the two N-methylleucines and the proline.

In the light of this structural feature of fortuitine and the fact that derivatives of several synthetic hepta- and octapeptides were not volatile enough to give mass spectra, J. Van Heijenoort, working with Lederer's group, proposed that the factor limiting the volatility of peptide derivatives was intermolecular hydrogen-bonding through the -CO-NH- groups (ref. 14). It was found that acyl peptides could be quantitatively converted to N-methyl amides, -CO-NMe-, thus eliminating the possibility of such interaction (ref. 44). The expected increase in volatility of these N-permethylated derivatives resulted in useful mass spectra from much larger peptides (refs. 45 and 48). But now an unexpected problem became

TABLE III.

IDENTIFICATION OF SEQUENCING PEAKS.

Lederer, et al:	1965	ref. 36	long-chain acyl groups; mixed homologous acyl groups.
	1967	ref. 14	mixed CH_3CO/CD_3CO groups; mixed $CH_3(CH_2)_8CO/CD_3(CH_2)_8CO$ groups.
Prox:	1966	ref. 37	aryl groups containing a hetero-atom with fixed isotopic ratio.
McLafferty:	1966a	ref. 38	separate spectra of CH_3CO, CD_3CO derivatives; use of CF_2BrCO; high resolution measurement and computer interpretation.
	1966b	ref. 39	high resolution measurements and computer interpretation.
Biemann:	1966a	ref. 40	high resolution measurements and computer interpretation.
	1966b	ref. 34	high resolution measurements and computer interpretation; acyl group containing a halogen atom; aryl group.
Barber:	1966	ref. 41	high resolution measurements and computer interpretation.
	1967	ref. 42	automated metastable detection, high resolution measurements and computer interpretation.

apparent: successive sequencing peaks decreased in intensity with increasing mass and ions beyond a certain point (ten to twelve residues) were too weak to be observed (ref. 45).

This search for a means of increasing the volatility of peptide derivatives was to lead to an unexpected bonus: the mass spectra of permethylated peptide derivatives consist almost exclusively of sequence peaks resulting from cleavage of the peptide bond (ref. 45). It is this simplification, even more than the increase in volatility, that makes N-permethylation such an important technique in the sequence determination of peptides by mass spectrometry.

Several groups have adopted this procedure and unknown peptides, both naturally occurring and from proteins, have been successfully sequenced (see Table IV). Because of the simplified spectra of the N-methyl derivatives and the fact that the amino acid composition of the peptide was usually known, high resolution measurements were not taken. Lederer (ref. 43) has used mass spectrometry of permethylated derivatives to locate and identify artefacts in synthetic peptides; the nature of these artefacts, some residual N-methoxycarbonyl-peptide and a threonine present as a benzoate, was such that they would probably not have been found by normal degradative sequencing techniques.

Other Approaches:

As well as the developments outlined above, several other approaches to the mass spectrometry of peptides have been tried, with little success so far. Two research groups in particular should be mentioned, because of their continued work in the field.

A series of German papers on the mass spectrometry of N-trifluoroacetyl-peptide methyl esters have been published, based on the original work of Stenhagen (1961, ref. 19). GLC was used in conjunction with mass spectrometry to separate and identify small peptides (di-, tri- and tetra-peptides) from partial acid hydrolysates of oligopeptides, and the sequence of the original peptide was then

TABLE IV.

PERMETHYLATED UNKNOWN PEPTIDES.

Lederer's group:	1968a	ref. 43	pentadecapeptides; gramicidns A and B.
	1968b	ref. 47	tetrapeptidoglycolipid; mycoside C_{b1}.
	1968c	ref. 48	tetradecapeptide (10)*; stendomycic acid.
	1969a	ref. 46	heptapeptidolipid; esperinic acid.
	1969b	ref. 49	octadecapeptide (10); from pig immunoglobulin γ-chain.
	1969c	ref. 50	heptapeptide (6); from the zymogen of phospholipase A.
			docosapeptide** (6); from pig immunoglobulin γ-chain.
Geddes, et al.	1969	ref. 51	octapeptide; from silk fibroin.
Agarwal, et al.	1969	ref. 52	tetra- and octapeptides (7); from feline gastrin.
Hiramoto, et al.	1970	ref. 68	cyclodepsinonapeptidolipid; viscosin.

* the number in brackets indicates the number of residues successfully sequenced, if less than the total.

** docosapeptide, i.e. twenty-two amino acids.

reconstructed (refs. 53,67). The structure of one naturally occurring cyclodecapeptide has been deduced in this fashion (refs. 53,54,55). This method has also been used to check "failure sequences" in oligopeptides synthesised by the Merrifield solid phase technique (ref. 53), but such a degradative approach in the search for artefacts in synthetic peptides is questionable.

Shemyakin and his co-workers in the U.S.S.R. have also done a considerable amount of work in this field. A series of papers on the mass spectral fragmentations of synthetic compounds pioneered the study of depsipeptides by mass spectrometry (refs. 61,62).

In 1965 Shemyakin apparently independently realised the significance of mass spectral fragmentation at the peptide bond (Heyns and Grutzmacher, 1963. refs. 15,21) in the sequence determination of oligopeptides (ref. 63). This concept was developed in a number of papers in 1965 and 1966. The Russian workers then embarked on a systematic study of all the characteristic details introduced by each individual amino acid into the general pattern of the mass spectrum of acyl-peptide esters (ref. 56). Although a vast amount of work on synthetic peptides was done (ref. 59) and much useful chemistry of peptide derivatives was elucidated (refs. 57,58,64) there has been a singular lack of application to unknown peptides. Only two structural determinations have been published by the group: that of isariin (ref. 32), which has since been shown to be erroneous, and that of staphlomycin S (ref. 33). In a recent review article (1970, ref. 60), mention is made of the successful sequencing of three peptides from the partial hydrolysate of a protein; detailed results have not been published.

The aim of this group has been to develop mass spectrometry as a tool for the routine sequencing of small peptides from proteins. Reasons for doing this have been given: "mass spectrometry, with its great saving in time and in requirement of material used, is a major advance in the determination of the amino acid sequence of proteins" (Shemyakin, 1966 ref. 59). Any advantage of mass

spectrometry in protein analysis in either of these respects is dubious. In trying to compete on equal terms, rather than attack complementary problems not susceptible to normal chemical methods, Shemyakin and his co-workers have a difficult task. Only time will tell whether it proves to be impossible.

2. INTRODUCTION.

The aim of this investigation was to develop methods for the use of mass spectrometry in the sequencing of peptides for the determination of protein structure. When work began, the only unknown peptides successfully sequenced by mass spectrometry were a few naturally occurring peptidolipids and depsipeptides. These compounds were suitable for mass spectrometry without any modification and were available pure in relatively large amounts. Partial or complete structures had previously been proposed from ordinary chemical evidence in most cases (see Table II, between pages 9 and 11).

In protein chemistry, a routine sequencing procedure must be applicable to submilligram quantities of relatively impure free peptides. The method should be both simple and fast in order to cope with the large number of peptides produced in the study of each protein. Thus, much of the work that had been done on the mass spectrometry of natural peptide derivatives was not relevant to this investigation.

Several research groups had published the results of studies of synthetic peptides undertaken with a view to the use of mass spectrometry in protein sequence studies. There were three basic approaches, differing in the peptide derivatives used. Shemyakin et al. (ref. 56) used n-decanoylpeptide methyl esters and had embarked on a study of the individual contributions of each amino acid residue to the mass spectra of the peptides; the German group (ref. 53) used GLC and mass spectrometry of N-trifluoroacetyl-peptide methyl esters from the partial acid hydrolysates of oligopeptides; finally, Lederer's group had recently studied the mass spectrometry of permethylated acyl-peptide deriviatives of some naturally occurring and synthetic peptides (ref. 44,45,47, 48). None of these procedures had been applied to free peptides isolated from proteins.

The first approach we considered was the use of a series of consecutive

chemical pretreatments to modify each possible type of polar functional group in a peptide. The use of permethylation by Lederer's group eliminated the need for such individual steps; it seemed a simple procedure that would achieve the aim of blocking all functional groups and so increase the volatility of the peptide. It was decided to study the preparation and mass spectrometry of these N-permethylated derivatives.

The original Kuhn peptide permethylation procedure using silver oxide and methyl iodide (ref. 44) could not be applied to very small amounts of peptide, despite improvements in experimental conditions (ref. 45). A second technique, due to Hakomori (ref. 104) and Vilkas (ref. 47), seemed more promising in this respect as well as offering other advantages (ref. 65). This technique used the dimethylsulfinyl carbanion of Corey and Chaykovsky (ref. 70) and methyl iodide. A detailed study of this permethylation procedure and its application was undertaken.

The relevance of this line of investigation has been considerably increased by subsequent developments. Three research groups have reported the application of mass spectrometry of permethylated peptide derivatives to protein chemistry (refs. 49,50,51,52). Such derivatives have been found to give very simple mass spectra consisting almost exclusively of sequencing peaks due to fragmentation at the peptide bond (ref. 45). Mass spectrometry of other types of peptide derivatives has not been successfully applied to the determination of protein structure. Moreover, although other permethylation procedures were developed and used (refs. 49,52,71), that of Hakomori and Vilkas is considered to be the method of choice as the only one in which no artefact formation has been reported to occur (Lederer's group, Sept. 1969; ref. 66). No free peptides from natural sources have been sequenced using derivatives permethylated by this procedure.

Note added in proof: Lenard and Gallop (ref. 72) have reported the partial sequences of two heptapeptides from enzymatic digests of a protein, determined by mass spectrometry of derivatives permethylated using the Hakomori-Vilkas procedure.

3. MODIFICATION OF PEPTIDES FOR PERMETHYLATION.

A. FUNCTIONAL GROUPS NEEDING PRIOR MODIFICATION:

Permethylation of peptides containing free amino groups results in the formation of quaternary ammonium salts. Because of their charged character, these compounds have low volatility and decompose pyrolytically in the mass spectrometer (refs. 43,45). Using the Hakomori-Vilkas permethylation procedure, the quaternary ammonium iodide would result. On heating under vacuum in the mass spectrometer, the tertiary amine would be released. This in itself may not necessarily hinder the production of a mass spectrum, as is shown by the normal behaviour of histidyl residues which are quaternised in the side-chain (ref. 43). However, the presence of a charged group at one end of the permethylated peptide molecule would give it detergent-like solubility properties which would prevent its isolation by extraction into chloroform in the work-up after permethylation.*

Such quaternisation reactions can be prevented by acylation of the free amino groups prior to permethylation. In the past, the acyl group introduced at the N-terminal residue had a dual function: to eliminate the zwitterion character of the free peptide and hence increase its volatility, and to act as a marker identifying ions containing the N-terminal residue. This latter function was necessary because the many fragmentation modes of acyl-peptide esters led to complex mass spectra. A variety of N-acyl groups were tried, each offering some advantage in identifying the "sequence peaks" (ref. 73; see Table II, between pages 9 and 11). The mass spectra of permethylated peptide derivatives are so simple that use of an N-acyl group as a marker is no longer necessary. The N-acetyl derivative is adequate for permethylation.

Other functional groups in the side-chains of amino acids introduce complications. Prior to permethylation, arginine-containing peptides must be modified by treatment with hydrazine to give the corresponding ornithine containing peptides (ref. 43), and sulphur-containing peptides desulfurised with

* The peptide Ac-His-Ser-Gln-Gly-Thr-Phe was permethylated; mass spectrometry of the chloroform extract failed to show evidence of any product (Exp. (53)).

Raney nickel (refs. 43,74). Peptides containing cysteic acid behave normally (ref. 50). In this investigation we have not dealt with peptides containing arginine or sulphur amino acids. Hydroxyl groups are O-methylated, while free carbxyl groups in sidechains and at the C-terminal residue are esterified in the permethylation reaction (ref. 43). In spite of this, esterified acyl-peptides have been used for permethylation.

B. DERIVATIVES PREVIOUSLY USED:

Three groups of research workers have published mass spectra of permethylated derivatives of naturally occurring free peptides. Lederer's group have prepared acetyl-peptides using methanol + acetic anhydride to selectively acetylate amino groups while any hydroxyl groups present remain free (ref. 45). Geddes et al. (ref. 51), and Agarwal et al. (ref. 52) permethylated the acetylpeptide esters produced by treatment of the peptide with HCl in methanol followed by acetic acid + acetic anhydride. The methods used by these three research groups were duplicated as carefully as possible and the derivatives produced from a simple peptide examined.

Reaction with methanol + acetic anhydride: This is known to give simultaneous acetylation and partial esterification of peptides (refs. 73,75,76,77,78), while transacylation involving the hydroxyl group of serine residues has also been shown to occur (ref. 77). Use of this reagent is not considered to give cleavage of peptide bonds (ref. 78); Alpin found no evidence of cleavage of peptide bonds, though mass spectrometry of the crude reaction products showed ions of mass greater than the expected molecular ion (ref. 73).

Mass spectrometry of the products from the reaction of methanol + acetic anhydride with Leu-Gly-Leu and DL-Leu-Gly-Phe showed evidence of the introduction of two acetyl groups into the molecule (Exps. (1) and (2)). This was not subsequently observed and can be attributed in this case to the use of temperatures up to 50° in the vacuum evaporation of the reagents. Mass spectrometry of the crude reaction products from treatment of Leu-Gly-Leu with

methanol + acetic anhydride indicated that rupture of at least one peptide bond in the molecule had occurred.

Reaction with a simple amino acid, phenylalanine, gave three products. Acetylation was complete in less than an hour at room temperature, but the formation of the other two products continued. Preparative TLC yielded acetylphenylalanine and acetylphenylalanine methyl ester. The third compound could not be isolated (Exps. (4) and (5)).*

Treatment of DL-Ala-Gly-Gly with methanol + acetic anhydride for five days at room temperature led to the formation of six ninhydrin-negative products. Preparative TLC, followed by mass spectrometry of the isolated compounds, identified Ac-Ala-Gly-Gly-OMe, Ac-Ala-Gly-OMe and Ac-Gly-OMe. Two of the isolated compounds would not yield mass spectra (this had previously been noted in other cases; (see Exp. (54)). After permethylation, one of these compounds gave a mass spectrum showing it to be Ac-Ala-Gly-Gly; the other did not give an interpretable mass spectrum, though an Ac-Ala-Gly-Gly- residue was clearly present in the molecule. The last of the six products could not be isolated as it was unstable in methanol solution. Analytical TLC showed that it decomposed to give Ac-Ala-Gly-OMe and Ac-Gly-OMe (Exp. (6)).

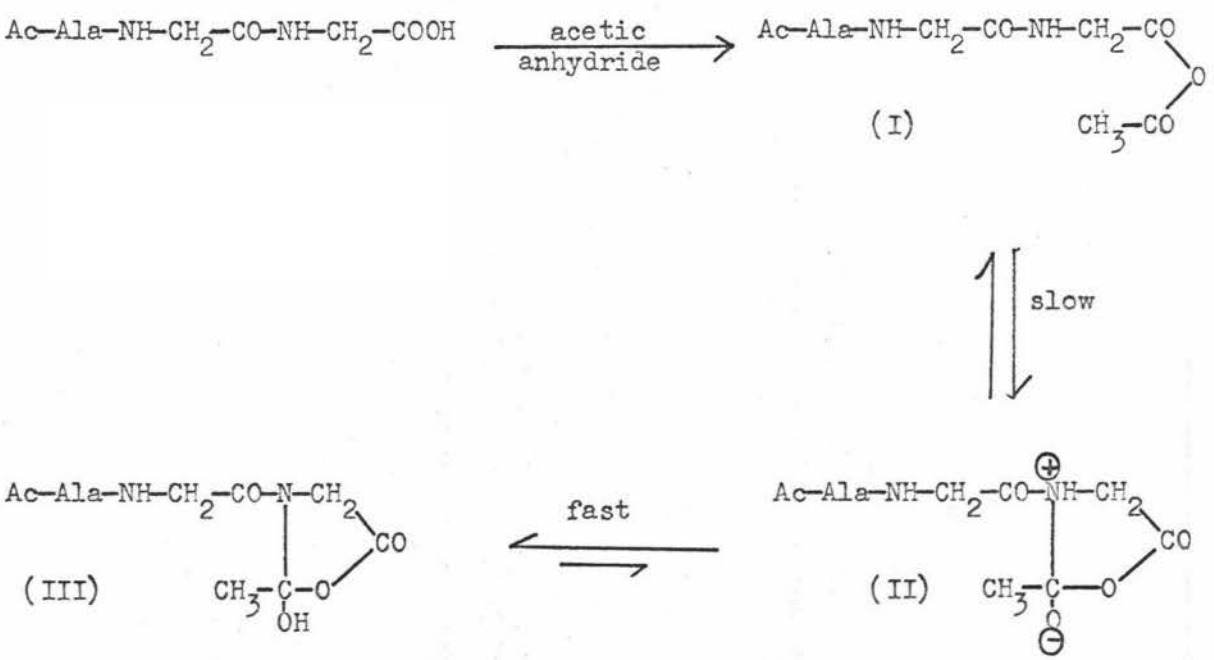
Studies of the formation of these byproducts on treatment of DL-Ala-Gly-Gly with methanol + acetic anhydride showed that acetylation was complete in one hour at room temperature and that the levels of other products became significant after three hours (see plate 13 and 14, Exp. (11)). Complete esterification did not occur even after several days.

In general terms, reaction of a peptide with methanol + acetic anhydride gives complete acetylation within an hour at room temperature; some esterification also occurs, as had been previously noted. A novel observation is that the C-terminal residue of the peptide is specifically cleaved to yield

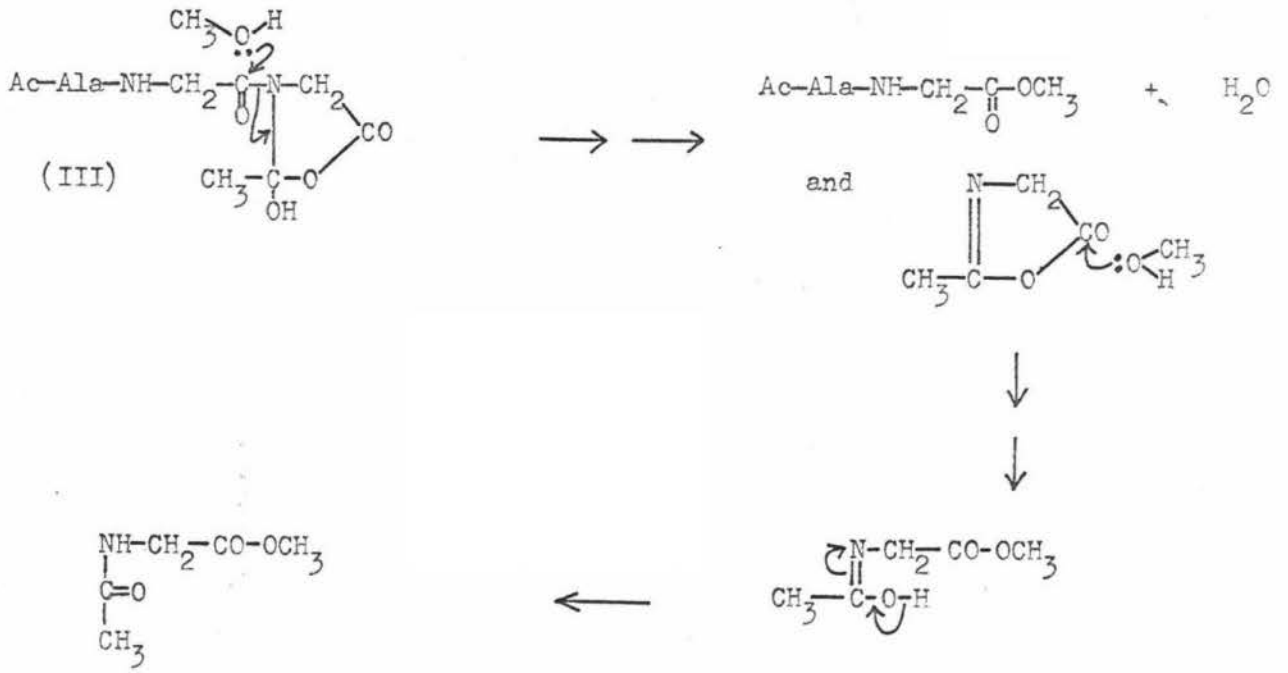
* Use of the Rydon-Smith chlorine-starch/potassium iodide spray (ref. 80) involved the exposure of the TLC plates to gaseous chlorine. Mass spectrometry of the subsequently isolated compounds showed the N-chloro compounds, which were remarkably stable.

an acetyl-amino acid methyl ester and the acetyl-peptide ester of the remainder of the molecule:

Ashley and Harrington (ref. 75) postulated that esterification of amino acids with methanol + acetic anhydride involved the formation of the azlactone. A similar mechanism could explain the selective cleavage of the C-terminal residue of a peptide:



Reaction of the mixed anhydride (I) with methanol would yield the acetyl-peptide ester. Reaction of methanol with (III) could proceed as follows:



The unstable compound that decomposed in methanol solution to give Ac–Ala–Gly–OMe and Ac–Gly–OMe could have the structure (III).

Esterification followed by acetylation: Both Geddes et al. and Agarwal et al. used methanolic HCl to esterify the free peptides. The strength of the HCl was not specified by Geddes et al; this was taken to indicate that they did not consider it to be critical and 6.2N HCl was used in duplicating their procedure. Agarwal et al. specified 0.1N HCl in methanol.

Esterification using HCl in methanol was first used on amino acids by Emil Fischer in 1906 (ref. 81) who used a saturated solution, about 13N HCl. The procedure has frequently been used since and complete esterification can be attained using much lower concentrationsof HCl (ref. 82). The rate and extent of esterification depend on the concentration of HCl and on the temperature; 0.1N HCl gives complete esterification of carboxyl groups in 24 hours at room temperature (ref. 79). Methanolysis of peptide bonds occurs to a sign-

ificant extent even under these conditions.

Esterification of DL-Ala-Gly-Gly according to Geddes and Agarwal was performed under conditions rigorously excluding moisture. Esterification was virtually complete but methanolysis had occurred in both cases, though far more extensively with the use of 6.2N HCl (Exps. (19) and (20)).

The products of the esterifications were acetylated under the conditions specified by each author. Analytical TLC of the final products showed that the expected Ac-Ala-Gly-Gly-OMe made up about 70% of the total products when Agarwal et al.'s procedure was used and a much smaller proportion in the other case. The level of byproducts corresponded to the extent of methanolysis observed in the esterification step, being much worse where 6.2N HCl in methanol had been used (Exps. (7) and (8)).

To summarise, the three methods previously used to prepare peptide derivatives for permethylation all give byproducts. Use of methanolic HCl, even in low concentration, has caused extensive methanolysis of the peptide. Treatment of the peptide with methanol + acetic anhydride can yield the pure acetyl derivative, if conditions are strictly controlled, though a variety of side reactions can occur.

C. ACETYLATION OF FREE PEPTIDES:

As already noted, it is not necessary to esterify free carboxyl groups of the peptide prior to permethylation; acetylation alone is necessary.

The characteristics of a suitable acetylation procedure are as follows: the chemical steps necessary must be applicable to small amounts of peptide, there must be an absence of side-reactions and the product must be readily isolated, preferably by evaporation of the coproducts and excess reagents. It is desirable that the acetylation should be complete in only a few hours. Several room-temperature acetylation procedures using volatile reagents were tried on DL-Ala-Gly-Gly and the products examined.

Use of equal amounts of acetic acid and acetic anhydride proved entirely satisfactory; however, acidic peptides such as Phe—Asp—Ala—Ser—Val would not dissolve in this reagent without the addition of water and the heterogeneous reaction was very slow (Exp. (47)). Addition of water to acetic acid + acetic anhydride also gave a low level of byproducts, but the reaction did not go to completion and the isolated product contained significant amount of free peptide (Exp. (12)). The most satisfactory procedure was the use of equal amounts of water and acetic anhydride. Side-reactions did not occur to a significant extent and reaction was rapid and quantitative at room temperature. On addition of the acetic anhydride to the aqueous solution of peptide, a heterogeneous mixture was formed. Shaking at room temperature resulted in a clear, homogeneous solution after about 20 minutes when acetylation was also complete (Exp. (13)). It was possible that cleavage of the C—terminal residue could have occurred by a mechanism similar to that with methanol + acetic anhydride, giving Ac—Ala—Gly—OH and Ac—Gly—OH as byproducts. These compounds were not detected. The products of reaction for 21 hours at room temperature were esterified with diazomethane and re-examined by analytical TLC for the corresponding esters; these were not present (Exp. (13a)).

Use of water + acetic anhydride to selectively acetylate the amino group of tyrosine was reported as long ago as 1932 (ref. 123). It is a standard procedure for the selective acetylation of amino functions in the presence of hydroxyl groups (ref. 83). It possessed this advantage of Thomas' method using methanol + acetic anhydride, while not giving the byproducts of that method for the peptide studied. Conditions for routine acetylation with water + acetic anhydride are given in the experimental section, p.64.

D. ACETYL-PEPTIDE ESTERS:

All three groups of research workers who have published mass spectra of permethylated peptide derivatives have used acyl-peptide methyl esters for

permethylation. Lederer's group also permethylated free acetyl-peptides which they considered equally suitable for permethylation (ref. 44). This unnecessary use of the esters of acyl-peptides was perhaps due to the previous use of the unpermethylated acyl-peptide esters as the standard derivatives for mass spectrometry.

A rapid method for synthesising pure esters of acetyl-peptides on a micro-scale was required in order to study their permethylation products. The procedures of Geddes et al. and Agarwal et al. had been shown to be unsatisfactory (see previous section, p.25). Several other esterification procedures were examined.

HCl in methanol is the standard reagent for esterifying amino acids, peptides and proteins (ref. 84). The reagent is prepared by bubbling dry HCl gas into absolute methanol, by the addition of acetyl chloride to absolute methanol (ref. 85), or by addition of concentrated aqueous HCl to methanol (ref. 86). Various concentrations of HCl have been used, the saturated (13N HCl) solution of Fischer giving way to 0.01N HCl (ref. 82). Methanolysis of the peptide is always observed (refs. 84,87,88), although the use of 0.1N HCl in absolute methanol was found to minimise methanolysis while giving quantitative esterification in 24 hours at room temperature (ref. 79). N to O acyl-migration involving the hydroxyl groups of serine and threonine also occurs (refs. 77,88).

The peptide Phe—Asp—Ala—Ser—Val was treated with methanol + acetyl chloride, then acetylated. Mass spectrometry showed the presence of Ac—Phe—Asp—OMe and Ac—Phe—Asp—Ala—OMe as well as the expected esterified acetyl-peptide
OMe OMe
(Exp. (14)). When DL-phenylalanine was treated with this reagent under identical conditions, only 50% esterification occurred (Exp. (15)). Complete exclusion of moisture and extension of the reaction time to $3\frac{1}{2}$ days with DL-Ala-Gly-Gly left little unreacted peptide, but four ninhydrin-positive products were formed (Exp. (16)). The reagent did not give complete esterification in a reasonable

time and resulted in significant degradation of the peptide.

Addition of 2,2' - dimethoxypropane to the methanolic HCl had been shown to accelerate the esterification of amino acids and fatty acids (refs. 86,89,90,91). This chemical acts as a water scavenger, generating methanol in the process and thus accelerating the esterification :

$$H_2O + (CH_3)_2C(OCH_3)_2 \longrightarrow 2CH_3OH + CH_3CO.CH_3$$

Methanol + acetyl chloride + 2,2' -dimethoxypropane was used to esterify DL-phenylalanine. No unreacted phenylalanine remained after 3 days, although the esterification resulted in the formation of several ninhydrin-positive products (Exp. (17)). This is possibly due to condensation of the phenylalanine methyl ester in the absence of water to yield oligopeptide esters, a process that should be accelerated by the presence of a water scavenger (cf. formation of the tetrapeptide ester on standing glycine ester in a suitable solvent at room temperature in the absence of water, (ref. 92a)). Also, a red gum was produced in increasing quantity throughout the reaction. Use of the same reagents on DL-Ala-Gly-Gly gave similar results (Exp. (18)). No unreacted tripeptide remained after three days at room temperature, but several ninhydrin--positive products were formed. These could have been due to methanolysis of the peptide, or to condensation reactions yielding polypeptides (cf. production of the hexapeptide ester from standing glycylglycylglycine methyl ester in methanol at room temperature, (ref. 92b)). A red gum was produced in increasing quantity throughout the reaction and was apparently identical with that from reaction with DL-phenylalanine.

Dry methanolic HCl was generated and used for esterification under conditions which rigorously excluded moisture. Two strengths, 0.1N HCl and 6.2N HCl, were used on DL-Ala-Gly-Gly at room temperature. The progress of the esterification was monitored by TLC. No unreacted peptide remained after 6 hours and 3 hours respectively. In both cases several ninhydrin-positive

products were formed by this time; this was attributed to methanolysis of the peptide and was more extensive with the stronger HCl solution (Exps. (19),(20), (21) and (22)). Another method for the esterification of peptides was used by Wilcox (ref. 78) and Hormann (ref. 77). They used methanol + acetic anhydride for twenty to thirty hours at room temperature to produce acetylated, esterified peptides. Under these conditions the reagent gives extensive artefact formation, as we have seen (p.22); moreover, it is known to give only partial esterification, as was observed on p. 22 (refs. 76,77). An N to O acyl-migration involving serine residues has also been observed (ref. 77).

Diazomethane is known to esterify rapidly and quantitatively in solution at room temperature. Its use to esterify amino acids and peptides had been reported as unsatisfactory owing to methylation of the nitrogen atom (refs. 93,94), although it had been used to esterify trifluoroacetylamino acids for GLC, apparently quite satisfactorily (ref. 95). The N to O acyl-shift observed with other esterification procedures does not occur with diazomethane (ref. 88).

Esterification of Gly-Val and DL-Ala-Gly-Gly with diazomethane gave rise to products with several extra methyl groups in the molecule (Exps. (23) and (24)). There was no indication of the site of this methylation, which could in theory occur either at the enol form of the amide carbonyls or on the free amino group. Acetylation of DL-Ala-Gly-Gly was followed by treatment with excess diazomethane. Analytical TLC showed the only significant product to be Ac-Ala-Gly-Gly-OMe (Exp. (25)); no unesterified peptide remained. Mass spectrometry of the crude reaction products showed the major product to be Ac-Ala-Gly-Gly-OMe; there was a minor amount of compound with an additional methyl group, as well as a very small amount of another product not simply related to the acetyl--peptide ester (see Fig. 31 , Exp. (39)).

This was the most satisfactory preparation of the acetyl-peptide ester of all the methods investigated. Methanolysis did not occur, there was a low level

of byproduct formation and the esterification was rapid and quantitative.

4. THE PERMETHYLATION REACTION.

A. PREPARATION OF THE DIMETHYLSULFINYL CARBANION:

The dimethylsulfinyl (dimsyl) carbanion was first prepared by Corey and Chaykovsky in 1962 (ref. 70) as a cloudy yellow-grey solution in dimethylsulfoxide (DMSO). This carbanion is a very strong base; it reacts rapidly with oxygen, carbon dioxide and water, and these must be rigorously excluded in its preparation and subsequent use (ref. 96). Because of the low acidity of DMSO, estimated to be 10^{-4} that of triphenylmethane (refs. 97,98), generation of the carbanion requires the use of a strong base. The reagents most commonly used to prepare it are metal hydrides and metal amides (ref. 99); in this case, one of the products is a gas, hydrogen or ammonia, which is removed from the reaction system ensuring quantitative generation of the carbanion.

The dimsyl carbanion is usually prepared as its sodium salt in DMSO, according to the original method of Corey and Chaykovsky. This involves heating finely powdered sodium hydride in DMSO at 65° - 70° under nitrogen, until hydrogen evolution stops after about forty-five minutes. The carbanion solution is very sensitive to heat, and prolonged heating or the use of higher temperatures leads to extensive decomposition (refs. 70,100). The thermal decomposition of the dimsyl carbanion in DMSO has been studied by Price and Yukuta (ref. 101). They found that heating 1 M solutions at 80°C for several hours led to extensive decomposition: the solutions turned dark red-brown, a white precipitate separated and volatile products were evolved. These volatile products were identified as mixed dienes and dimethyl sulfide. The rate of decomposition at 97°C was such that after thirty minutes 17% decomposition had occurred, while after five hours 60% of the carbanion had decomposed. At temperatures above 100°C, the initial reaction became violent. Solutions of dimsyl sodium are relatively stable at room temperature under nitrogen, losing about 8% of their activity per week (ref. 100). The carbanion has also been generated without external

heating, using sodium hydride in DMSO and an ultrasonic generator to disperse the reagent (ref. 102). The chemistry of the dimethylsulfinyl carbanion has recently been reviewed by Durst (ref. 99).

Generation of the dimsyl carbanion for permethylation has been described by Thomas (refs. 46, 65) and involves reaction of sodium hydride and DMSO at 100°C for five minutes. This could be expected to lead to partial decomposition of the carbanion. We found that permethylation of DL-Leu-Gly-Phe using dimsyl sodium in DMSO generated at 110°C gave a partially methylated product (Exp. (26)).

Since it was intended to carry out many permethylations, the dimethylsulfinyl carbanion was generated on a larger scale according to Corey and Chaykovsky at temperatures not exceeding 68°C (Exp. (27)). Over-heating to 100°C led to decomposition of the carbanion as evidenced by the strong odour of organic sulfides and the appearance of a yellow colour. Use of a heating mantle led to localised overheating and similar decomposition. The dark-grey solution of dimsyl sodium was stored under nitrogen at -20°C (ref. 100).

Dimsyl sodium in DMSO was also generated on a smaller scale "in situ" immediately prior to use, at temperatures not exceeding 70°C (experimental section, p.64).

B. REACTION CONDITIONS:

Because the first step in the permethylation involves deprotonation, the reaction must be performed in an aprotic solvent such as DMSO. The dimsyl carbanion is an exceptionally strong base and deprotonation reactions involving it will be rapid; virtually instantaneous deprotonation of an acid as weak as triphenylmethane has been reported (ref. 100). The subsequent reaction with methyl iodide will also be favoured, as anions have been shown to be weakly solvated and very reactive in DMSO. Considerable rate enhancement has been observed in many similar reactions using DMSO as solvent (ref. 103).

Use of the dimsyl carbanion in permethylation was first described by

Hakomori (ref. 104), who used it to O-methylate complex carbohydrates; it gave a completely methylated product where standard methods failed. Vilkas (ref. 47) adapted its use to the O- and N-methylation of a peptidoglycolipid for mass spectrometry. Both of these authors used relatively long equilibration times (ten minutes to an hour) with equivalent amounts of carbanion, prior to the addition of the methyl iodide. Thomas (refs. 46,65,66) used dimsyl sodium and methyl iodide to permethylate a wide variety of peptide derivatives; addition of the peptide derivative to a large excess (ten to fifty-fold) of dimsyl carbanion was followed immediately by addition of methyl iodide. Reaction times following the addition of the methyl iodide ranged from twenty minutes (Hakomori) to twelve hours (Vilkas); Thomas used a time of one hour. In all cases the reaction was terminated by the addition of water.

As difficulty had been encountered with the preparation of pure acetyl-peptides, acetylphenylalanine methyl ester was used to examine permethylation conditions. A solution of dimsyl sodium in DMSO which had been stored for eight weeks under nitrogen at -20°C was used. The equilibration time with dimsyl carbanion was varied from thirty minutes to two minutes before addition of the methyl iodide and the subsequent reaction time with methyl iodide, from sixty minutes to four minutes (Exps. (28),(29) and (30)). Although complete N-methylation was observed in all cases, mass spectrometry of the reaction products failed to show significant amounts of the expected molecular ion from Ac-Phe-OMe. A variety of ions of higher mass were observed.
Me
Use of dimsyl sodium in DMSO prepared "in situ" immediately prior to use gave a similar result (Exp. (31)), eliminating the possibility that decomposition of the stored carbanion was at fault.

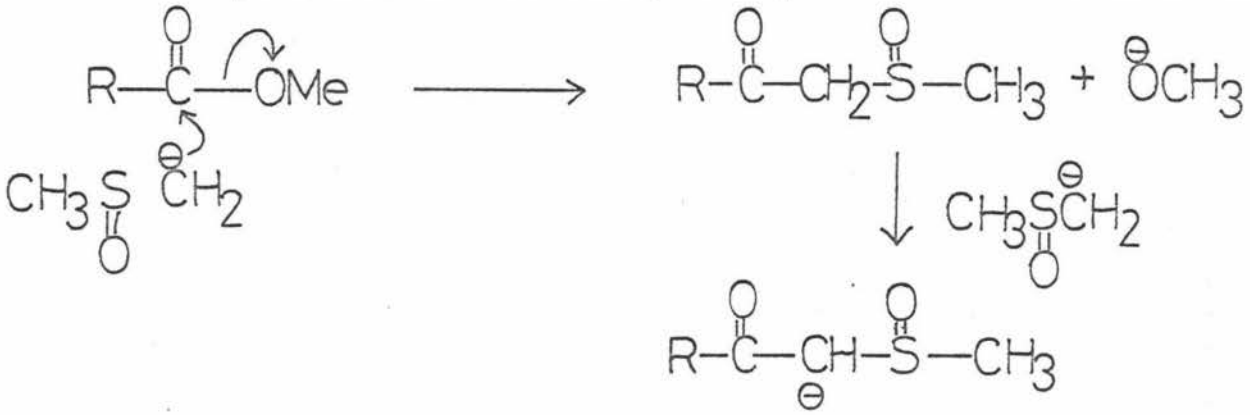
Permethylation of acetylphenylalanine under the same conditions and examination of the products by mass spectrometry showed molecular ions due to Ac-Phe-OMe and a homologue with an extra methyl group in the molecule (Exp.(32)).
Me

There was no evidence of significant amounts of the products obtained from permethylation of acetylphenylalanine methyl ester.

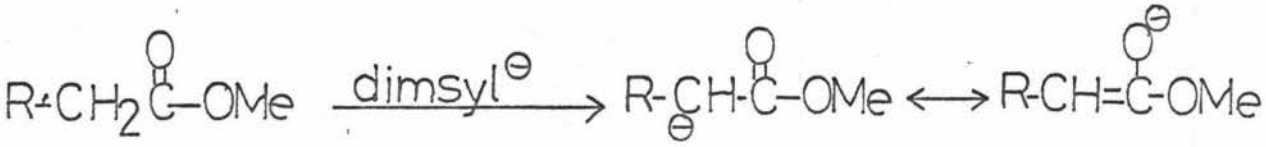
Addition of the peptide derivative to the dimsyl carbanion followed by immediate addition of methyl iodide and a subsequent reaction time of about ten minutes seemed satisfactory reaction conditions: these were routinely used throughout the remainder of the investigation and in every case gave complete N- and O- methylation.

C. PERMETHYLATION OF ACETYL-PEPTIDE ESTERS:

The dimethylsulfinyl carbanion reacts with esters in two ways (ref. 96). The carbonyl group of the ester can undergo nucleophilic attack by the dimsyl carbanion to yield the β-keto sulfoxide; two equivalents of carbanion are used:

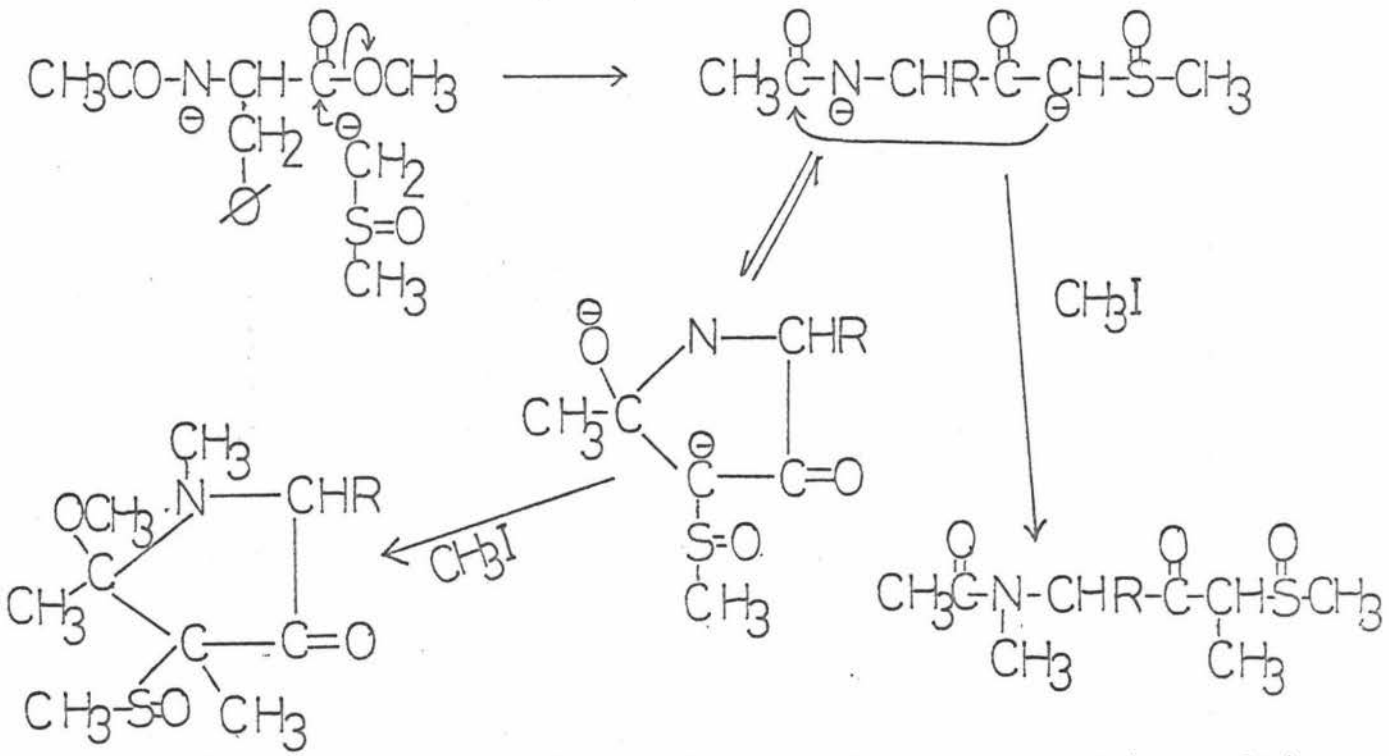


Such β-keto sulfoxides are readily alkylated at the α-carbon atom (ref. 105). On the other hand, if the α-carbon atom of the ester has a labile proton or if carbonyl addition is sterically hindered, then deprotonation predominates:



Many esters undergo both reactions. On addition of methyl iodide, the anions will be methylated.

It was found that permethylation of acetylphenylalanine methyl ester failed to give the expected Ac—Phe—OMe, and the mass spectra indicated some
Me
form of substitution at the ester group (Exps. (28) to (31)). The exact nature of the products could not be deduced from the mass spectrum, although there was evidence of sulfur-containing ions, which could have arisen as follows:



After a suitable esterification procedure had been devised (see p.29) acyl-peptide esters were permethylated and the products examined. Mass spectrometry of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OMe showed the expected molecular ion accompanied by an ion of equal intensity 10 mass units (m.u.) higher. The spectrum contained the expected sequencing peaks up to the C-terminal residue (Exp. (37)). Recognition of the molecular ion of the expected product would not be possible because of additional intense ions in the same region.

Permethylation of Ac—Phe—Asp—Ala—Ser—Val—OMe resulted in a very
OMe
complex mass spectrum, with indications that the ester group of the aspartyl residue had also undergone side reactions (Exps. (33),(34) and (35)). In both cases, permethylation of the free acid of the peptide resulted in the expected product. The spectra are compared in Figs. 1 and 2, overleaf.

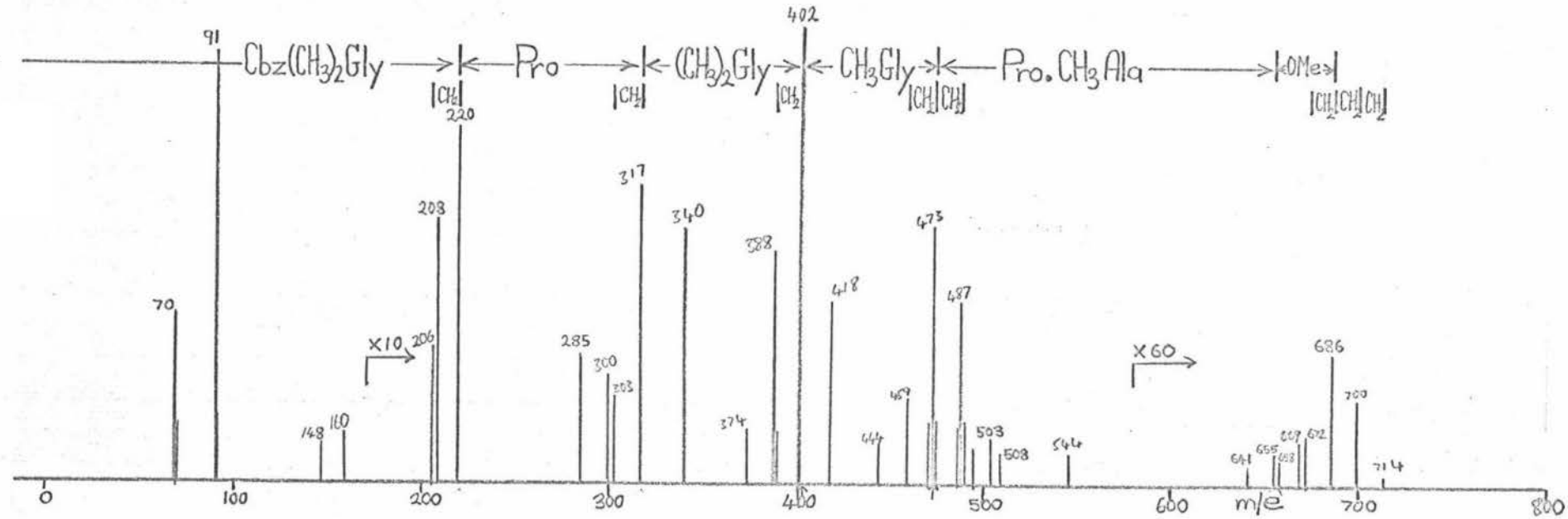


Fig. 1(a) Mass spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OH (Exp. (43)).

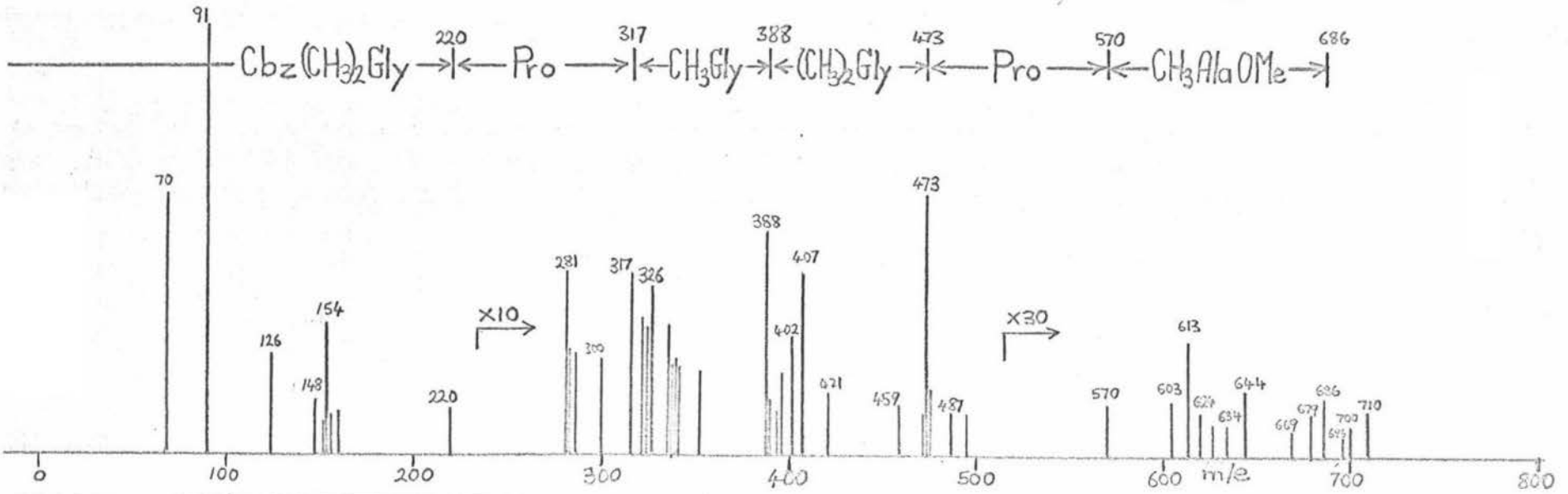


Fig. 1(b) Mass spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OMe (Exp. (37)).

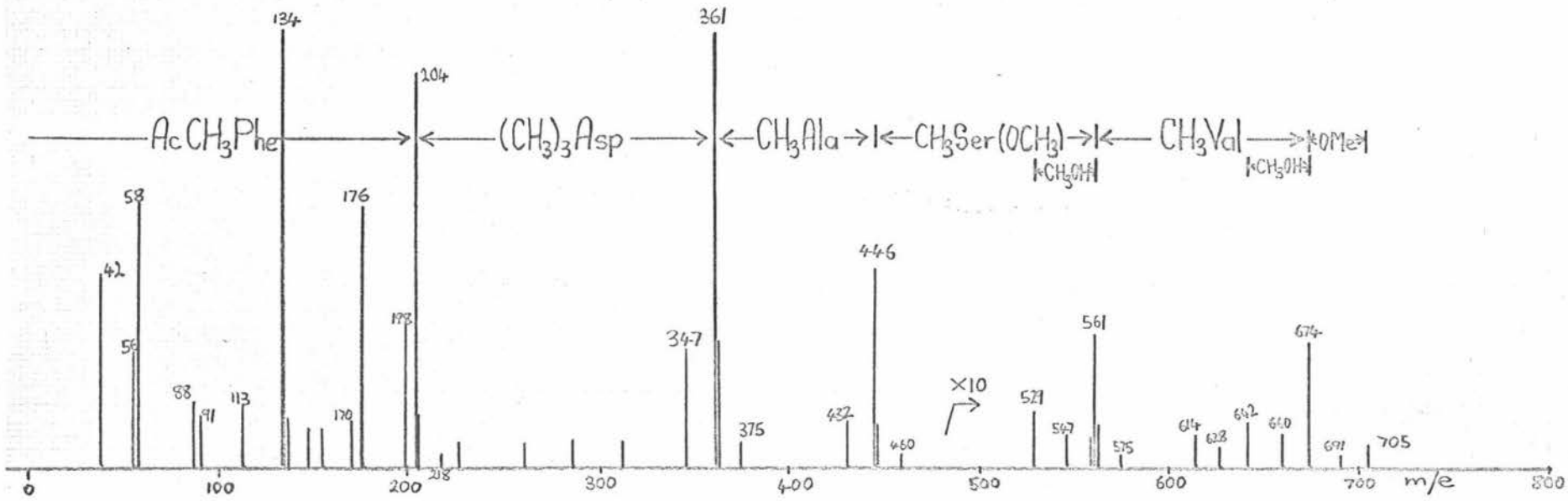


Fig. 2(a) Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val-OH (Exp. (47)).

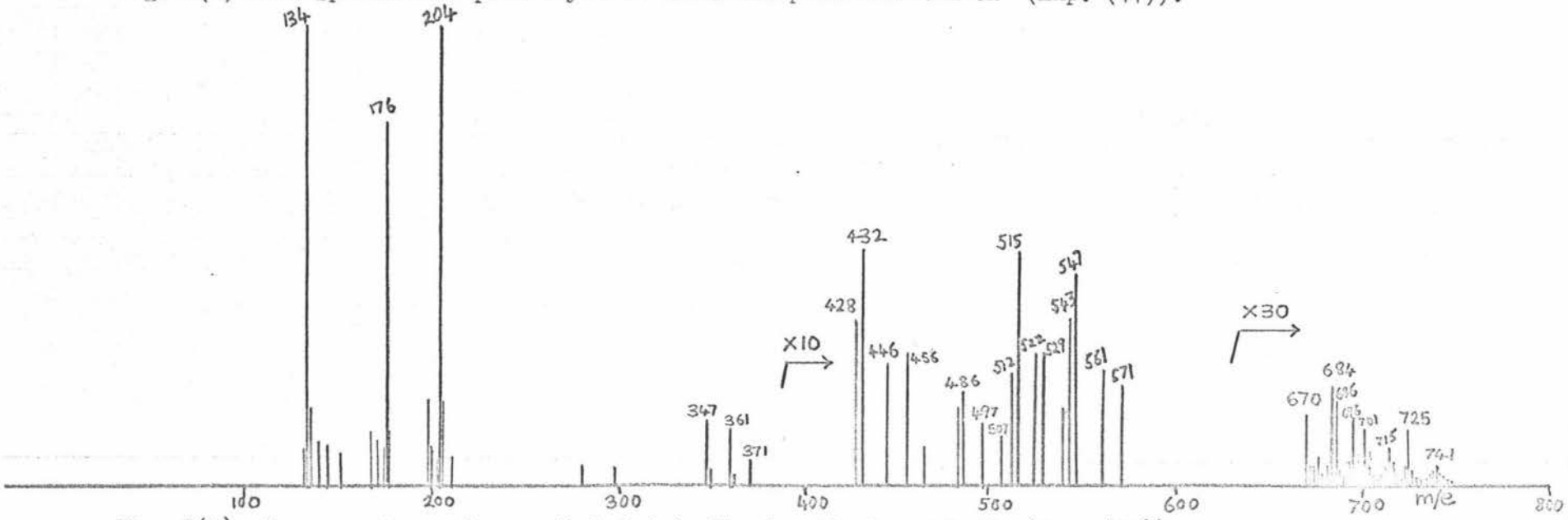
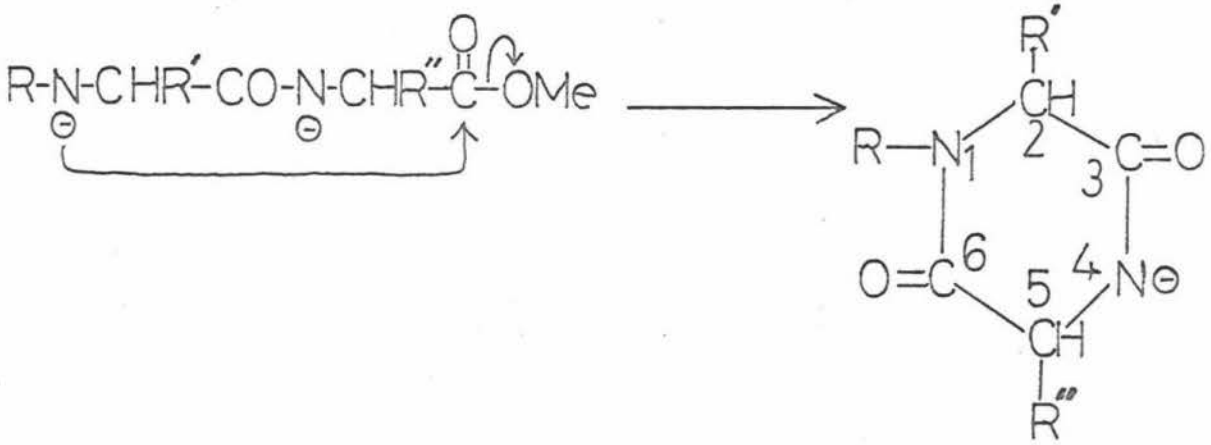


Fig. 2(b) Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val-OMe (Exp. (35)).

Mass spectrometry of permethylated Ac-Ala-Gly-Gly-OMe gave two spectra with molecular ions at m/e 329 and m/e 339 (Exp. (39)). However, in this case the spectrum of permethylated Ac-Ala-Gly-Gly-OH (Exp. (38)) showed the molecular ion at m/e 329, 28 m.u. higher than the mass of the theoretical product, Ac-Ala-Gly-Gly-OMe. Both these products were hydrolysed
Me Me Me
in acid. Analysis showed N-methylglycine and N-methylalanine plus small amounts of the free amino acids and several other ninhydrin positive compounds. The analyses were identical and did not help explain the unusual nature of the parent compounds (Exp. (40)).

The permethylation of acyl-peptide esters is further complicated by the possibility of intra-molecular nucleophilic attack, yielding cyclic products such as:

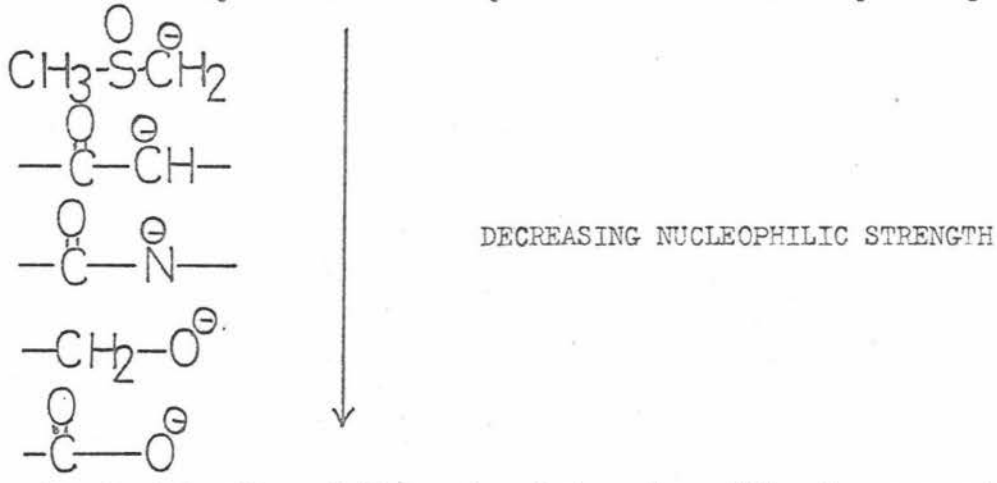


This compound would be susceptible to further methylation at the N-4 and C-2 atoms. Evidence was obtained of such cyclisation at both ester groups in the permethylation of Ac-Phe-Asp-Ala-Ser-Val-OMe (Exps. (33),(34) and (35)).
OMe
High resolution mass measurements on the ions 10 m.u. higher than the expected molecular ions in the above examples showed that these ions could have resulted from intramolecular cyclisation followed by the introduction of four additional methyl groups. On this assumption, mechanisms could be written for many observed metastable transitions.

In an effort to characterise the artefacts produced in the permethylation of esters using the dimsyl carbanion, methyl benzoate was permethylated

under the usual conditions and the crude reaction products analysed by mass spectrometry (Exp. (42)). As well as unchanged methyl benzoate, several other products were observed; one of these was the dimethylated β-keto sulfoxide. Other products were not identified; they did not occur when benzoic acid was permethylated (Exp. (41)).

Although it was not possible to characterise artefacts from the use of acyl-peptide esters, their formation could be prevented by permethylation of the free acid. In all cases, this gave excellent mass spectra with the expected molecular ions accompanied only by higher homologues due to the introduction of more than the expected number of methyl groups. In the strongly basic dimsyl sodium solution, the carboxyl group will be completely deprotonated. Nucleophilic attack cannot occur at the carbonyl of the carboxylate anion. On introduction of methyl iodide into the mixture of peptide--anion and dimsyl carbanion, competitive reaction will occur, preferentially with the strongest nucleophile, the dimsyl carbanion. The weakly nucleophilic $-COO^{\ominus}$ will react very much more slowly than the other nucleophiles present:



Significant esterification of the carboxylate anion will not occur until virtually all of the dimsyl carbanion has reacted.

The use of acyl-peptide esters for permethylation and mass spectrometry should be avoided. Artefact formation obscures the molecular ion region in all cases, while the presence of more than one ester group in the molecule results

in complex and generally uninterpretable mass spectra. Unchanged ester remains, offering some hope of successfully sequencing peptides containing pre-existing ester groups, although knowledge of likely artefacts is essential.

D. INTRODUCTION OF EXTRA METHYL GROUPS:

In our experience, use of the Hakomori-Vilkas permethylation always resulted in the introduction of more than the required number of methyl groups into the peptide molecule. This "extra-methylation" has also been observed using other permethylation procedures. Agarwal et al. used the anion of dimethylacetamide with methyl iodide in dimethylacetamide. They observed partial extra-methylation of a glycine residue in a tetrapeptide; this was prevented by reducing the amount of base present in the methylation reaction (ref. 52). Thomas, working with Lederer's group, used a simple heterogenous mixture of methyl iodide, sodium hydride and acyl-peptide in dimethylformamide to prepare permethylated peptides (ref. 49). He observed extra-methylation of a number of different residues in a variety of peptides; extra-methylation could not be avoided using this reagent. Thomas found that the Hakomori-Vilkas permethylation procedure gave permethylated peptide derivatives with no indication of extra methyl groups (ref. 66).

He also noted that there are two possible sites at which an additional methyl group could be introduced into an N-methylamino acid residue: O-methylation of the enol form, or methylation of the α-carbon atom. He eliminated the possibility of O-methylation on the grounds that it could not explain the two methyl groups occasionally introduced in glycine residues (ref. 66). Agarwal had stated it to be C-methylation, but published no supporting evidence (ref. 49).

It was found that permethylation of acetylphenylalanine using fresh dimsyl carbanion resulted in extra-methylation (Exp. (32)), and mass spectrometry indicated that an extra methyl group had been introduced on the α-carbon atom. Permethylation of Cbz-Gly-Pro-Gly-Gly-Pro-Ala gave a

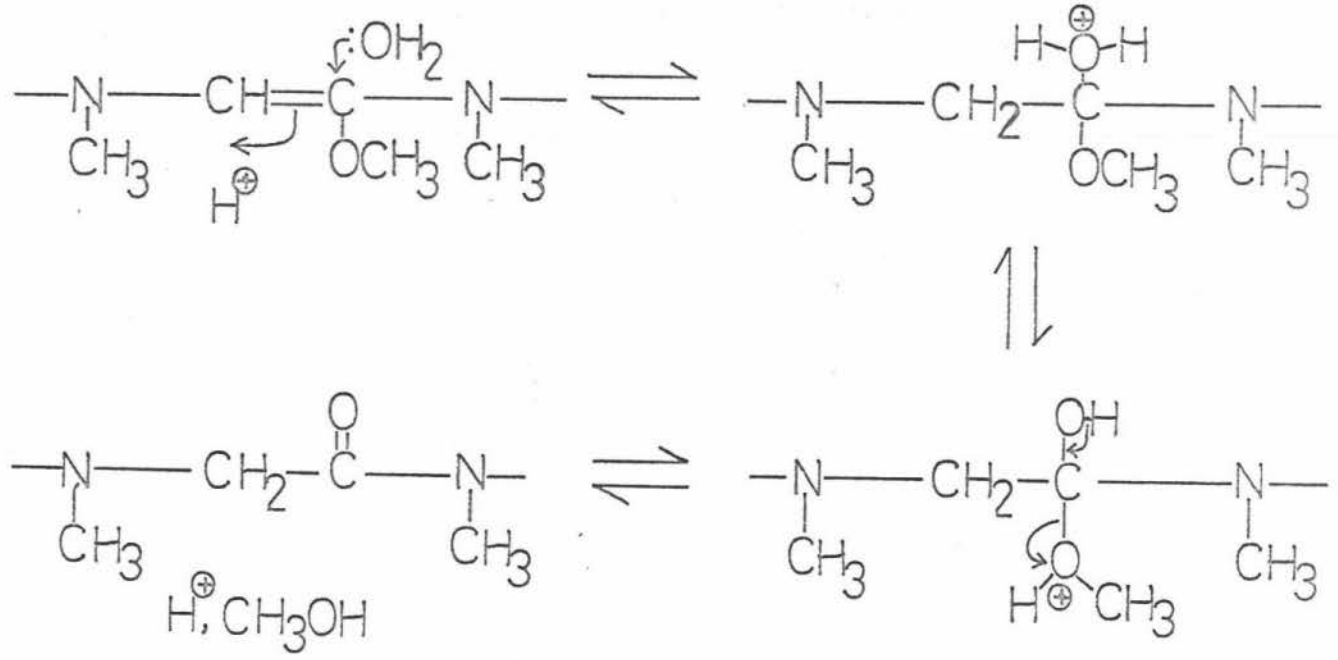
mixture of products showing molecular ions at m/e 672, 686, 700 and 714 (Exp. (43)). The theoretical product has mass 658. Use of deuteriomethyl iodide under the same conditions showed that up to nine methyl groups had been introduced into the molecule during the permethylation, compared with the five theoretical sites for methylation (Exp. (44)). Permethylation of Ac-Phe-Asp--Ala-Ser-Val followed by mass spectrometry showed the principal product to contain a single extra-methyl group on the aspartyl residue; extra-methylation had occurred to only a very minor extent on the other residues (Exp. (47)).

Site of Extra Methylation:

In order to determine the site of extra-methylation, Ac-Gly-Gly was permethylated and examined by mass spectrometry. About thirty percent of the product had an extra methyl group in the C-terminal residue. The product was hydrolysed in acid and N-methylglycine and N-methylalanine identified in the ratio of 2-3 : 1, (Exp. (49)), indicating that the C-terminal glycine residue had been methylated on the α-carbon atom.

In another experiment, acetylglycine was repeatedly permethylated, and mass spectrometry showed that about twenty percent of the product contained an extra methyl group. Examination of the mass spectrum indicated that the extra methyl group was on the α-carbon atom. Acid hydrolysis and analysis identified N-methylglycine and N-methylalanine in the ratio of 2-3 : 1 (Exp. (50)), confirming C-methylation.

The analysis of acid hydrolysates of extra-methylated compounds would not be expected to show direct evidence of the O-methylated compounds. These are sensitive to acid and would be demethylated in the hydrolysis: (see over)



Thus extra O-methylation cannot be ruled out. However, the weight of evidence suggests that it does not occur: there was no evidence of an additional O-methyl group in the mass spectra of permethylated Ac-Gly-Gly, Ac-Gly and Ac-Phe; furthermore, the amount of N-methylalanine found in the acid hydrolysates of permethylated Ac-Gly-Gly and Ac-Gly was sufficient to account for the amount of extra-methylation detected by mass spectrometry and detailed examination of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala showed that the N-terminal glycine residue was methylated on the α-carbon atom, not on the enolate O-atom (Exp. (46)).

Residue-Specific Extra Methylation:

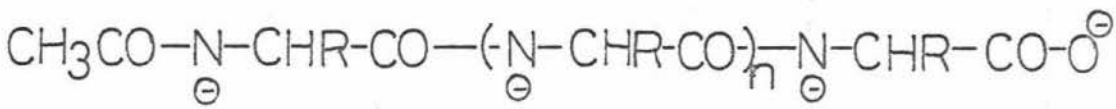
Re-examination of the mass spectra of the permethylated derivatives of Cbz-Gly-Pro-Gly-Gly-Pro-Ala and Ac-Phe-Asp-Ala-Ser-Val showed that two types of extra-methylation had occurred. The residues underlined had been specifically extra-methylated to an extent of at least eighty percent; as well as this, several of the other residues had been extra-methylated to a much lesser extent. For example, the N-terminal phenylalanine of the second peptide was about five percent extra-methylated, while the glycine residue in position 3 of the Cbz-hexapeptide was about thirty percent extra-methylated. This much less extensive methylation was termed "random" extra-methylation.

It was thought that extra C-methylation might be due to deprotonation of the α-carbon atom after methylation of the previously deprotonated nitrogen

atoms. This sequential ionization is suggested because a multiple anion involving neighbouring atoms is unlikely on energy considerations and initial deprotonation of the amide nitrogen would be expected to be more favourable than deprotonation of the α-carbon atom. The dropwise addition of methyl iodide to the solution of dimsyl carbanion and peptide hitherto employed would accentuate extra-methylation according to this mechanism. By the same token, if the solution of dimsyl carbanion and peptide in DMSO was slowly added to a large excess of methyl iodide, the rapid preferential methylation of excess dimsyl carbanion would minimise sequential ionization.

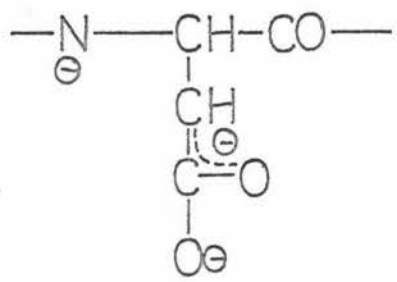
A solution of dimsyl sodium and Cbz-Gly-Pro-Gly-Gly-Pro-Ala was added to vigorously stirred methyl iodide, using a micrometer syringe. Mass spectrometry of the permethylated product indicated that extra-methylation had occurred almost exclusively at the glycine residues in positions 1 and 4. High resolution mass measurement and metastable detection indicated that the N-terminal glycine residue was permethylated at the α-carbon atom, only (Exp. (46)). Ac-Phe-Asp-Ala-Ser-Val was permethylated under similar conditions and gave a mass spectrum indicating that extra-methylation had occurred at the aspartyl residue to the extent of about fifty percent: the other residues were not extra-methylated (Exp. (48)). Thus the so-called "random" extra-methylation was eliminated in this way.

In practice, it was found that rapid addition of a large excess of methyl iodide to the vigorously stirred solution of dimsyl carbanion and peptide in DMSO was more convenient and equally efficient in this respect. Under these conditions, the permethylation apparently proceeds via a "multiple anion" mechanism. The dimsyl carbanion deprotonates the peptide molecule:

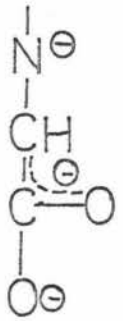


On addition of methyl iodide, excess dimsyl carbanion would be neutralised and the peptide methylated at the anionic sites.

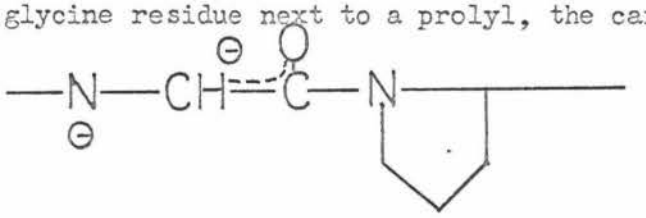
Residue-specific extra-methylation occurs at carbon atoms that are more easily deprotonated because of their environment within the molecule. In the aspartyl residue, the carbanion:



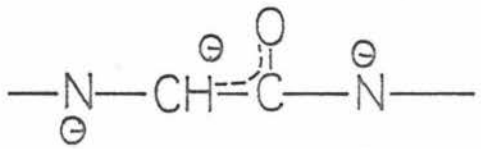
is more stable than



which could be formed at a C-terminal glycine residue. Similarly, at the glycine residue next to a prolyl, the carbanion



is more stable than the carbanion



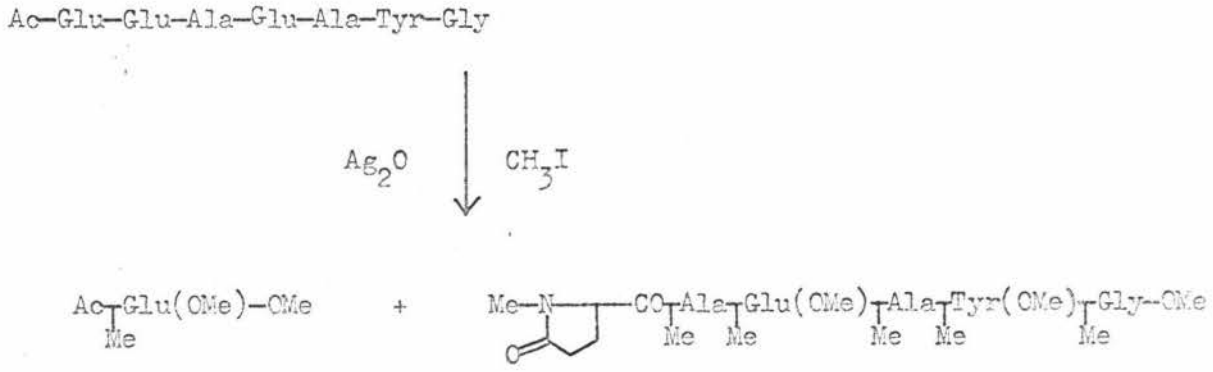
which could be formed at other glycine residues. Extra-methylation of glutamic acid residues would also be expected.

It was observed that even at these sites, extra C-methylation was not quantitative. Sufficient destabilisation of the carbanions must still exist in the multiple anion of the peptide to prevent their complete formation in the presence of dimsyl carbanion. The use of a sodium hydride/acyl-peptide/methyl iodide mixture for permethylation would ensure complete reaction at these sites via a sequential mechanism in which the readily ionizable groups (-NH-, -OH, -COOH) would first be methylated, followed by deprotonation of the carbon atoms in the favoured sites; these would be completely methylated, as Thomas observed

(ref. 66). Partial or complete methylation at even less favoured sites could also be expected, but this would take place slowly under the reaction conditions he used. This method is more prone to C-methylation because it involves a different (sequential) reaction mechanism.

E. SPECIFIC CHAIN-CLEAVAGE:

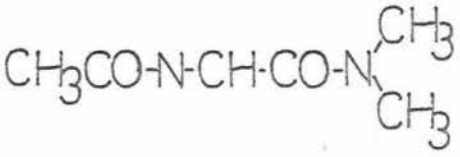
Partial cleavage of the peptide chain in the permethylation reaction has been observed by Thomas (ref. 65) and Agarwal (ref. 106); in both cases, the Kuhn permethylation procedure employing silver oxide and methyl iodide was used. The cleavage occurred at glutamic acid residues and involved the formation of a pyrrolidone:

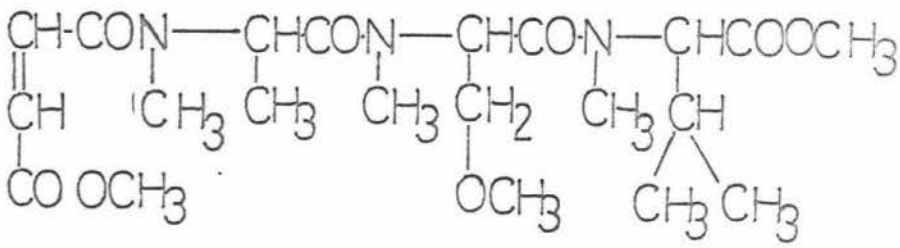


Products due to cleavage at the other glutamic acid residues were also formed (ref. 106). Thomas and Lederer at first ascribed this to use of silver oxide contaminated with methanol (refs. 43,45), but subsequently observed the same cleavage using pure silver oxide (ref. 65). Artefact formation at aspartic acid residues was also observed by both groups under these conditions, apparently due to cyclisation of the aspartyl side chain followed by extra-methylation (ref. 106).*

* cf. cyclisation artefacts at aspartyl residues using the Hakomori-Vilkas permethylation procedure, p.36.

We have observed chain cleavage at aspartyl residues. Permethylation of Ac-Phe-Asp-Ala-Ser-Val under the usual conditions was followed by mass spectrometry (Exp. (47)). Three distinct spectra were observed as the sample was heated. This was attributed to specific cleavage of the peptide chain, the first two spectra being due to the products formed. High resolution mass measurements enabled structures to be proposed for these:





Permethylation of the original acetyl-peptide was repeated, using deuteriated methyl iodide, when three analogous spectra were also obtained (Exp. (51)). Thorough investigation of the first two spectra confirmed the structures shown above. In both cases, the third spectrum was that of the expected product, extra-methylated in the aspartyl side-chain.

Partial chain cleavage had occurred specifically at the aspartyl residue. There was no evidence of extra-methylation in either of the fragments formed. The spectrum of the lower mass fragment was obtained "probe-out" at room temperature, indicating that the cleavage had not occurred on heating the sample in the mass spectrometer. The cleavage was not observed on permethylation of Ac-Phe-Asp(OMe)-Ala-Ser-Val-OMe under the usual conditions (Exp. (35)) , and occurred to a reduced extent under conditions minimizing sequential ionization (Exp. (48)).

The structures of the products formed, together with these observations point towards a base-catalysed Hofmann elimination resulting in cleavage of the peptide chain (see Fig. 3, overleaf) . This may proceed via the quaternary amide (III), in the presence of the strong base $B^{\ominus}$, in this case the dimsyl carbanion.

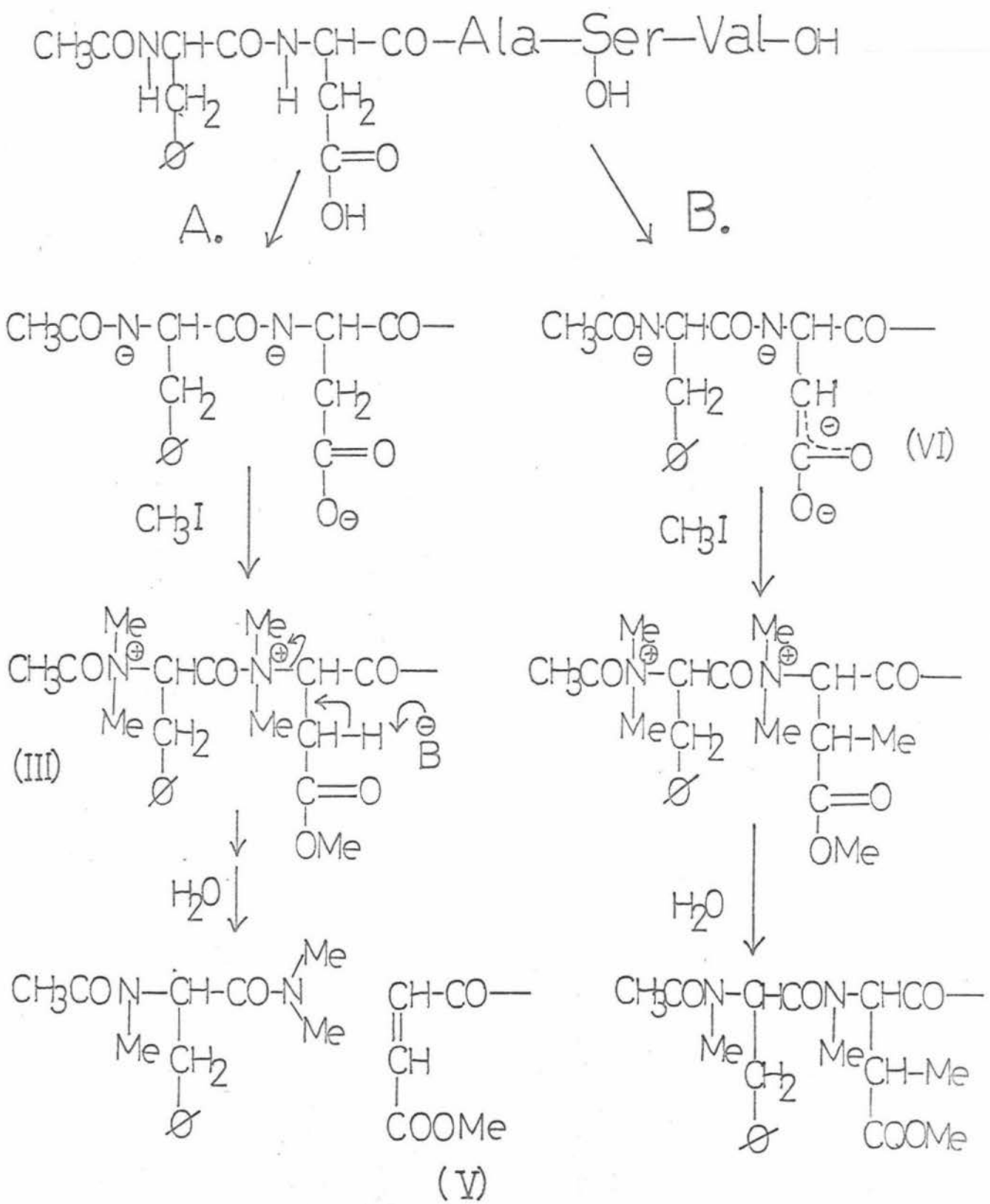


Fig.3 Permethylation of the aspartyl-containing peptide.

Pathway A: chain-cleavage via the quaternary amide

Pathway B: C-methylation of the aspartyl side-chain

The specific chain cleavage would be due to an unusually labile proton on the carbon atom β to the amide nitrogen in the aspartyl residue. The absence of extra C-methylation at this site in the fragment (V) indicates that chain cleavage of the quaternised peptide occurs simultaneously with deprotonation of the carbon atom.

On mixing the acyl-peptide with the dimsyl carbanion, some initial carbanion formation in the peptide will occur, leading to a second reaction pathway (see Fig. 3, B). Formation of uncleaved permethylated peptide, with an extra methyl group in the aspartyl side chain could be explained by this deprotonation of the carbon atom β to the amide nitrogen, prior to the addition of methyl iodide. This would result in extra-methylation of this carbon atom; the positive inductive effect of the introduced methyl group could prevent abstraction of the remaining proton.

Quaternised amides have been reported (ref. 107); they are sensitive to water, regenerating the alkylated amide. It is possible that every amide nitrogen atom in the peptide is quaternised in the permethylation and that injection of water yields the observed N-methyl products. The small amount of expected product, the uncleaved permethylated peptide without extra methyl groups. formed in the above reaction could be taken to indicate either that quaternisation is not complete, or that not all quaternised peptide (III)(Fig. 3) undergoes the elimination reaction.

It now becomes apparent that the formation of the carbanion (VI) (Fig. 3) which leads to C-methylation of this peptide is less favourable than at first thought. The proportion of the acyl-peptide which did not form a carbanion is represented by both the cleavage products and the intact permethylated peptide. From the total ion current observed for the three spectra, it was estimated that only 20-30% of the acyl-peptide was C-methylated (cf. p. 40).

The observation that the aspartyl-ester peptide is not significantly cleaved reinforces this interpretation. Such an ester with a labile proton on

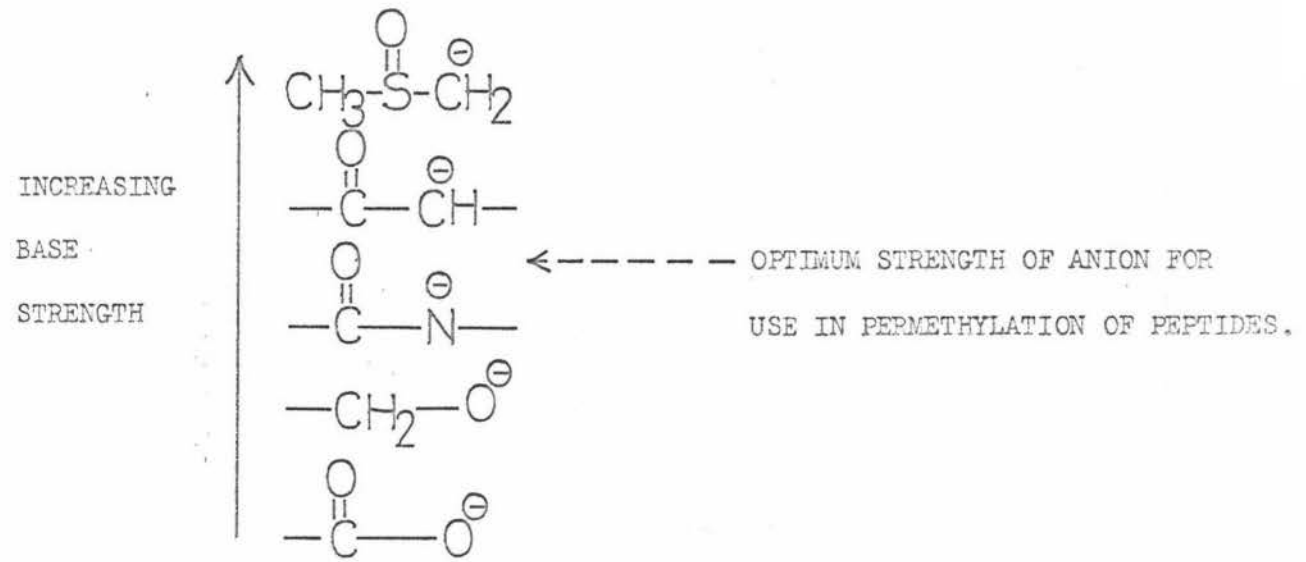
the α-carbon atom would be quantitatively deprotonated on mixing with the dimsyl carbanion (ref. 96). Further evidence supporting the mechanism proposed is the increased chain cleavage observed on addition of dimsyl sodium to a mixture of peptide ester and methyl iodide (Exp. (36)). Such reaction conditions would favour the sequential elimination reaction.

F. SUMMARY:

Clearly, the Hakomori-Vilkas permethylation procedure is not as problem-free as an examination of the literature would suggest. Thomas (ref. 66) reported that "none of the artefacts produced by the Kuhn or Coggins and Benoitin methods have been encountered". Here it has been shown to produce these artefacts of chain-cleavage and extra-methylation, as well as artefact formation at ester groups. However, in contrast to the methods examined by Thomas, artefact formation is not an unavoidable side-reaction of the Hakomori-Vilkas permethylation technique. The side-reactions are not quantitative and, with an understanding of the probable mechanism of their occurrence, reaction conditions can be chosen which will minimise, if not entirely eliminate them.

Artefact formation at ester groups was eliminated by the use of the free acid of the acetyl-peptide. Both extra C-methylation and specific chain cleavage are due to excess dimsyl carbanion and under the usual reaction conditions occur only partially; a reduction in the amount of base used from the present fifty-fold excess to a small two or three-fold excess should prevent these side reactions. The dimsyl carbanion solution used by Thomas in his investigation of the Hakomori-Vilkas procedure was prepared at 100°C (refs. 46,65), and would therefore have been partly decomposed. This inadvertant use of a much weaker solution of the carbanion probably explains why he did not observe artefact formation. Generation of the carbanion at a lower temperature with a smaller excess of sodium hydride would allow precise control of the amount of carbanion used.

All the evidence suggests that N and O-methylation of peptides is not as difficult as at first thought (ref. 44) and that the dimethylsulfinyl carbanion is a much stronger base than is necessary. A weaker anion of the correct strength would permit complete N and O-methylation, while eliminating the side-reactions of chain cleavage and C-methylation:



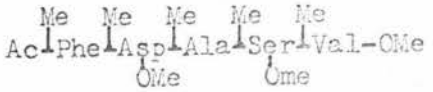
Agarwal used the anion of dimethylacetamide for permethylating peptide derivatives; extra C-methylation was observed which could be prevented by reducing the amount of base used (ref. 52). The anion of dimethylacetamide apparently suffers the same problem as the dimsyl carbanion in that it is far too strong a base. Use of weaker anions such as the dimethylsulfonyl carbanion of Corey and Chaykovsky (ref. 96) should be investigated.

5. MASS SPECTROMETRY OF PERMETHYLATED PEPTIDE DERIVATIVES.

The side reactions observed do not prevent use of the Hakomori-Vilkas procedure to prepare permethylated peptide derivatives, provided conditions are chosen to minimise them. Optimum conditions involve the use of the free acid of the acetyl-peptide, conveniently prepared with acetic anhydride + water, followed by permethylation using a small excess of dimsyl carbanion. Addition of the acetyl-peptide to the dimsyl sodium solution should be followed immediately by rapid addition of excess methyl iodide.

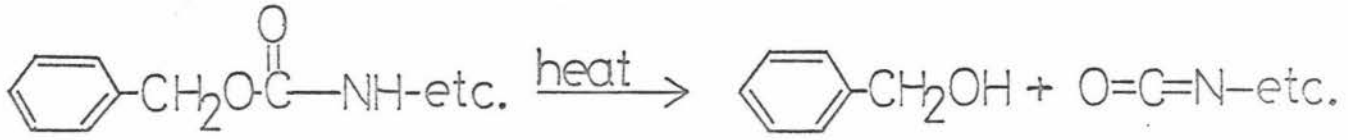
A. FRAGMENTATION MODES:

Permethylated peptides show an extreme simplification of fragmentation modes in the mass spectrometer when compared with acetyl-peptide esters, as first observed by Thomas who stated: "Because of the resultant simplification of mass spectral fragmentation, N-permethylation can even be advantageous for the sequence determination of simple peptides where volatility poses no problem" (ref. 45). The mass spectra of acetyl-peptide esters are complex and special techniques are required to recognise the peaks containing sequencing information; in general, these sequencing peaks are not the most prominent peaks (ref. 56). There is a large volume of literature on the fragmentation of acetyl-peptide esters; the data has been reviewed by Lederer and Das (ref. 108) and Shemyakin (ref. 58), but these results are not generally applicable to the mass spectrometry of permethylated derivatives.

The principal fragmentation mode of permethylated peptides is rupture of the peptide bond; the ion corresponding to subsequent loss of the carbonyl group is not observed. The spectra thus consist almost exclusively of sequencing peaks resulting from cleavage of the amide bond. This simplicity in the resulting spectrum is dramatically illustrated by a comparison of the mass spectra of Ac-Phe-Asp-Ala-Ser-Val-OMe and the permethylated derivative 

(Fig. 4 overleaf; Exps. (14) and (47)).

Lederer's statement that "the simplification of the spectra obtained by permethylation is certainly in part at least due to the decrease of pyrolytic reactions as a result of the lower temperature used " (ref. 43) is not an adequate explanation of the dominance of the sequencing fragmentation mode in permethylated peptides. The example given by Lederer (refs. 43,45) was a benzyloxycarbonyl-octapeptide, and the complexity of the spectrum of the unpermethylated ester can now be recognised as due to thermal fragmentation of the protecting group. Aplin et al (ref. 109) have since shown that the aryl carbamate is pyrolysed to benzyl alcohol and the isocyanate at temperatures greater than 200°C:



Permethylation of the octapeptide enabled volatilisation at a much lower temperature and the absence of thermal fragmentation of the benzyloxycarbonyl group resulted in a simplified mass spectrum. The two spectra shown in Fig. 4 were obtained at sample temperatures of 230°C and 220°C respectively; such a slight lowering in temperature would not be expected to result in significantly less pyrolysis.

Ions of the type $R\text{-}\underset{Me}{N}\text{-}CHR'\text{-}C\overset{\oplus}{O}$ are more abundant in spectra of N-methylated peptides, while ions of the type $R\text{-}\overset{\oplus}{\underset{Me}{N}}\text{=}CHR'$ appear to be entirely absent. Shemyakin (ref. 58) found that the second "aldimine" type of ion was not formed directly from the molecular ion in the mass spectra of acetyl-peptide esters. The relative abundance of an ion is dependent on both its ease of formation and the ease with which it undergoes further fragmentation, so that the absence of aldimine-type ions in the mass spectra of permethylated peptides could be due

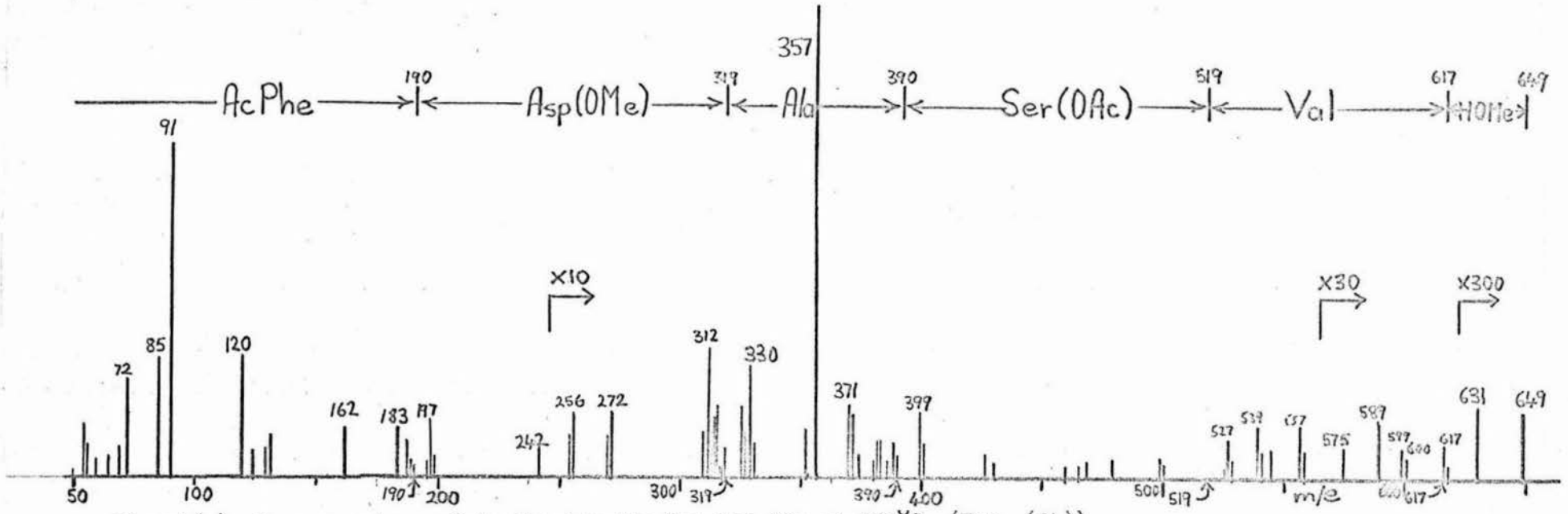


Fig. 4(a) Mass spectrum of Ac-Phe-Asp-Ala-Ser-Val-OMe at 230°C (Exp. (14)).

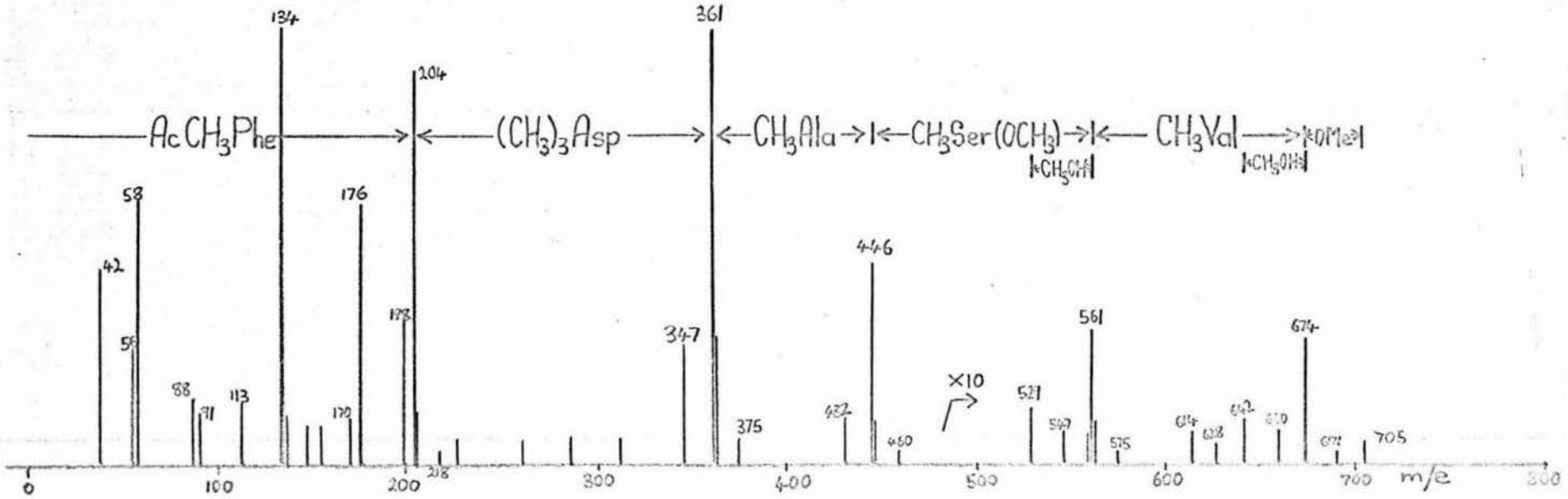
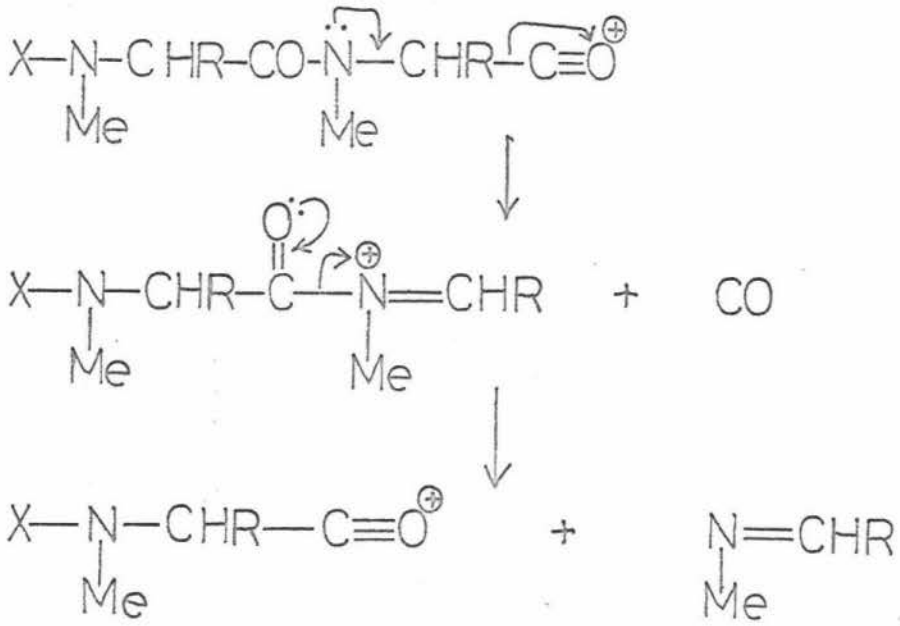
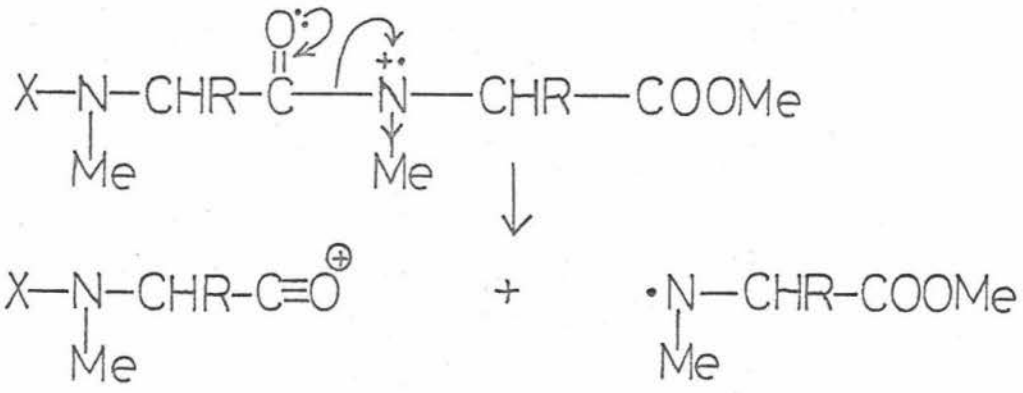


Fig. 4(b) Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val at 220°C (Exp. (47)).

either to a decreased tendency for the fragments formed by cleavage of the peptide bond to lose a carbonyl group, or to an increased tendency for the aldimine-type fragments to undergo further fragmentation, or to a combination of both. The two reactions concerned are:



The introduction of an N-methyl group with its negative inductive effect (ref. 111) would therefore decrease the tendency for the first reaction and increase the tendency to further fragmentation; it is possible that this latter effect predominates as significant loss of CO is observed at the N-terminal residue. The relative abundance of aldimine-type fragments would be lowered by both effects, while at the same time increasing the intensity of fragments corresponding to rupture of the peptide bond. An electron-withdrawing group at the nitrogen atom of the amide bond would also favour the initial peptide bond cleavage in the molecular ion:



Thus, the predominance of amino acyl type sequencing peaks in the spectra of N-methylated peptide derivatives can be rationalized in terms of the recently discovered negative inductive effect of the methyl group; although the methyl group was regarded as electron-donating, it is now thought the observed phenomena are due to solvation effects in the reactions studied (ref. 111). The effect of a methyl group on unimolecular reactions in the gas phase is that of an electron-withdrawing substituent.

Only two other fragmentation modes are normally significant in the mass spectra of permethylated peptide derivatives: loss of OCH_3 from the C-terminal ester of the molecular ion, and loss of $HOCH_3$ from the side-chains of serine and threonine methyl ethers. The loss of OCH_3 (31 m.u.) from the molecular ion can be contrasted with the loss of $HOCH_3$ (32 m.u.) from the molecular ion of acyl-peptide esters. An analogous loss of OCH_3 from the side chains of -Asp- and -Glu- does not occur in the permethylated derivatives. The loss of $HOCH_3$ from the side chains of -Ser- and -Thr- is also observed in the mass spectra of acyl-peptide esters (refs. 43,58).

B. INTERPRETATION OF SPECTRA:

The mass spectra of permethylated peptides are readily interpreted in terms of the amino acid sequence; knowledge of potential artefacts from the permethylation is essential to a correct and reliable sequence determination. Briefly, the mass of each N-methylaminoacyl residue is unique, with the exception of leucine and isoleucine which possess isomeric side chains. The mass differences between the intense ions due to rupture of the peptide bonds therefore correspond to particular amino acid residues in the original peptide. Usually, the interpretation is performed starting with the N-terminal acylamino acid fragment ion and working upwards through the spectrum, bearing in mind the other possible individual fragmentation modes.

The first (low mass) sequencing ion will show an accompanying peak

28 m.u. lower due to loss of carbon monoxide, which will be the major peak for an N-terminal pyrrolidone (ref. 50). Serine or threonine residues will lead to peaks 32 m.u. below the sequencing fragments for those residues and all others up to the molecular ion. Sequencing peaks from the rupture of peptide bonds involving the carbonyl group of proline residues are sometimes weak or entirely absent (refs. 43,112; cf. Exps. (43) and (46)).

The molecular ions of permethylated peptide derivatives are often very weak (e.g. ref. 66; Exps. (48) and (52)) and sometimes entirely absent (ref. 52)*. The molecular ion is located by searching for peaks 31 m.u. apart, resulting from loss of OCH_3 from the C-terminal ester group of the molecular ion. There is a general dropping off in the intensity of successive sequencing peaks towards higher mass (ref. 45) and the combination of this with the lower intensity of the molecular ion makes the identification of the C-terminal residue difficult. There will often be some uncertainty as to its identity.

The sequence deduced from the mass spectrum can be confirmed and ambiguities resolved by repetition of the permethylation using deuteriated methyl iodide. In this way, naturally occurring N-methylamino acid residues will also be identified (ref. 113). High resolution measurements are not necessary for the determination of the amino acid sequence (ref. 45) and at the relatively high mass range involved could do little more than confirm the interpretations already made. However, the identity of the N-terminal residues can be readily checked by accurate mass measurement of the first few sequencing peaks. In the case of new or unexpected amino acids, high resolution mass measurements may be necessary.

If sufficient sample is available, the detection of metastable transitions (ref. 42) for the sequencing peaks would be useful, especially in locating parent ions. The probable molecular ion is often too weak for this

* In this latter case, failure to recognise the molecular ion can probably be attributed to the use of the acetyl-peptide ester for permethylation.

technique to be used; in any case, the absence of metastable transitions would only be supporting negative evidence as these may be absent even for fragment ions.

C. UNKNOWN PEPTIDES:

The techniques developed in this investigation were applied to a naturally occurring free peptide isolated from an alcohol extract of cheese by gel filtration, followed by ion exchange chromatography using a pyridine-acetic acid buffer. High voltage paper electrophoresis at pH 6.4 indicated that the peptide was homogeneous. Amino acid analysis of a twenty-hour acid hydrolysate indicated that it contained Asp_2 Ser_2 Glu_3 Gly_2 Ala_1 Val_2 Leu_3.*

One milligram of the freeze-dried peptide was acetylated and permethylated using deuteriated methyl iodide (Exp. (52)). On mass spectrometry of the product, a series of complex spectra were obtained. Examination of the mass spectra indicated that three peptides were present. Because of the very similar volatilities of the peptide derivatives, there was considerable overlap in the spectra; by multiple scanning of the spectrum as the temperature of the sample increased it was possible to identify the individual spectra, which are shown overleaf, Figs. 5,6 and 7. It was estimated that the peptides (I),(II) and (III) were present to the extent of about 20%, 70% and 10% respectively, in order of decreasing volatility.

Sequences could be readily assigned to the three peptides, although the molecular ions were very weak and uncertainty as to the identity of all three C-terminal residues existed. High resolution mass measurements on key ions confirmed the N-terminal residue for each peptide. Another sample (2mg) of the peptide was acetylated and permethylated using methyl iodide. Mixed spectra were again obtained; the three spectra obtained by repetitive scanning

* The co-operation of Barry Richardson of the New Zealand Dairy Research Institute in supplying this cheese peptide is gratefully acknowledged.

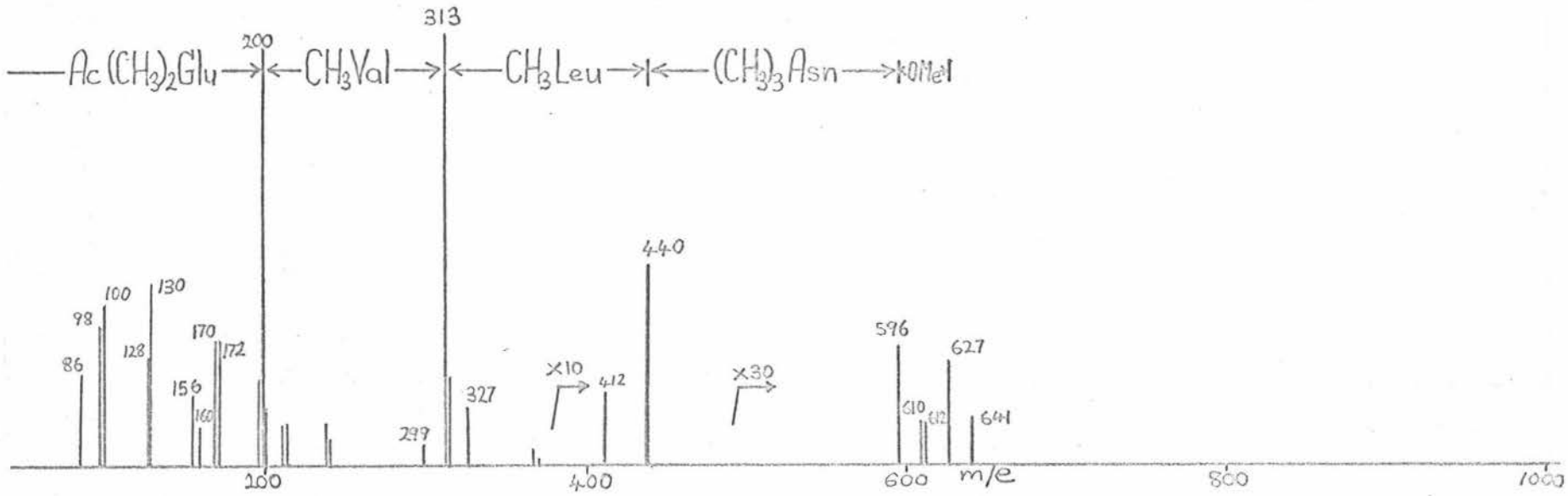


Fig. 5 (a) Mass spectrum of the permethylated cheese peptide (I) (Exp. (52)).

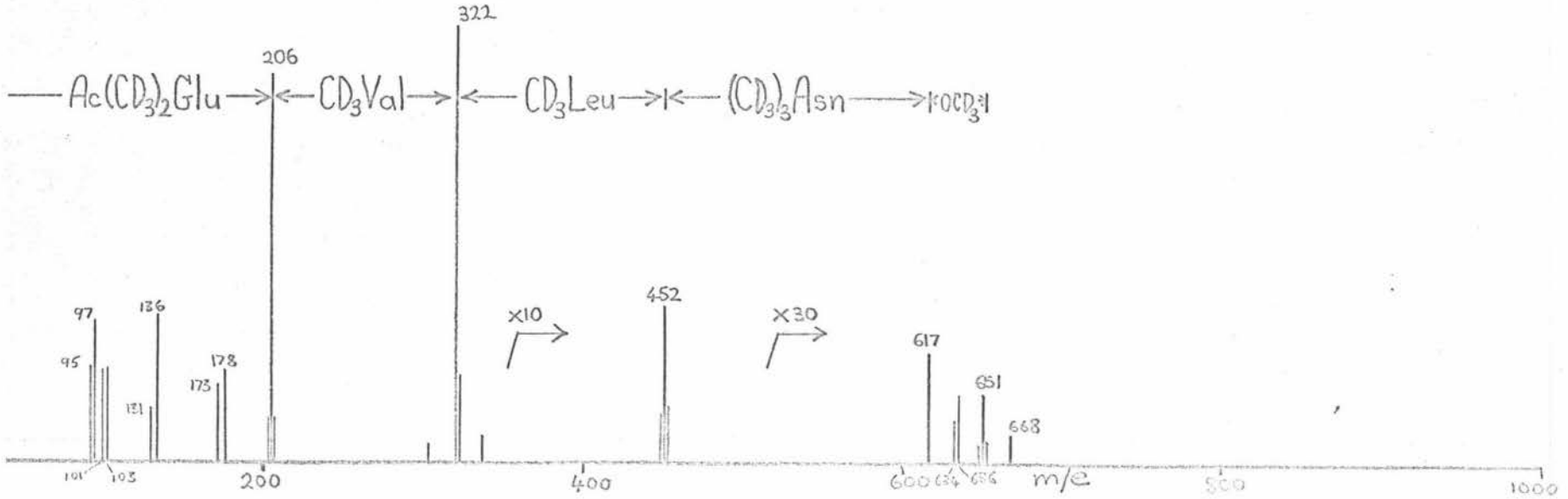


Fig. 5(b) Mass spectrum of the perdeuteriomethylated cheese peptide (I) (Exp. (52)).

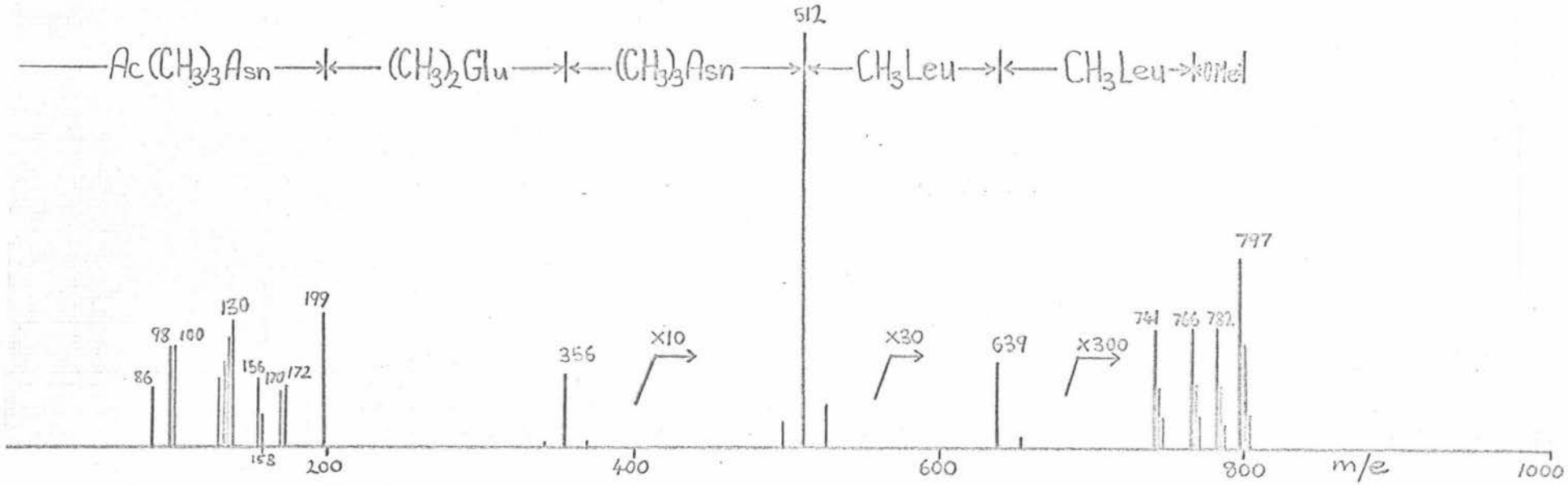


Fig. 6(a) Mass spectrum of the permethylated cheese peptide (II) (Exp. (52)).

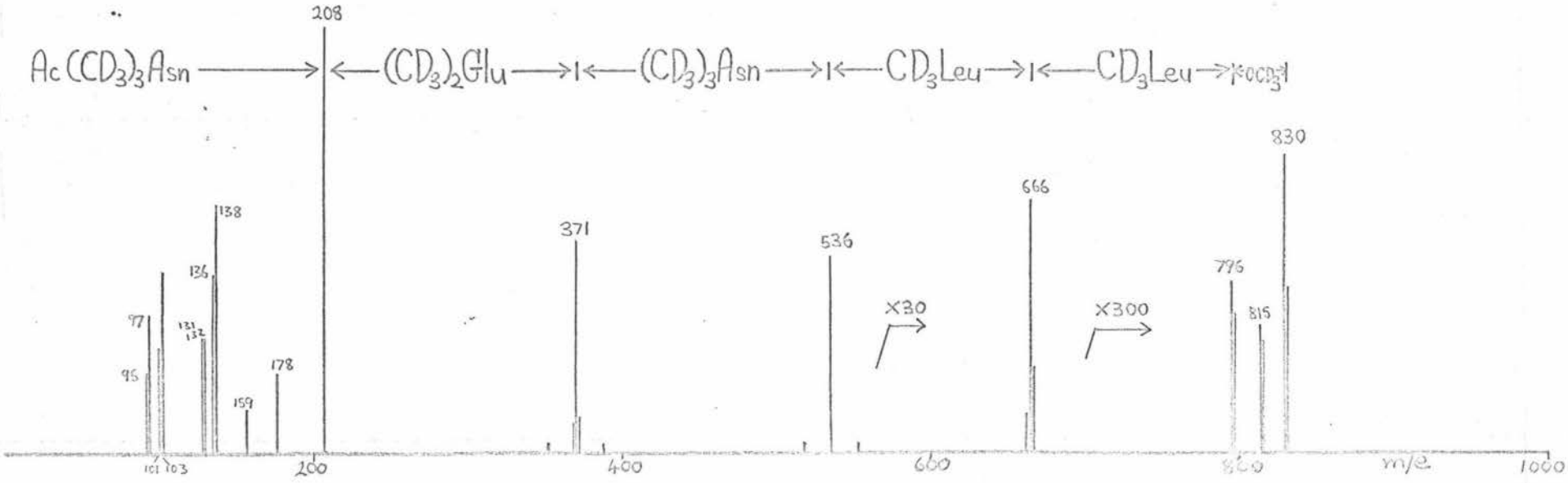


Fig. 6(b) Mass spectrum of the perdeuteriomethylated cheese peptide (II) (Exp. (52)).

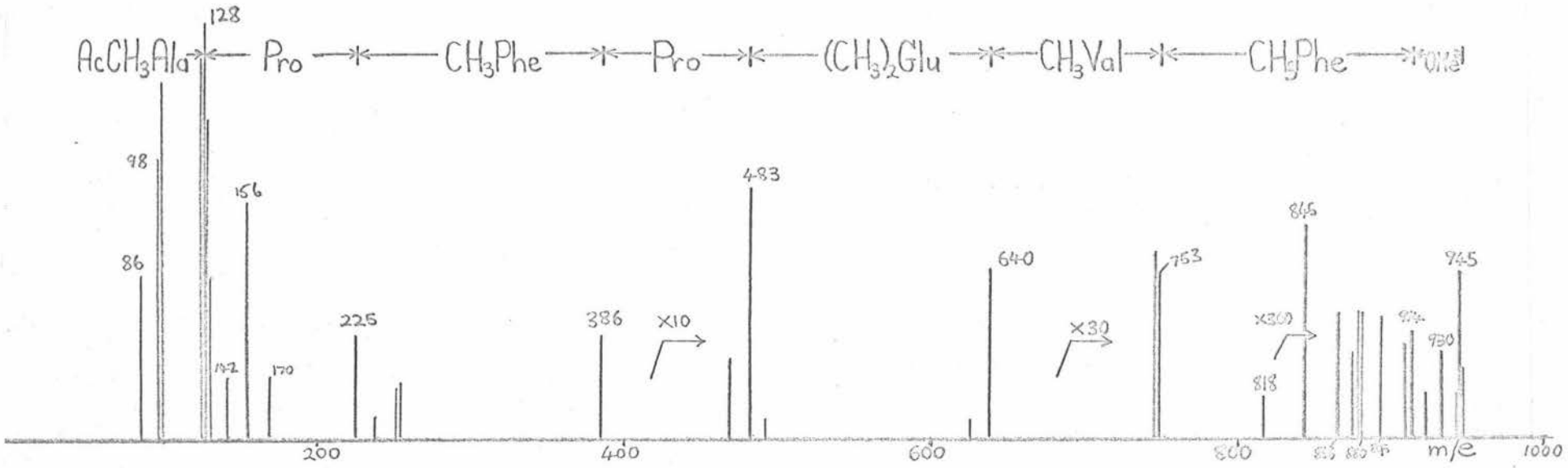


Fig. 7(a) Mass spectrum of the permethylated cheese peptide (III) (Exp. (52)).

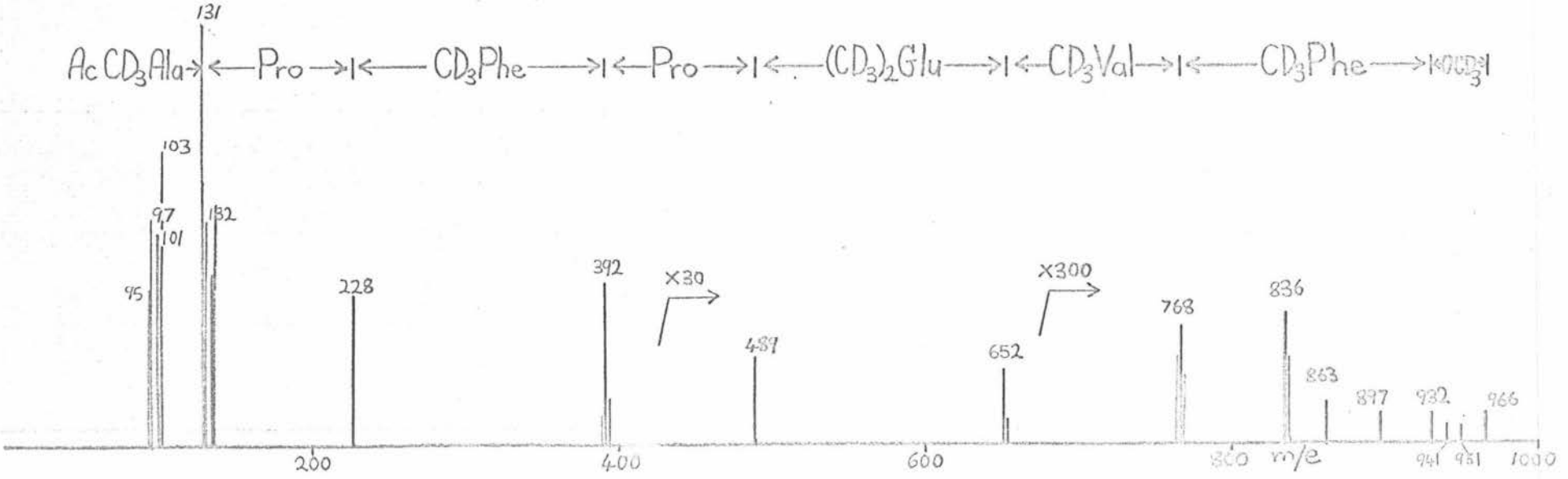


Fig. 7(b) Mass spectrum of the perdeuteriomethylated cheese peptide (III) (Exp. (52)).

showed the expected mass shifts for the proposed sequences and are also shown in Figs. 5,6 and 7. Metastable detection confirmed the sequence of the third least volatile peptide, present in minor amount.

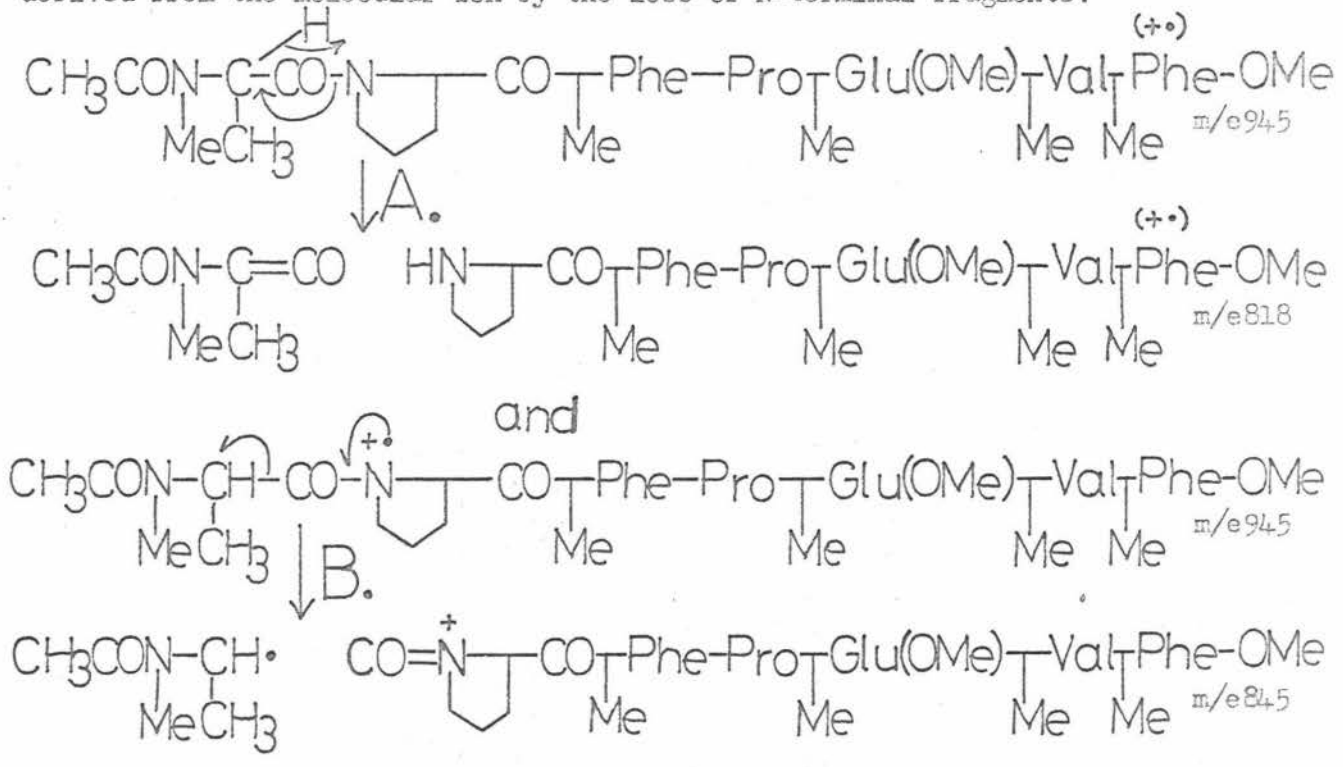
(I) Glu-Val-$\overset{*}{\text{Leu}}$-Asn

(II) Asn-Glu-Asn-Leu-Leu

(III) Ala-Pro-Phe-Pro-Glu-Val-Phe

(* Leu or Ile; these cannot be ditinguished by mass spectrometry.)

An unusual feature of the mass spectrum of permethylated acetyl-peptide (III) (Fig. 7) requires some comment. The relatively intense ions at m/e 818 and m/e 845 are not normal sequencing peaks; they occur at intervals of 65 m.u. and 92 m.u. from the preceeding sequencing peak at m/e 753, mass differences which do not correspond to an N-methylamino acid residue. The mass shift in the two spectra of the peptide indicated a content of 18 deuterium atoms in each fragment, compared with the 18 deuterium atoms in the $(M-31)^+$ peak at m/e 914. These facts led to the conclusion that m/e 818 and m/e 845 are derived from the molecular ion by the loss of N-terminal fragments:



Geddes et al. (ref. 51) have reported a phenomenon similar to the fragmentation A. An intense non-sequencing peak in the high mass region of the mass spectra of two permethylated acetyl-octapeptides was in each case apparently due to loss of the two N-terminal residues of the peptide, involving a hydrogen transfer to the higher mass fragment which retained the charge; this was confirmed by high resolution mass measurement. Obviously, such anomalous fragmentation could seriously interfere with sequence determination from low resolution spectra.

The peptides (I) and (II) had a high content of the trifunctional amino acids glutamic acid and asparagine, resulting in a higher mass and lower volatility than might have been expected for the number of residues in each. The presence of a single free glutamic acid residue in each of the three peptides accounts for their identical mobilities in the high voltage electrophoresis and for their concurrent isolation by ion-exchange chromatography.

Of the possible artefacts of the Hakomori-Vilkas permethylation procedure, only C-methylation was observed. This occurred to a significant extent only at the C-terminal asparagine residue in peptide (I). The possibility that chain cleavage at aspartyl residues had given several fragments from a larger peptide was excluded by high resolution mass measurement of the N-terminal acetylamino acid residue fragment ion of each compound, together with the correlation between the spectra derived from the use of deuteriated and undeuteriated methyl iodide. Asparagine behaved satisfactorily and was unequivocally located; its location is not straightforward using normal chemical techniques (ref. 114).

These sequences were determined without prior knowledge of the amino acid content of each peptide. In fact, the analysis of an acid hydrolysate cannot distinguish asparagine (and glutamine) residues from the corresponding

free acids, and could not have been expected to detect the amino acids derived from the minor peptides, (I) and (III). On reflection, it is probable that a fourth, much larger peptide was present in the original sample. Gel filtration had indicated a molecular weight of two or three thousand for the peptides applied to the ion exchange column; amino acid analysis of the isolated peptide showed serine and glycine to be present in large amounts, but these were not in the peptides sequenced; finally, a proportion of the acetylated peptide preparation would not dissolve in DMSO for permethylation.

The ease with which the individual sequences were determined, in spite of the similar volatilies of the permethylated derivatives and the wide difference in the amounts present indicates the sensitivity of mass spectrometry and demonstrates that the technique can be applied in protein analysis, where relatively impure peptide preparations are the rule rather than the exception.

6. CONCLUSION.

As yet, the use of mass spectrometry to determine peptide sequences is not a routine method in protein chemistry. Several groups of research workers have used mass spectrometry to sequence the permethylated derivatives of free peptides from natural sources (Table IV, between pages 14 and 16). Four permethylation techniques are in use, all of which present problems. Derivatives can be made from small amounts of impure peptides.

This investigation has examined in detail some aspects of the most promising method for the preparation of peptide derivatives for mass spectrometry (ref. 65): that is, acetylation of the free peptide, followed by permethylation using the Hakomori-Vilkas technique (refs. 47, 104). Several artefacts were found and reaction conditions adjusted to minimise their occurrence; three naturally occurring free peptides were sequenced. With the knowledge gained, there is no theoretical objection to the use of mass spectrometry of these permethylated derivatives in the structural determination of proteins.

The limitations of the mass spectrometry of permethylated peptides as a method of sequence determination are no greater than those of the normal chemical techniques. They are complementary procedures: mass spectrometry has been used to sequence peptides with blocked N-terminal residues (ref. 50), to distinguish acidic amino acid residues from their amides where the dansylation technique failed (ref. 49) and to confirm a sequence proposed on equivocal chemical evidence (ref. 51).

Mass spectrometry can normally be used to determine the sequence of about ten amino acid residues in a peptide. Though peptides containing up to twenty-two amino acids have yielded mass spectra (ref. 50), the intensity of each successive sequencing peak decreases towards higher mass and ions beyond a certain point, about m/e 1400-1600, were too weak to be detected (ref. 45).

Although it is desirable to know the amino acid content of a peptide, with the use of permethylated derivatives this is no longer necessary for sequence determination, as we have illustrated (cf. Shemyakin, ref. 56). The sequencing peaks are readily recognised as the principal ions in the spectrum.

The main restriction on the use of N-methyl peptide derivatives is due to the residues which must be modified prior to permethylation: the sulfur containing amino acids cysteine and methionine, and the basic amino acid arginine. Techniques exist for their modification, applicable to the small amounts of peptides usually encountered in protein chemistry (refs. 43, 74, 115), though these do not appear to be completely satisfactory.

The realisation of the potential of mass spectrometry in the sequencing of peptides appears to lie with the development of the use of permethylated derivatives. Other approaches, such as the use of decanoyl-peptide esters or trifluoroacetyl-peptide esters, offer no advantages in any respect: the preparation of the derivatives requires as many pretreatment steps, they are less volatile and give complex mass spectra that are difficult to interpret. Even with the relatively simple spectra obtained from permethylated peptides, there is no substitute for wide experience in the use of the technique and the resulting intuitive judgment.

Each peptide is chemically unique and the potential pitfalls are well illustrated by the case of the pentadecapeptides, gramicidins A and B. Lederer (ref. 43) found that the permethylated derivatives gave mass spectra up to and including the twelfth residue, but that the tryptophan residue in position 9 had a mass 30 m.u. greater than expected, due to the introduction of an extra -OMe group. Extensive investigation ruled out the possibility of a pre-existing hydroxyl group in the residue. The tryptophan in position 11 in the same molecule behaved normally. Permethylation of synthetic gramicidin A gave an identical result. However, use of another permethylation procedure yielded the expected derivative in position 9 (ref. 65). In this case, the

sequence of the peptide was known; in the case of an unknown peptide, the result would not have been recognised as an artefact.

Thus, although mass spectrometry of permethylated peptide derivatives may be routinely used to sequence unknown peptides, the results must be regarded with caution; the method is probably more reliable than the existing chemical sequencing techniques which can also be misinterpreted.

One disadvantage of mass spectrometry for peptide sequencing is the high initial cost of the instruments. The equally acceptable "wet" chemical techniques available for protein analysis, on the other hand, may be set up in any laboratory, simply and at low cost. With both methods, considerable experience is necessary to obtain routine, reliable results (ref 1). Mass spectrometry is unlikely to become the standard method of sequencing peptides until equipment costs are drastically reduced.

There are several possible developments in the application of mass spectrometry that could occur in the near future. When improved instruments with increased sensitivity become available, we can look forward to the complete sequencing of the large peptides than can now be volatilised; for example, a mass spectrum has been obtained from the complete A-chain of insulin showing the five N-terminal residues (ref. 50); the ions beyond that point were too weak to be detected. Alternatively, increased ion intensity may be attained by the use of ionization methods other than electron-impact. Cyclopeptides and simple peptide derivatives have recently been studied using a field-ionization source (refs. 116, 117); this forms ions of lower excitation energy and usually results in an increase in the intensity of the molecular ion. The intensity of ions resulting from simple fragmentation processes is also increased at the expense of fragmentations involving rearrangements. Permethylated peptides have not yet been examined by field ionization mass spectrometry, but the required increase in intensity of the sequencing ions and the molecular ion may be obtained in this way. Chemical

ionization (ref. 124) is potentially able to offer these same advantages, together with an increased ionization efficiency.

It is possible to obtain high resolution measurement of complete mass spectra in a very short time using an on-line computer to process the output from the mass spectrometer. These installations are available and their widespread use in conjunction with high resolution double-focussing mass spectrometers is not far off. The advantages of complete high resolution data in the sequencing of peptides are twofold; such measurements are valuable confirmation of the elemental compositions of the sequencing ions and hence of the deduced sequence; further, the data can be used to unequivocally distinguish individual sequences in mixed spectra derived from permethylated peptides of identical volatility (ref. 51).

Another combination that has been available for some time is gas-liquid chromatography on-line to a mass spectrometer. Using GLC-MS, mixtures of volatile compounds can be separated and the mass spectrum of each component obtained. This technique has already been applied in the sequence analysis of peptides, principally by German workers (refs. 53,55,67). The mixture of peptides in the partial acid hydrolysate of an oligopeptide were converted to the trifluoroacetyl-peptide esters, and examined by GLC-MS. From the low resolution spectra, the sequences of the di- and tripeptides could be determined and the sequence of the parent nonapeptide derived (ref. 53). Even in the most favourable cases, the upper limit for the GLC of these trifluoroacetyl-peptide esters is a tetrapeptide. The use of permethylated acetyl--peptides for GLC has not been investigated; they are considerably more volatile than simple derivatives of peptides and their separation according to mass on a gas chromatograph using temperature programming should be routinely possible up to hexapeptides at least. This would be a more efficient version of the partial separation of mixtures obtained by fractional distillation in the mass spectrometer. The effluent could be directly

analysed by mass spectrometry, or the fractions collected and examined. The use of such purified permethylated derivatives would greatly reduce the amount of peptide required for successful mass spectrometry. This could be combined with rapid high resolution mass measurement of the complete spectra; computer--aided interpretation of the resulting data would probably be desirable, especially with the mixed spectra likely to result from each GLC fraction.

Clearly the mass spectrometry of permethylated peptides has great potential for automated sequencing of proteins. In the ultimate, it may be possible to acetylate and permethylate the enzymatic digest of a protein, separate many of the peptides by GLC and determine their structures by mass spectrometry. Once set up, use of such a method would be simple and rapid and the elimination of techniques such as ion exchange chromatography and gel filtration would minimise the amount of asmple needed.

In the more immediate future, the opinion expressed by Schroeder (ref. 118) that "It may well be ... that high resolution mass spectrometry will become the method of choice for sequence determinations within a few years" can become reality only when the methods of making derivatives of peptides for mass spectrometry become sufficiently reliable and well understood for routine and universal application.

EXPERIMENTAL.

Sources of amino acids and peptides are quoted in the text. Abbreviations used are: "N.B. Co." is the Nutritional Biochemical Corporation, "Sigma" is the Sigma Chemical Company, "M.R." is the Mann Research Laboratories Division of Becton-Dickinson and Company, "BDH" is British Drug Houses Ltd., England. All solvents and volatile reagents were purified by distillation. Melting points were determined using a Reichert hot-stage microscope. Literature values quoted are from Heilbron's "Dictionary of Organic Compounds", 4th edition 1965, unless otherwise stated. Reactions and work-up of the products were performed at room temperature to minimise side-reactions, unless otherwise stated. "Dried under vacuum" indicates that the product was evacuated to $\leqslant$0.5mm Hg on a rotary oil pump with a liquid-air vapour trap; the pumping was maintained throughout, usually for 20 minutes to 30 minutes.

Thin-layer chromatography, development in a single direction, was used to check the identity of reaction products and estimate the progress of reactions. Rf values are quoted relative to the solvent front=Rf100. All layers were dried overnight at room temperature and were not activated prior to use. The chromatograms were developed in filter-paper lined tanks pre-equilibrated overnight with the developing solvent. Solvent-systems used and their abbreviations are (ref. 119):

solvent A: chloroform (85) methanol (10) acetic acid (5)

solvent P: ethyl acetate (5) pyridine (5) acetic acid (1) water (3)

solvent Q: 1-butanol (65ml) isopropanol (15ml) water (20ml) chloroacetic acid (3g)

solvent BAW, 4:1:1v/v: n-butanol (4) acetic acid (1) water (1)

solvent BEW plus 1% HAc:

The developed chromatograms were dried in an air-stream at room temperature, prior to spraying. Two spray reagents were used:

"ninhydrin/$Cd(OAc)_2$" according to Bieleski and Turner (ref.120),

and "Cl_2- 1% starch/1%KI", according to Rydon and Smith (ref.80).

These spray reagents could be used consecutively, the ninhydrin/$Cd(OAc)_2$ first, though this resulted in a high background colour with the Cl_2 - 1% starch/1%KI spray.

Copies of the chromatograms were taken using ammonia-developed blue-print paper. The plates were placed layer-down on the paper and exposed under a 400 watt mercury-vapour lamp at about 8" for 20 seconds. The copy was developed in an atmosphere of ammonia and was stable over the entire period of research. Where necessary, the chromatograms were marked before copying.

For the analysis of hydrolysed permethylated peptides, two dimensional TLC on cellulose layers was used. A modification of the method according to Haworth and Heathcote (ref. 121) proved satisfactory for the identification of N-methylamino acids. After development, the chromatograms were sprayed with ninhydrin/$Cd(OAc)_2$; on heating the sprayed chromatogram to less than 100°C or standing overnight at room temperature, N-methylglycine gave a slow-developing colour and N-methylalanine was not detected (compare with ref. 122). On heating to 130°C both these compounds were detected. Colours were much slower to develop on spraying after development in the acid (first) solvent only, though a lower background colour was obtained. Spraying after development in the ammonia-containing (second) solvent gave a high background colour. The N-alkyl-amino acid spray according to Sheehan (ref. 122) was also used. Free amino acids were not detected. N-methylglycine gave only a faint yellow colour, while N-methylalanine gave an orange-red colour.

All mass spectra were obtained using an AEI MS902 mass spectrometer. All reported metastable transitions occurred in the field-free region between the earth-accelerating plate and the electrostatic analyser. All mass measurements were within $\pm$ 3 parts per million of the theoretical value for the elemental compositions listed. Where mixtures of homologous compounds were examined, the proportions of the constituents were estimated from the ratios of the molecular ion intensities.

Free peptides were routinely acetylated using (per mg peptide) water (1ml) and acetic anhydride (1ml); the heterogeneous solution was shaken at room temperature for 2 hours, then evaporated to dryness at room temperature under vacuum ($\leqslant$ 1mm Hg). The residue was dissolved in water and re-evaporated.

Peptide derivatives were permethylated using the dimsyl carbanion of Corey and Chaykovsky. All operations were carried out under dry O_2-free, CO_2-free nitrogen until the reaction was stopped by addition of water. Details of the conditions of particular permethylations are given in the text; the following is a more detailed description of a typical permethylation.

Generation of the dimethylsulfinyl sodium (dimsyl sodium) in dimethylsulfoxide (DMSO): NaH/oil dispersion (20mg, 50% NaH) in a two-necked 10ml B14 "Quickfit" round-bottomed flask was pre-rinsed three times with sodium-wire dried petroleum ether (80°-100°C fraction), decanting the supernatant. A teflon-coated stirring bar was added and the flask was connected to a vacuum/nitrogen line and the other neck was "Suba" sealed. Final traces of petroleum ether were removed by evacuating the flask on a liquid-air trapped vacuum pump to $\leqslant$ 0.5mm Hg. The flask was then filled with nitrogen by re-evacuating and flushing with nitrogen, twice. Dry DMSO* (0.20ml) was then injected onto the NaH, using a dry 1ml syringe. Magnetic stirring was maintained for 1 hour with the flask on an oil bath at 65°C. A paraffin filled U-tube was used as a non-return valve to release the hydrogen evolved. The resulting gray-green solution was removed from the oil bath, the by-pass valve sealed off and positive nitrogen pressure re-applied. The solution was cooled by stirring at room temperature for 5 to 10 minutes.

Permethylation: The dried acyl-peptide derivative (1-5mg) dissolved in DMSO (0.2ml), was injected into the cooled dimsyl sodium solution, rinsing the

* DMSO used was dry, specially prepared for silylation by Pierce Chemical Co., supplied in a "Suba" sealed bottle under nitrogen.

flask and syringe with DMSO (0.2ml). After 5 minutes stirring at room temperature, chilled I_2-free CH_3I (0.30ml) was injected rapidly into the vigorously stirred solution of dimsyl sodium and acyl-peptide in DMSO. The gray-green solution at once turned light brown. After a further 10 minutes stirring at room temperature, water (1ml) was injected into the reaction mixture. The two-necked flask was opened and the reaction mixture extracted with several small (1ml) lots of chloroform. The chloroform extract was washed with several small (2ml) lots of water and the chloroform evaporated at room temperature under water-pump vacuum. The residue, usually a light yellow oil, was applied to the direct insertion probe for mass spectrometry. If necessary, the product was applied as a solution in chloroform.

ACETYLATION WITH METHANOL ACETIC ANHYDRIDE.

Thomas, et al. (ref.112): "The peptide is treated with methanolic acetic anhydride (4:1 v/v)... the N-acetyl derivative is isolated after a few hours at room temperature simply by evaporation of the solvent and the reagent".

Evidence of diacetylation:

Exp. (1) A small amount of DL-Leu-Gly-Phe (N.B. Co.) was applied to the direct insertion probe for mass spectrometry. A spectrum was obtained at about 200°C, and the identity of the molecular ion and several expected fragments was confirmed by mass measurements. A small amount of compound m/e346 (i.e.M + 11) was also detected.

To DL-Leu-Gly-DL-Phe (2.2mg) was added methanol (2ml) and acetic anhydride (0.5ml). The solution was kept at room temperature for 26 hours, then evaporated under vacuum at 50°C. The product was applied to the direct insertion probe for mass spectrometry.

The spectrum obtained showed ions at m/e 391, corresponding to Ac-Leu-Gly-Phe-OMe and m/e 433 diAc-Leu-Gly-Phe-OMe in almost equal amount (Fig. 18).

Exp. (2) A small amount of free Leu-Gly-Leu (M.R.) was applied to the direct insertion probe for mass spectrometry. Distinct spectra were obtained: that of leucine ($M^{+\cdot}$ at m/e 131) and that of Leu-Gly-Leu ($M^{+\cdot}$ at m/e 301). A small amount of material with m/e 312, i.e. M + 11 was also detected.

To Leu-Gly-Leu (M.R.) (4.4mg) was added methanol (4.0ml) and acetic anhydride (1.0ml). A clear solution was formed after ten minutes stirring at room temperature. This solution was kept at room temperature for 20 hours and evaporated at room temperature. The product was dissolved in ethyl acetate and applied to the direct insertion probe for mass spectrometry.

As the temperature of the ion source was raised the spectra of the following compounds appeared in the order:

N-acetylleucine methyl ester *
N-acetylleucine
N-acetylglycylglycine methyl ester
N-acetylleucylglycine methyl ester
N-acetylleucylglycylleucine methyl ester *

(* There was evidence of M+42 (i.e. diacetyl) compounds in these spectra.)

Although Ac-Leu-OMe could have arisen from free leucine contaminating the tripeptide (see Exp. (3) below), the dipeptides were not present initially and were due to methanolysis of the peptide.

Exp. (3) Leu-Gly-Leu M.R. (2.7mg) was dissolved in 0.15N aqueous ammonia (200μl). Analytical TLC (250μ silica gel G; solvent P; sprayed ninhydrin/$Cd(OAc)_2$ showed two ninhydrin-positive components of Rf's 57 and 65. A single streak (36 μl) of the peptide solution was applied for preparative TLC (250μ, silica gel G, pre-run; solvent P; sprayed with mask, ninhydrin/$Cd(OAc)_2$). Zones Rf 47-62, Rf 62-72 were marked, eluted into methanol, evaporated at room temperature and dissolved in 0.15N aqueous ammonia (100 μl). Analytical TLC (conditions as above) showed complete separation of the ninhydrin positive components. The compound from the zone Rf47-62 had Rf 58 and co-chromatographed with authentic L-Leucine (M.R.). Thus, Leu-Gly-Leu (M.R.) was contaminated

with free leucine.

Isolation and Identification of the Products of Reaction with DL-phenylalanine:

Exp. (4) To DL-phenylalanine (BDH)(5.1 mg) dissolved in methanol (4 ml) was added acetic anhydride (1 ml). The solution was kept at room temperature; aliquots (1.0ml) were taken at once, 1 hour, 2 hours, 3 hours and 24 hours after the addition of the reagent, evaporated at room temperature and dissolved in 0.15N aqueous ammonia (100 µl).

Duplicate analytical TLCs (250µ silica gel G; solvent P; sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2- 1% starch/ 1%KI) showed three ninhydrin-negative products, Rf 70, 85 and 95. The 2 hour and 3 hour aliquots were pooled and preparative TLC was performed (250µ silica gel G; solvent P;). The layers were partly masked and sprayed (Cl_2- 1% starch/ 1%KI). The unsprayed areas of the three zones Rf 68-78, 82-92, 92-100 were eluted with chloroform and taken to dryness at room temperature. The products were dissolved in ethyl acetate (50 µl) and analytical TLC (conditions as previously) was performed. The compounds in all three fractions chromatographed at the solvent front and were yellow-green in colour. Apparently these were the N-chloro compounds (see mass spectrometry, below). Each fraction was applied to the direct insertion probe in ethyl acetate for mass spectrometry.

Fraction Rf 70: gave mixed spectra of N-acetylphenylalanine and N-acetyl--N-chlorophenylalanine.

Fraction Rf 85: gave mixed spectra of N-acetylphenylalanine methyl ester and N-acetyl-N-chlorophenylalanine methyl ester.

Fraction Rf 95: gave no mass spectrum.

Exp. (5) Attempted isolation of minor product Rf 95 in solvent P, Rf 45 in solvent A.

To DL-phenylalanine (BDH) (10.2 mg) was added methanol (4 ml) and acetic anhydride (1 ml). The solution was kept at room temperature. Aliquots (0.5 ml) were taken at 20 minutes, 50 minutes, 1 hour 20 minutes, 2 hours 20

minutes, 4 hours 20 minutes, 6 hours 20 minutes, 25 hours, 72 hours and 11 days. The aliquots were taken to dryness at room temperature, then dissolved in 0.15N aqueous ammonia (100μl).

Analytical TLC (250μ silica gel G; solvent A; sprayed ninhydrin/ $Cd(OAc)_2$ and Cl_2-1% starch/1%KI) showed three ninhydrin-negative products, Rf 40, 45 and 70. The 6 hour, 25 hour and 72 hour aliquots were pooled and preparative TLC was performed (500μ silica gel H, pre-run; solvent P; sprayed with mask with 0.05% methanolic morin) and zones of Rf60-75 and Rf80-100 were marked and unsprayed areas eluted with chloroform, evaporated at room temperature and dissolved in ethyl acetate for analytical TLC (250μ silica gel G; solvent P; sprayed Cl_2-1% starch/1%KI). The product from the zone Rf80-100 contained a single compound of Rf90 co-chromatographing with Ac-Phe-OMe; the product from the zone Rf 60-75 contained a single compound of Rf72 co-chromatographing with Ac-Phe. There was no sign of the expected minor product of Rf95.

The ethyl acetate solution of the product from the zone of Rf80-100 was rechromatographed (500μ silica gel H, pre-run; solvent A; sprayed with mask with 0.05% methanolic morin). Zones Rf 15-50, Rf50-7-, Rf75-90 were marked and the unsprayed areas eluted with chloroform, evaporated at room temperature and dissolved in ethyl acetate for analytical TLC (250μ silica gel G; solvent A; sprayed Cl_2-1% starch/1%KI) A compound of Rf 65, co-chromatographing with Ac-Phe-OMe was found from zone Rf 50-70 and to a lesser extent in the other two zones. There was no evidence of the expected minor compound of Rf 40-50 (Solvent A) in any zone.

Isolation and Identification of Products of Reaction with DL-Ala-Gly-Gly:

Exp. (6) To DL-Ala-Gly-Gly (Sigma) (102mg) was added methanol (40ml) and acetic anhydride (10ml) and magnetic stirring was maintained until a clear solution formed (40 minutes). After $5\frac{1}{2}$ days at room temperature, the solution was

taken to dryness on the rotary evaporator under vacuum ($\leq$ 0.3 mm Hg) at room temperature. A yellow oil remained.

The product was dissolved in absolute methanol (10.0ml) and two-dimensional analytical TLC was performed (4μl aliquot; 250μ silica gel G; solvents 1st Dimension A, 2nd dimension BEW plus 1% H Ac; sprayed ninhydrin/ $Cd(OAc)_2$ and Cl_2-1% starch/1%KI) (see plate 1). Six ninhydrin-negative products were detected.

Preparative TLC (500μ silica gel H, pre-run; solvent A; sprayed Cl_2-1% starch/1%KI) was performed using 600μl (6mg) of the products in methanol for each plate. Plates were double developed in pairs. One plate from each pair was sprayed and zones marked accordingly on the other (see plate 2). Zones were eluted with methanol, taken to dryness at room temperature and dissolved in methanol (100μl). Analytical TLC (250μ silica gel G; solvent A double developed; sprayed Cl_2-1% starch/1%KI)(see plate 3) showed that fractions 1, 2, 3, 5, and 6 were reasonably pure. Fraction 4, from the zone of Rf 40-45 apparently containing two compounds, was found to contain roughly equal amounts of the products in fractions 2, 4, 5 and 6. It did not contain the compound in fraction 3. Contamination from the other zones must be ruled out, especially because of the absence of product 3 with the most similar Rf value.

The isolated fractions were applied in methanol to the direct insertion probe for mass spectrometry.

Fractions 1 and 2: did not give mass spectra.

Fraction 3: gave the spectrum of acetylalanaylglyclyglycine methyl ester (Fig. 8).

Fraction 4: analytical TLC showed this to be a mixture not suitable for mass spectrometry.

Fraction 5: gave the spectrum of acetylalanylglycine methyl ester (Fig. 9).

Fraction 6: gave the spectrum of acetylglycine methyl ester (Fig.10).

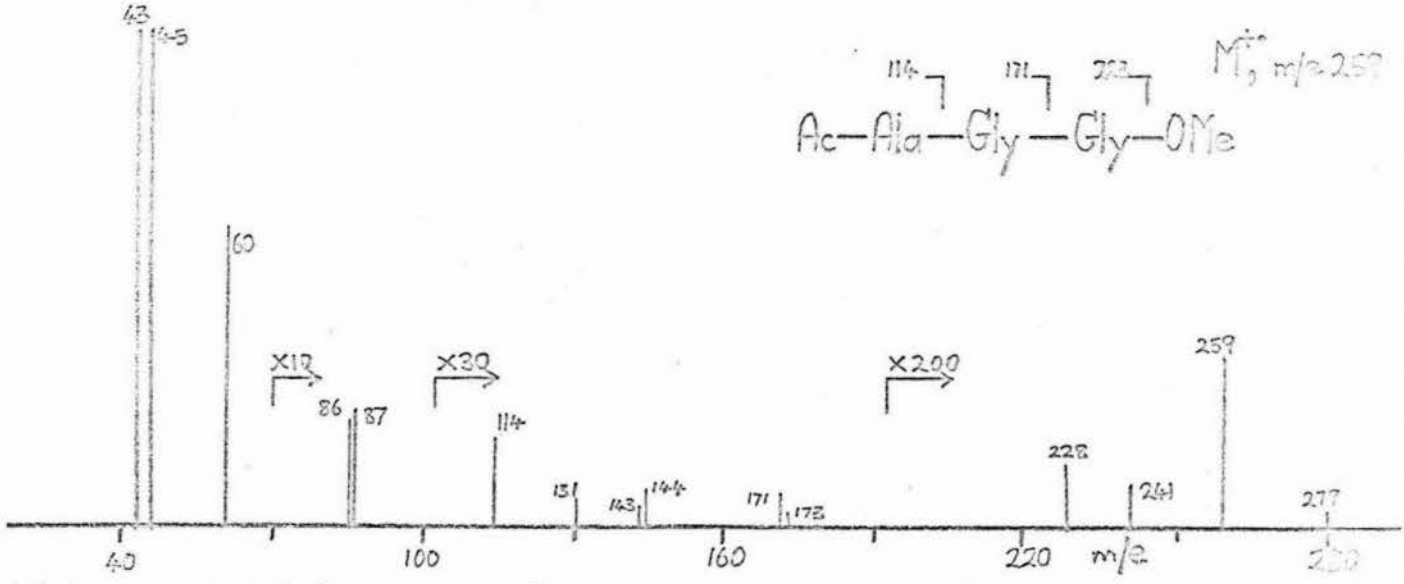


Fig. (8) Mass spectrum of "Fraction 3" from the action of methanol + acetic anhydride on DL-Ala-Gly-Gly (Exp. (6)).

High Resolution Data:

m/e 259 = $C_{10}H_{17}N_3O_5$

m/e 171 = $C_7H_{11}N_2O_3$

m/3 114 = $C_5H_8NO_2$

Expected Elemental Compositions:

$C_{10}H_{17}N_3O_5$

$C_7H_{11}N_2O_3$

$C_5H_8NO_2$

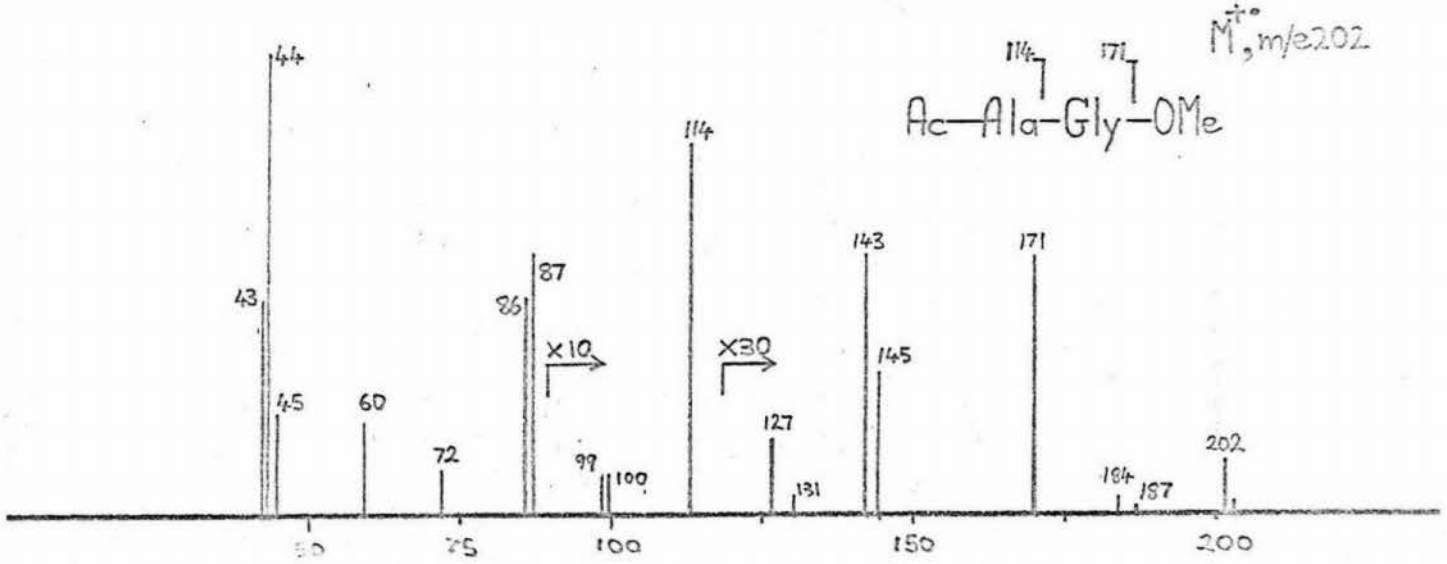


Fig. (9) Mass spectrum of "Fraction 5" from the action of methanol + acetic anhydride on DL-Ala-Gly-Gly (Exp. (6)).

High Resolution Data:

m/e 202 = $C_8H_{14}N_2O_4$

Expected Elemental Compositions:

$C_8H_{14}N_2O_4$

Metastable Transitions:

m/e202 ⟶ m/e171 ⟶ m/e143 ⟶ m/e114

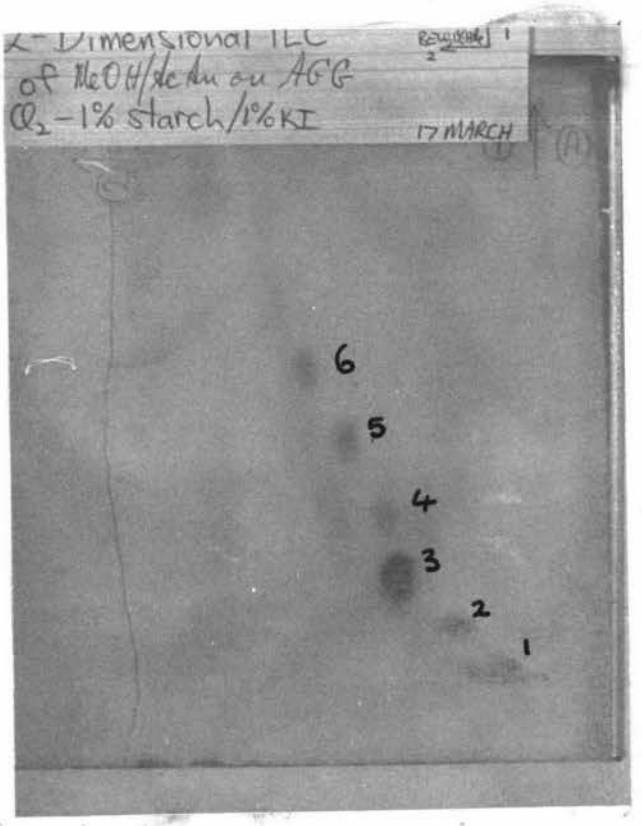


Plate 1. Products of reaction of Ala-Gly-Gly with methanol plus acetic anhydride (Exp. (6)).

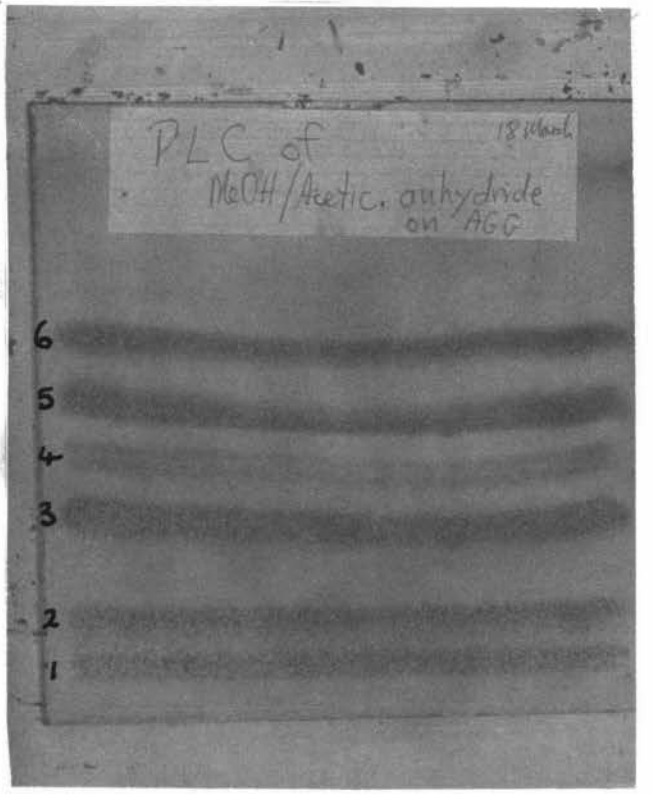


Plate 2. Preparative TLC of the same reaction products (Exp. (6)).

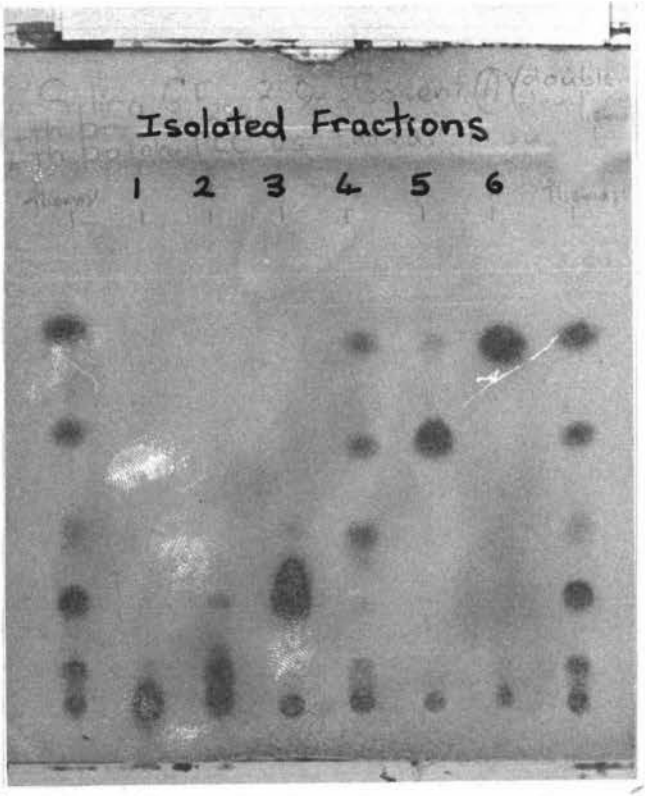


Plate 3. Analysis of isolated products of reaction of methanol + acetic anhydride with Ala-Gly-Gly (Exp. (6)).

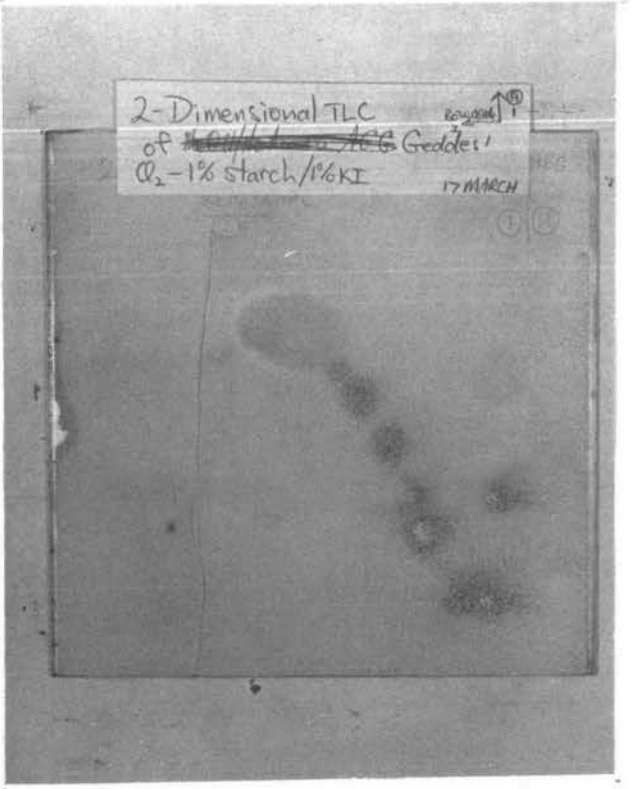


Plate 4. Esterified, acetylated Ala-Gly-Gly according to Geddes, et al. (Exp. (7)).

Permethylation of fractions 1 and 2.

To NaH/oil dispersion (23mg, 50% NaH) pre-rinsed three times with dry petroleum ether and dried under vacuum, was added dry DMSO (0.2ml). Magnetic stirring under nitrogen (65 min. 70°-55°C) gave a grey-green solution of dimsyl sodium in DMSO which was cooled briefly (5 min.) at room temperature. Fraction 1 or 2 was thoroughly dried under high vacuum on a liquid air trapped pump, dissolved in dry DMSO (0.2 ml) and injected into the DMSO/dimsyl sodium solution. After gentle magnetic stirring at room temperature (5 min), chilled I_2-free CH_3I (0.3ml) was injected very rapidly into the vigorously stirred reaction mixture. After a further 10 min. stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed well and evaporated at room temperature.

Products from the permethylation were applied in chloroform to the direct insertion probe for mass spectrometry.

permethylated fraction 1: gave a spectrum identical with that of permethylated acetylalanylglycylglycine (Fig. 11).
permethylated fraction 2: gave no interpretable spectrum.

ESTERIFICATION AND ACETYLATION.

Two sets of slightly different conditions have been employed.

A. Geddes, et al. (ref. 51):

"The peptide methyl esters were obtained by dissolving the peptide (about 10 micromoles) in excess of reagent (1 ml) prepared by bubbling dry HCl through methanol. The solutions were kept in sealed ampoules overnight at room temperature. The ampoules were opened and the reagents taken off under vacuum.

" N-acetylation was achieved by dissolving the residue (10 micromoles) in excess (1 ml) of acetic acid-acetic anhydride (1:1, v/v). The solutions were kept in sealed ampoules at room temperature for 4 hours. The reagents were taken off under vacuum in the precence of NaOH.

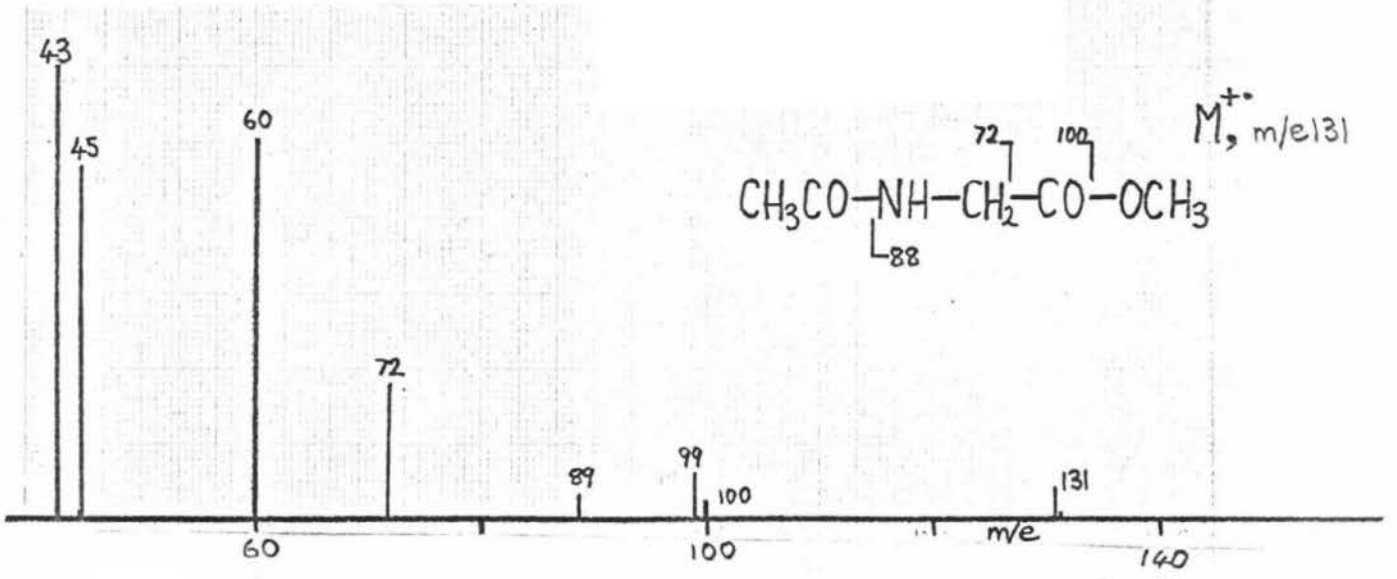


Fig. (10) Mass spectrum of "Fraction 6" from the action of methanol + acetic anhydride on DL-Ala-Gly-Gly (Exp. (6)).

High Resolution Data:	Expected Elemental Compositions:
m/e131 = $C_5H_9NO_3$	$C_5H_9NO_3$
m/e99 = $C_4H_5NO_2$	$C_4H_5NO_2$
m/e89 = $C_3H_7NO_2$	$C_3H_7NO_2$
m/e72 = C_3H_6NO	C_3H_6NO

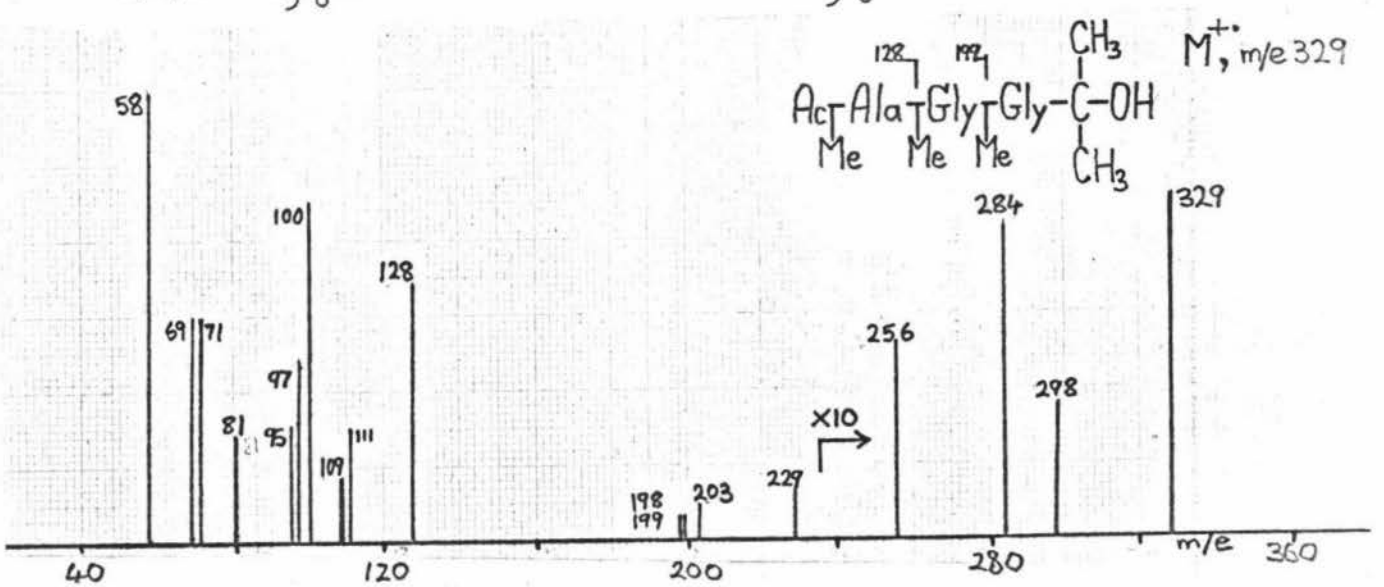


Fig. (11) Mass spectrum of permethylated "Fraction 1" from the action of methanol + acetic anhydride on DL-Ala-Gly-Gly (Exp. (6)).

High Resolution Data:	Expected Elemental Compositions:
m/e329 = $C_{15}H_{27}N_3O_5$	--
m/e284 = $C_{13}H_{22}N_3O_4$	--
m/e229 = $C_{10}H_{17}N_2O_4$	--

Compare with Fig. (30) between pages 92 and 93.

" The resulting N-acetyl-peptide methyl esters were subjected to permethylation".

Exp. (7) Trial on DL-Ala-Gly-Gly (Sigma)(2.0 mg); for the esterification dry methanolic HCl (6.2N) was used as described in Exp. (19). After analytical TLC (see plate22), the methanol solution was evaporated at room temperature and thoroughly dried ($\leqslant$ 0.5mm Hg, liquid-air trap, 20 min).

To the dried residue was added acetic acid (1.5 ml) and acetic anhydride (1.5 ml). The solution was stirred for one hour at room temperature. After a further $3\frac{1}{2}$ hours, the solution was evaporated at room temperature. The product was dissolved in methanol (100 μl). All analyses were performed within two days and showed no change in the pattern or proportions of products over that time.

Analytical two-dimensional TLC (250 μ silica gel G; 1st solvent A, 2nd solvent BEW plus 1% HAc: sprayed Cl_2-1% starch/1%KI)(see plate 4) showed six products present in roughly equal amounts.

B. Agarwal, et al.(ref. 52):

"The peptide was esterified (0.1N methanolic HCl at 20° for 12 hr), acetylated (acetic anhydride + acetic acid (1:1) at 20°C for 12 hr) them permethylated".

Exp. (8) Trial on DL-Ala Gly Gly (Sigma)(2.0mg); for the esterification dry methanolic HCl (0.1N) was used at room temperature for 12 hours as described in Exp. (20). After analytical TLC (see plate 22), the methanol solution was evaporated at room temperature and thoroughly dried ($\leqslant$ 0.5 mm Hg, liquid-air trap, 20 minutes).

Acetic acid (1.5 ml) and acetic anhydride (1.5 ml) were added to the dried residue. The reaction mixture was stirred at room temperature for $12\frac{1}{2}$ hours, evaporated at room temperature, redissolved in distilled water (1 ml) and re-evaporated. The product was dissolved in methanol (100 μl). All analyses were performed within two days and showed no change in products over that time.

Analysis of the Reaction Products from Exps. (7) and (8):

Analytical TLC (250μ silica gel G; solvents A, BEW plus 1% HAc; sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2-1% starch/1% KI) (see plates 5 and 6) was performed using the product of acetic acid-water-acetic anhydride and diazomethane on DL-Ala-Gly-Gly as a reference compound (see Exp. (25)). No ninhydrin-positive material remained in either product. Both procedures had led to by-product formation, apparently principally due to methanolysis in the esterification step as the level of by products was much worse from the procedure of Geddes, et al. This is in agreement with the analysis of the esterification step (see plate 22). The expected product, Ac-Ala-Gly-Gly-OMe makes up about 70% of the total products using Agarwal, et al.'s procedure.

Comparison with the Products of Reaction with Methanol + Acetic anhydride:

Analytical TLC (250μ silica gel G; solvents A, BEW plus 1% HAc; sprayed Cl_2-1% starch/1% KI)(see plates 7 and 8) was performed using the products of Exps. (6),(7) and (8). Several of the reaction products are apparently identical in each case, with Ac-Gly-OMe and Ac-Ala-Gly-OMe common to all three.

COMPARISON OF FOUR ACETYLATION PROCEDURES:

Exp. (9) Four samples of DL-Ala-Gly-Gly (Sigma)(2.2 mg, 2.5 mg, 2.3 mg, 2.4 mg) were weighed out and the acetylating reagents were added to each one in the order given: methanol (3.1 ml) acetic anhydride (0.8 ml); acetic acid (1.5 ml) acetic anhydride (1.5 ml); water (1.5 ml) acetic anhydride (1.5 ml); acetic acid (1 ml) water (1 ml) acetic anhydride (1 ml). The four reaction mixtures were stirred for 2 hours 10 minutes at room temperature, evaporated at room temperature, dissolved in methanol (1 to 2 ml) and re-evaporated. Each product was dissolved in methanol (100 μl) for analytical TLC (250μ silica gel G; solvents A and BAW 4:1:1 v/v; sprayed ninhydrin/$Cd(OAc)_2$, Cl_2-1% starch/1% KI)

In all cases more than one ninhydrin-negative product was formed. Reaction with water + acetic anhydride and water + acetic acid + acetic anhydride

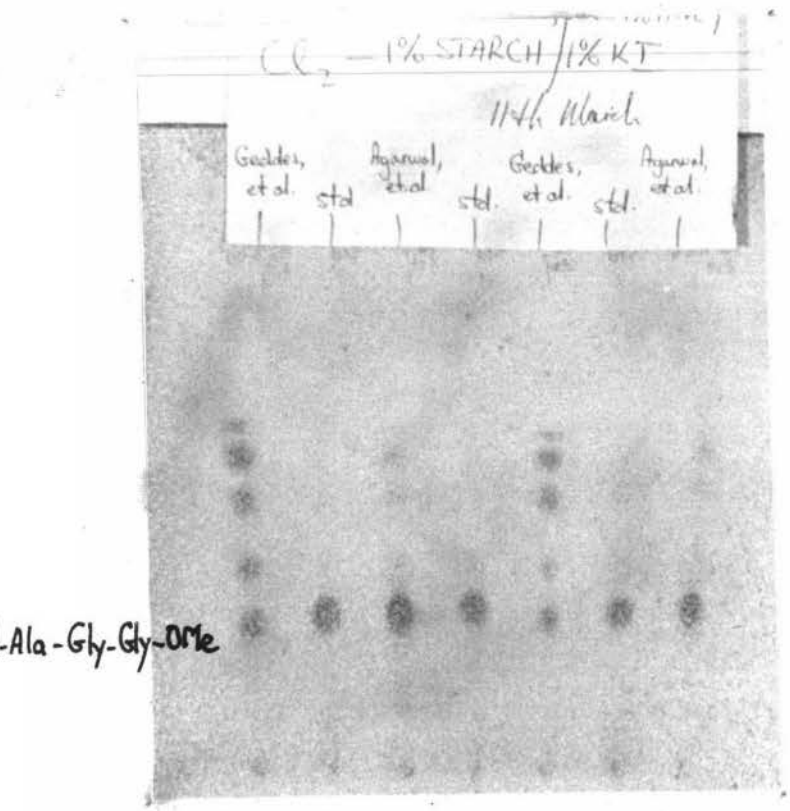


Plate 5. Esterified, acetylated Ala--Gly-Gly according to Geddes, et al. and Agarwal et al. Solvent A (Exps. (7) and (8)).

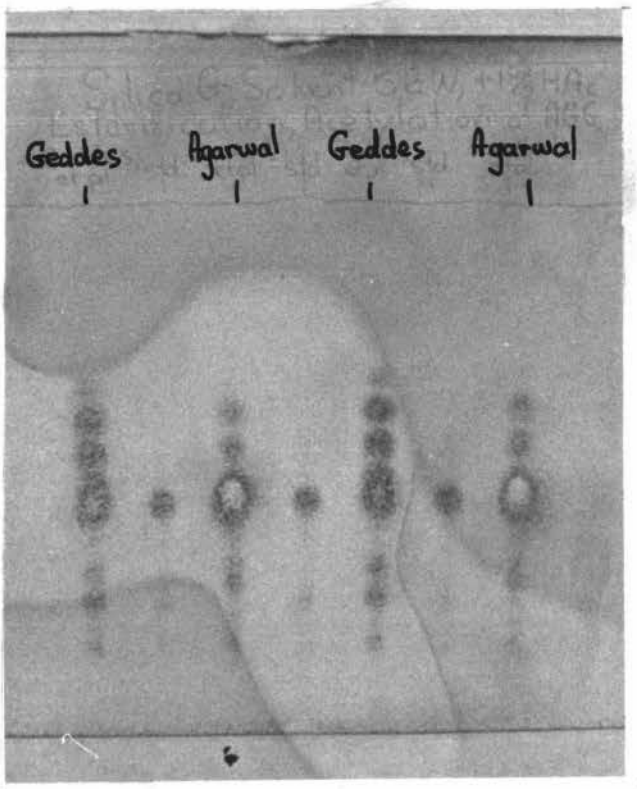


Plate 6. Esterified, acetylated Ala--Gly-Gly according to Geddes, et al. and Agarwal et al. Solvent BEW + 1%HAc (Exps. (7) and (8)).

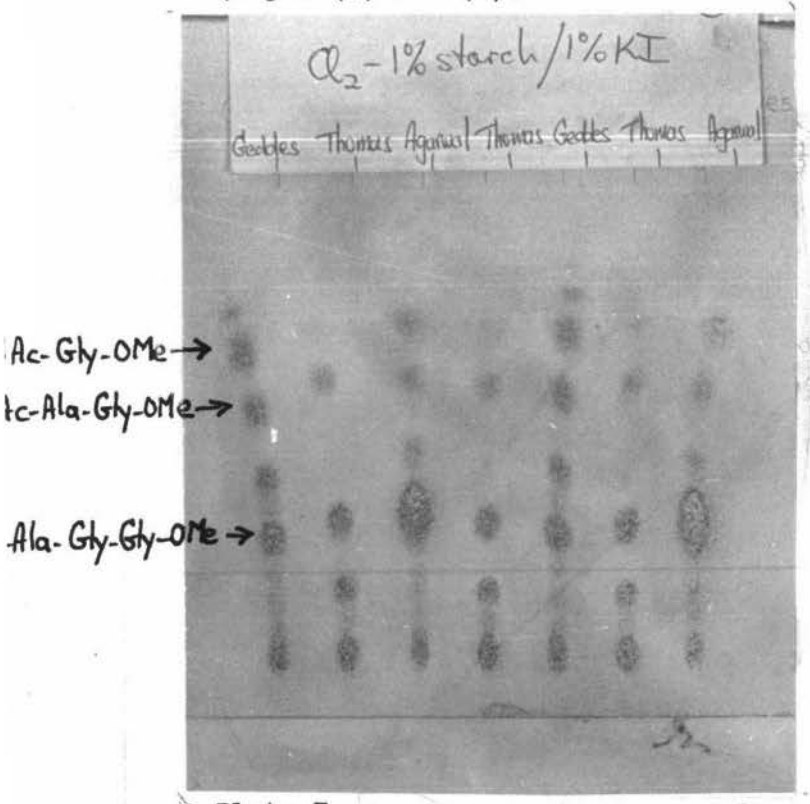


Plate 7.

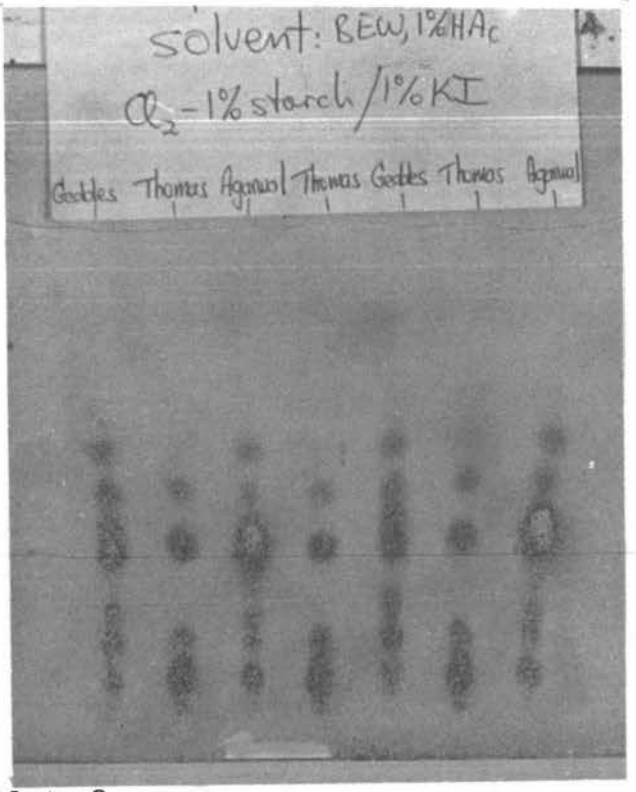


Plate 8.

Comparison of products from treatment of Ala-Gly-Gly with methanol + acetic anhydride and according to Geddes et al. and Agarwal et al. (Exps. (6),(7) and (8)). Solvents A and BEW + 1%HAc.

gave very low amounts of byproducts (Rfs 11,52 solvent A; 36,54 solvent BAW) as well as the expected N-acetyl-tripeptide (Rf 3 solvent A; Rf 25 solvent BAW). With water + acetic acid + acetic anhydride, there was a large amount of unreacted tripeptide remaining.

Compound Rf 25 solvent BAW, tentatively identified from its Rf value as Ac-Ala-Gly-Gly-OMe, was found in the products of reaction with acetic acid + acetic anhydride as well as with methanol + acetic anhydride.

Byproduct Formation in Acetylation Reactions:

Acetic acid plus acetic anhydride:

Exp. (10) To DL-Ala-Gly-Gly (Sigma)(5.0 mg) was added acetic acid (2.5 ml) and acetic anhydride (2.5 ml). After 8 minutes stirring at room temperature, a clear solution was formed. Aliquots (0.5 ml) were taken at the following times after the addition of the reagents: 8 minutes, 1 hour, 3 hours, 7 hours, 24 hours. The aliquots were evaporated at room temperature in less than 4 minutes, dissolved in distilled water ($\frac{1}{2}$ ml) and re-evaporated at room temperature. The product from each aliquot was dissolved in methanol (50 μl) for analytical TLC (250μ silica gel G; solvents A, BEW plus 1% HAc, BAW 4:1:1 v/v, Q); sprayed ninhydrin/$Cd(OAc)_2$, Cl_2-1% starch/1% KI)(see plates 9 to 12). Ninhydrin-positive material was only present in the 8 minute aliquot; only a very minor amount of byproduct formation was observed; the major product chromatographed as a single spot in all four solvents.

Methanol plus acetic anhydride:

Exp. (11) To DL-Ala-Gly-Gly (Sigma)(5.2 mg) was added methanol (4 ml) and acetic anhydride (1 ml). After 20 minutes stirring at room temperature, a clear solution was formed. Aliquots (0.5 ml) were taken at the following times after addition of the reagents: 20 minutes, 1 hour, 3 hours, 6 hours 10 minutes, 24 hours. The aliquots were evaporated at room temperature in less than 4 minutes, dissolved in distilled water ($\frac{1}{2}$ ml) and re-evaporated

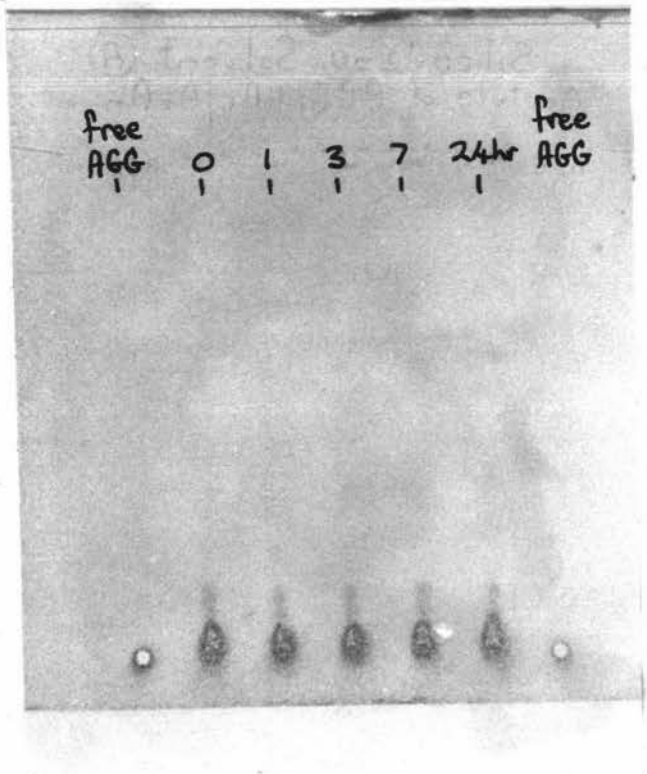


Plate 9. Reaction of acetic acid + acetic anhydride with Ala-Gly-Gly. Solvent A (Exp. (10)).

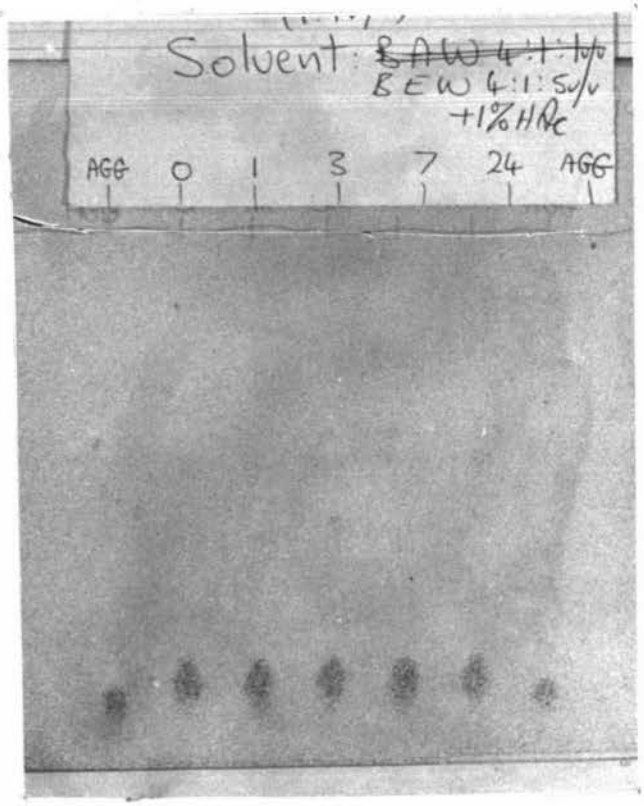
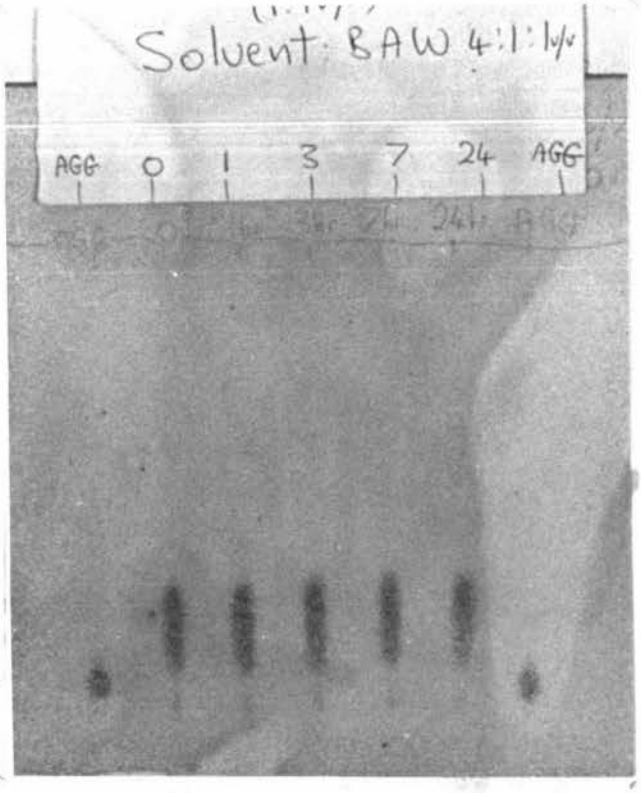


Plate 10. Reaction of acetic acid + acetic anhydride with Ala-Gly-Gly. Solvent BEW + 1%HAc (Exp. (10)).



ee Ala-Gly-Gly

Plate 11. Reaction of acetic acid + acetic anhydride with Ala-Gly-Gly. Solvent BAW 4:1:1 v/v (Exp. (10)).

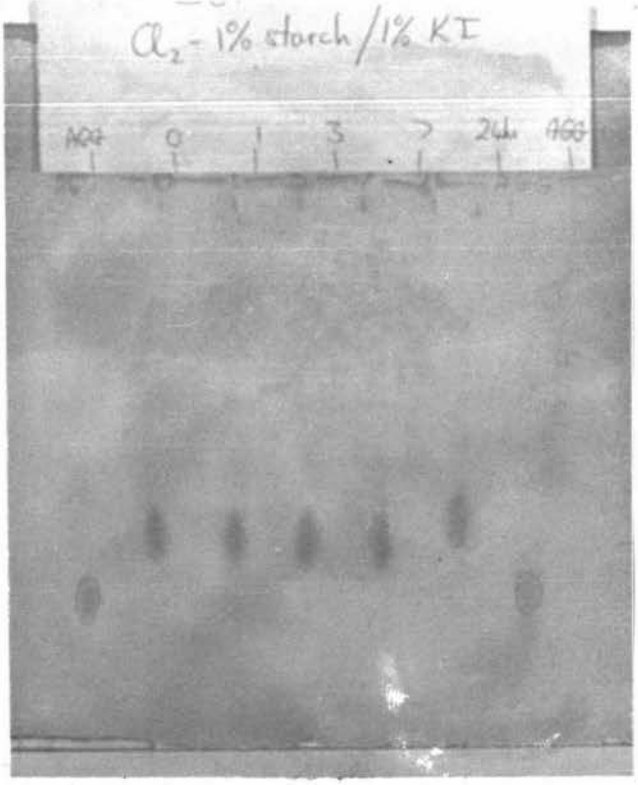


Plate 12. Reaction of acetic acid + acetic anhydride with Ala-Gly-Gly. Solvent Q (Exp. (10)).

at room temperature. The product from each aliquot was dissolved in methanol (50 µl). Analytical TLC (250µ silica gel G; solvents A, BEW plus 1% H Ac; sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2-1% starch/1% KI)(see plates 13 and 14) showed that no ninhydrin-positive material remained in any aliquots. The product first formed (Rf 3, solvent A) gradually decreased in concentration over the 24 hour period, while the levels of four other products (Rf 13,27,52,62; solvent A) increased. One product, Rf36 in solvent A, appeared to have a low but constant concentration throughout.

Water plus acetic acid plus acetic anhydride:

Exp. (12) To DL-Ala-Gly-Gly (Sigma)(4.4 mg) was added water (1.5 ml), acetic acid (1.5 ml) and acetic anhydride (1.5 ml). The peptide dissolved at once. The solution was kept at room temperature and aliquots (0.5 ml) were taken at the following times after addition of the reagents: 1 minute, 1 hour, 3 hours, 6 hours, 24 hours. The aliquots were evaporated at room temperature in less than 4 minutes, dissolved in distilled water ($\frac{1}{2}$ ml) and re-evaporated at room temperature. The product from each aliquot was dissolved in methanol (50 µl). Analytical TLC (250µ silica gel G; solvents A, BAW 4:1:1 v/v ; sprayed ninhydrin/$Cd(OAc)_2$, Cl_2-1% starch/1% KI)(see plates 15,16 and 17) showed that unreacted (ninhydrin-positive) peptide remained at a significant, constant level from 1 hour onwards. No byproducts were detected. The reaction gave only the desired product, but did not go to completion.

Water plus acetic anhydride:

Exp. (13) To DL-Ala-Gly-Gly (Sigma)(5.3 mg) was added distilled water (2.5 ml) and acetic anhydride (2.5 ml). Although the peptide dissolved at once, the reaction mixture was heterogeneous. After 20 minutes shaking at room temperature, a homogeneous solution was formed. Aliquots (0.5 ml) were taken at the following times after addition of the reagents: 10 minutes, 1 hour, 3 hours, 6 hours and 24 hours. The aliquots were evaporated at room temperature in

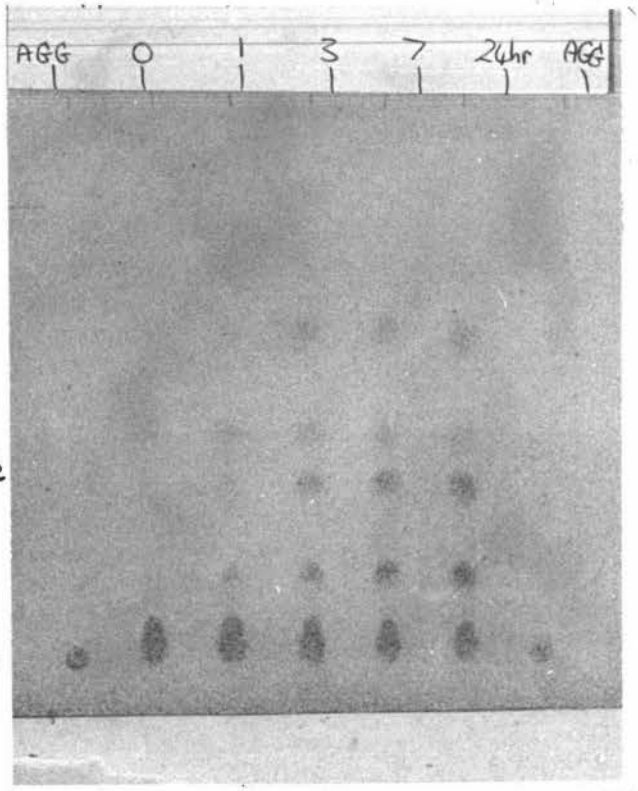


Plate 13. Reaction of methanol + acetic anhydride with Ala-Gly-Gly. Solvent A (Exp. (11)).

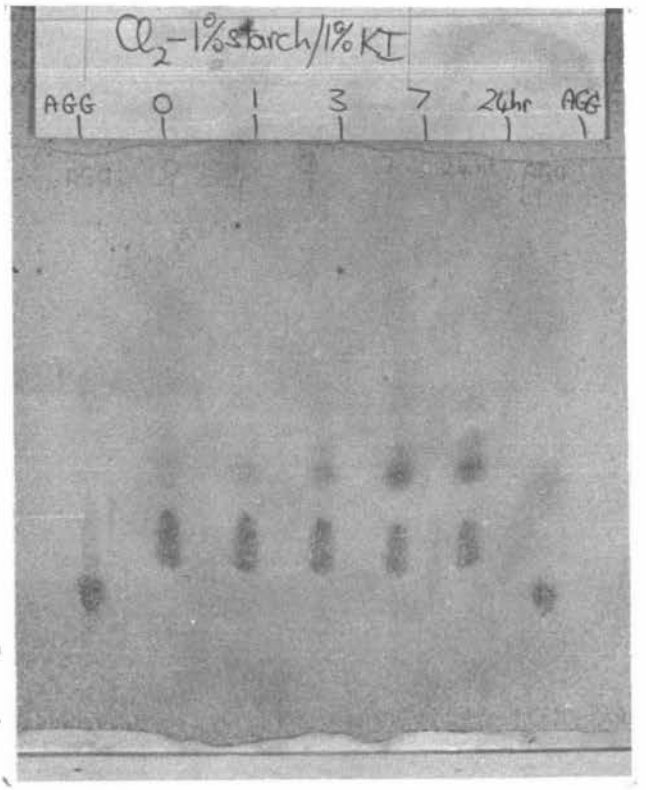


Plate 14. Reaction of methanol + acetic anhydride with Ala-Gly-Gly. Solvent BAW 4:1:1 v/v (Exp. (11)).

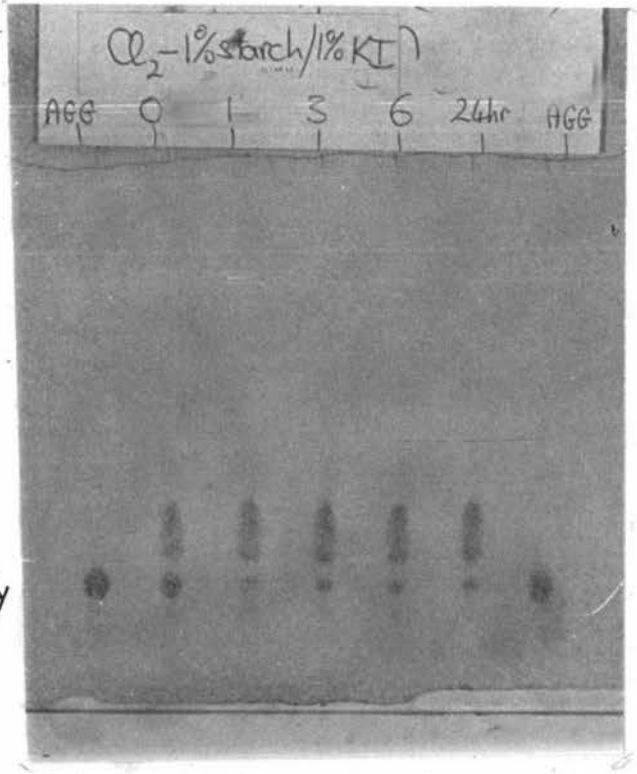


Plate 15. Reaction of water + acetic acid + acetic anhydride with Ala-Gly--Gly. Solvent BAW 4:1:1 v/v, sprayed Cl_2-1% starch1%KI (Exp. (12)).

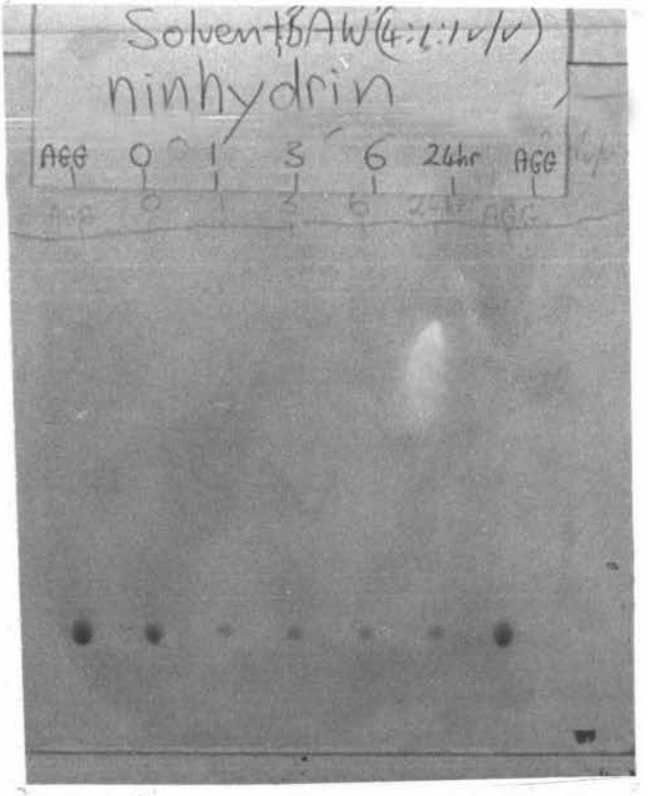


Plate 16. Plate 15, sprayed initially with ninhydrin/$Cd(OAc)_2$. Unreacted peptide remains at a constant level. (Exp. (12)).

less than 4 minutes, dissolved in distilled water ($\frac{1}{2}$ ml) and re-evaporated at room temperature. The product from each aliquot was dissolved in methanol (50 µl). Analytical TLC (250µ silica gel G; solvents A, BAW 4:1:1 v/v; sprayed ninhydrin/$Cd(OAc)_2$, Cl_2-1% starch/1% KI)(see plates 18 to 20) showed that no ninhydrin--positive material remained after 1 hour. Only the expected product (Rf 3, solvent A, Rf 28 solvent BAW 4:1:1 v/v) was detected. The reaction gave a high yield of the desired acetyl-peptide with minimum by-product formation.

Check for Chain-cleavage at the C-terminal residue:

Exp. (13a) Two samples of DL-Ala-Gly-Gly (Sigma)(5.3 mg, 6.3 mg) were weighed out and acetylating reagents added as follows: to the first, (5.3 mg) distilled water (2.5 ml) and acetic anhydride (2.5 ml); to the second, (6.3 mg) acetic acid (3.0 ml) and acetic anhydride (3.0 ml). Both reaction mixtures were stirred for 80 minutes at room temperature. After standing 21 hours, both solutions were evaporated at room temperature, the products dissolved in distilled water (2-3 ml), kept at room temperature for 1 hour, and re-evaporated to dryness at room temperature. The products were dissolved in methanol (500 µl, 600 µl) and aliquots (100 µl each) taken for analytical TLC. The remaining solutions were made up to about 1 ml with methanol, esterified with ethereal diazomethane (2 millimoles) for two hours at room temperature and evaporated at room temperature. The products were dissolved in methanol (200 µl, 250 µl). Analytical TLC (250µ silica gel G; solvent A, sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2-1% starch/1% KI)(plate 21) was carried out under identical conditions after the final methanolic saturations had been standing at 3°C for 1 day. No significant byproducts were detected in either case.

ESTERIFICATION PROCEDURES:

Methanol/acetyl chloride:

The Fischer esterification generating the methanolic HCl by addition of acetyl chloride to methanol (ref.85).

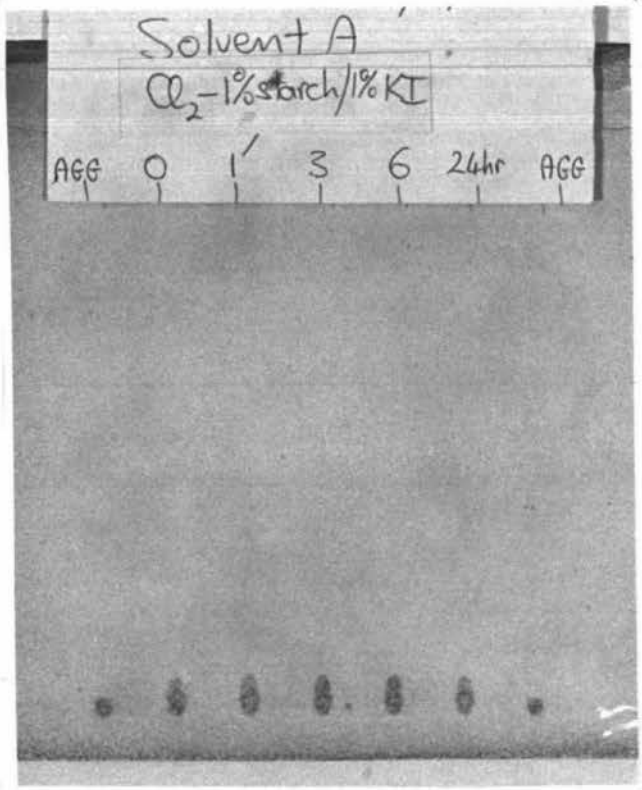


Plate 17. Reaction of water + acetic acid + acetic anhydride with Ala-Gly--Gly. Solvent A (Exp. (12)).

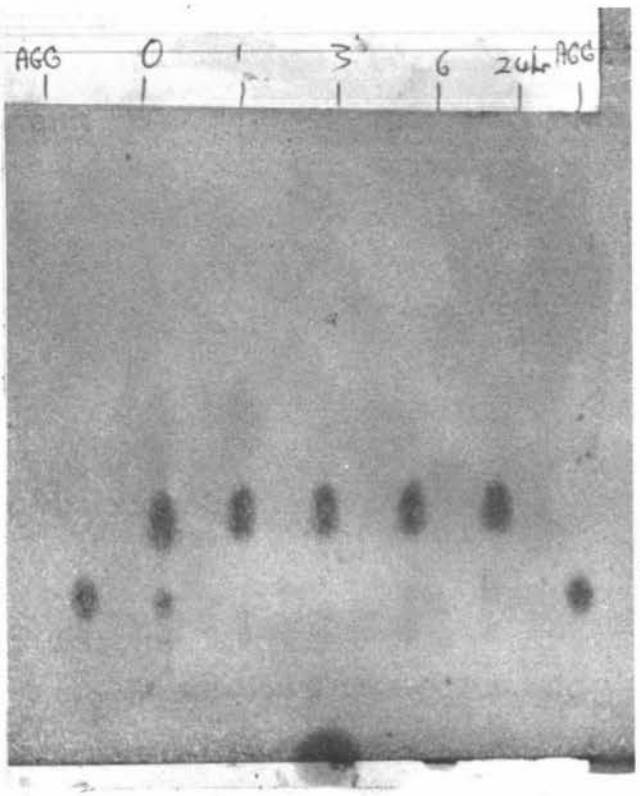


Plate 18. Reaction of water + acetic anhydride with Ala-Gly-Gly. Solvent BAW 4:1:1 v/v (Exp. (13)).

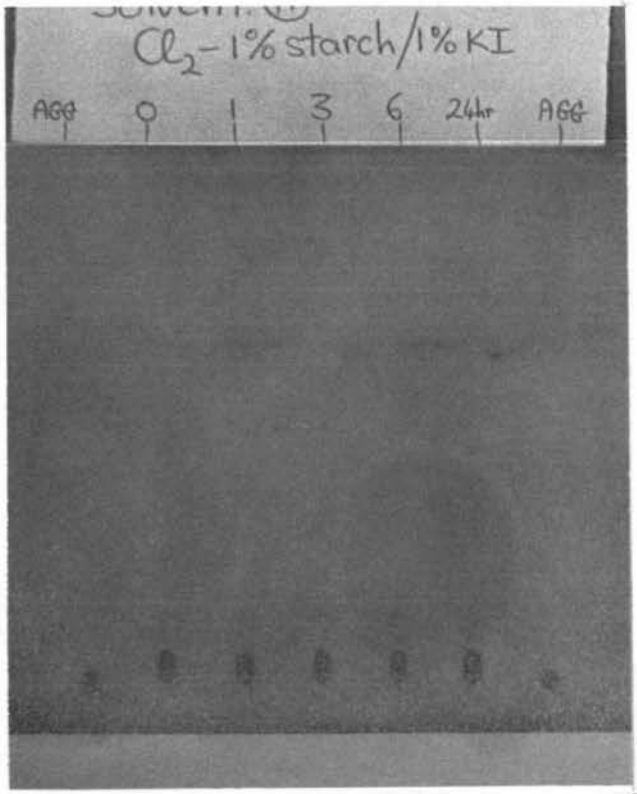


Plate 19. Reaction of water + acetic anhydride with Ala-Gly-Gly. Solvent A, sprayed Cl_2-1% starch/1%KI (Exp. (13)).

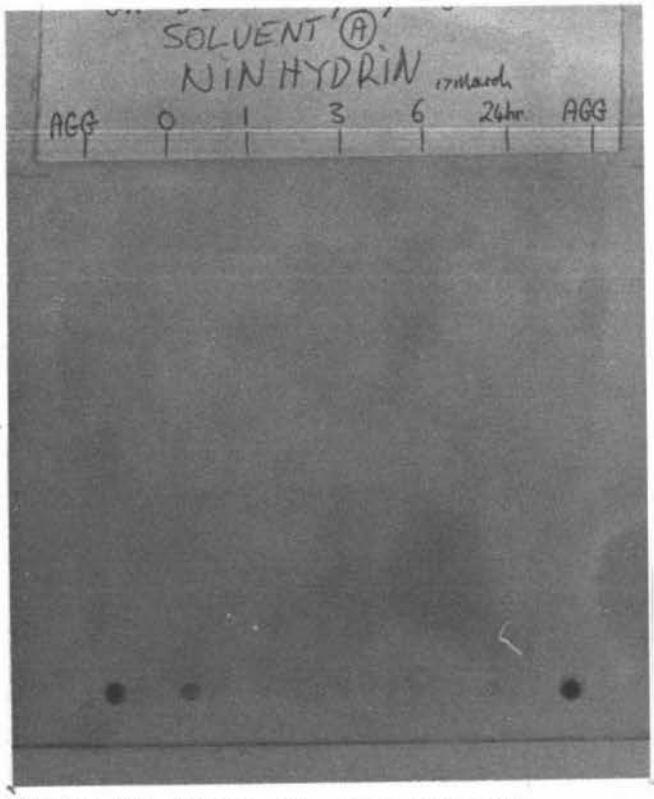


Plate 20. Plate 19, sprayed with ninhydrin/$Cd(OAc)_2$. No unreacted peptide remains after 1 hour (Exp.(13)).

Exp. (14) To Phe-Asp-Ala-Ser-Val (M.R.)(5.1 mg) was added methanol (5.0 ml) and acetyl chloride (100 µl). The peptide dissolved at once. The solution was kept at room temperature for 19 hours, evaporated at room temperature, dissolved in methanol and re-evaporated.

Acetic acid (1.25 ml) and acetic anhydride (1.25 ml) were added to the dried product. The reaction mixture was stirred at room temperature for two hours, evaporated at room temperature (15 minutes) and applied to the direct insertion probe for mass spectrometry.

A series of spectra were obtained as the temperature of the ion source increased (see Figs. 12,13 and 14):

at 170° - 190°C: mixed spectra of Ac-Phe-Asp-OMe (OMe) and Ac-Phe-Asp-Ala-OMe (OMe) were obtained.

at 200°C: the spectrum of Ac-Phe-Asp-Ala-OMe (OMe) was obtained.

at 230° - 240°C: A strong spectrum of the expected product, Ac-Phe-Asp-Ala-Ser-Val-OMe (OMe, OAc) was obtained.

No other products could be detected.

Exp. (15) To DL-β-phenylalanine (BDH)(2.5 mg) was added methanol (2.0 ml) and acetyl chloride (50 ml). The amino acid dissolved at once. The solution was kept at room temperature for 16½ hours, evaporated at room temperature and dissolved in 0.15N aqueous ammonia (200 µl). Analytical TLC (250µ silica gel G; solvent BEW plus 1% HAc; sprayed ninhydrin/$Cd(OAc)_2$) showed equal amounts of unreacted free phenylalanine (Rf 52) and phenylalanine methyl ester (Rf 72).

Exp. (16) To DL-Ala-Gly-Gly (Sigma)(3.9 mg) was added absolute methanol* (3.0 ml) and acetyl chloride (100 µl). The peptide dissolved at once. The clear solution was kept at room temperature for 3½ days, evaporated at room temperature, then dissolved in methanol and re-evaporated several times. The product was dissolved in methanol (500 µl). Analytical TLC (250µ silica gel G; solvent BAW 4:1:1 v/v; sprayed ninhydrin/$Cd(OAc)_2$) showed only a very small amount

* Absolute methanol was prepared according to Vogel, 3rd Edn, p. 169.

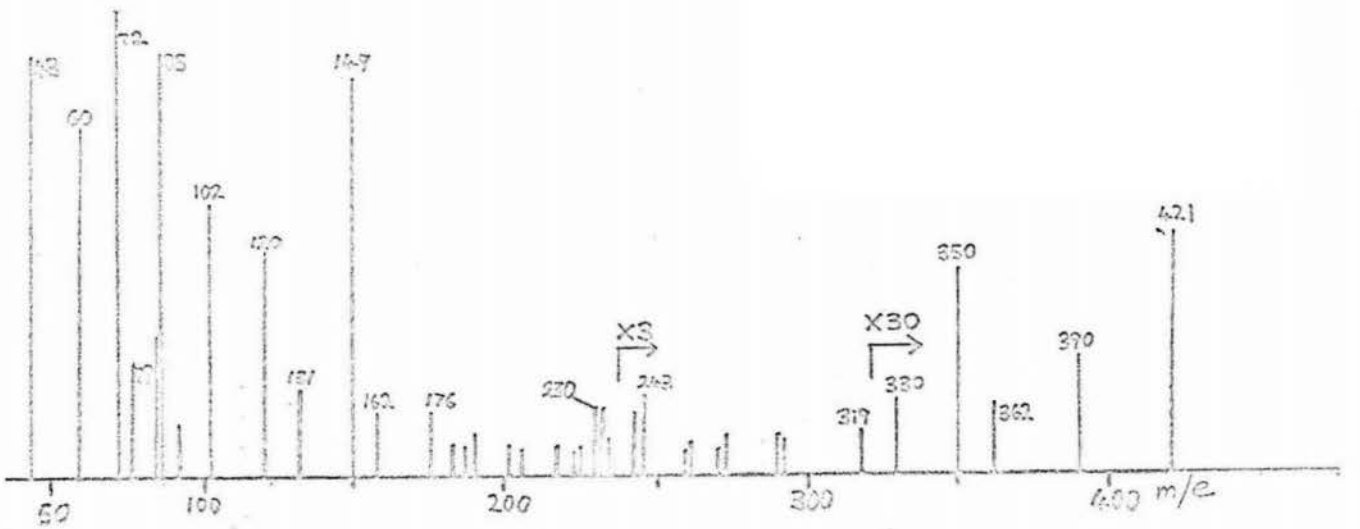


Fig. (12) Mixed spectra obtained at 195°C from the crude product from esterified, acetylated Phe-Asp-Ala-Ser-Val (Exp. (14)).

The spectrum contains two molecular species:

Ac-Phe-Asp-Ala-OMe molecular ion at m/e 421.
OMe

Ac-Phe-Asp-OMe molecular ion at m/e 350.
OMe

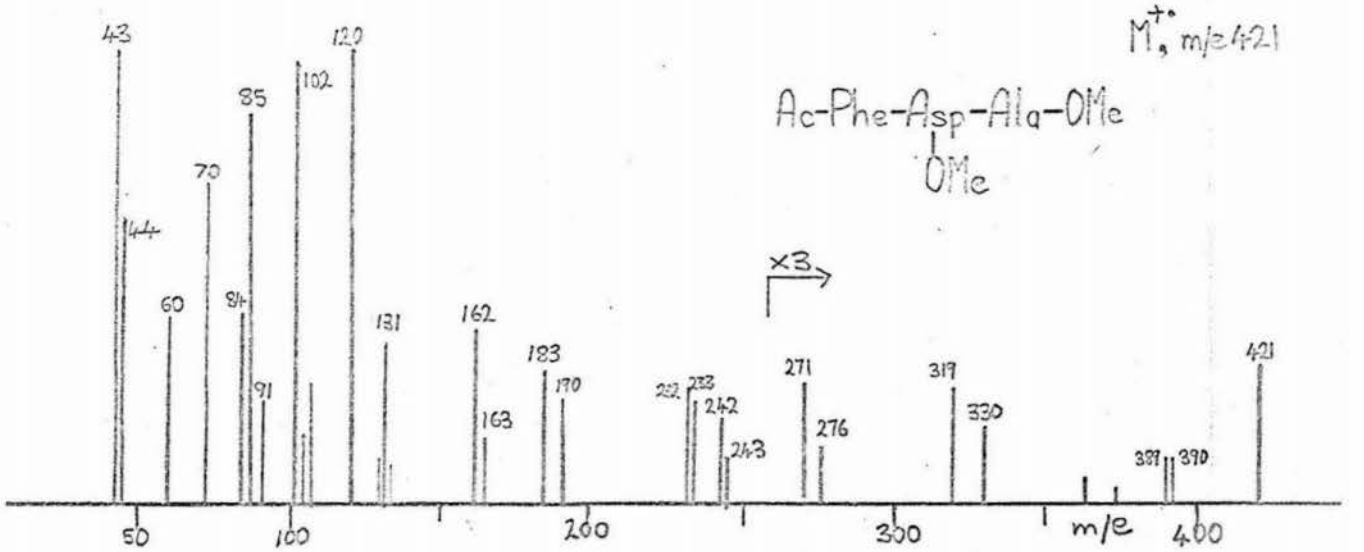


Fig. (13) Spectrum of obtained at 205°C from the crude product from esterified, acetylated Phe-Asp-Ala-Ser-Val (Exp. (14)).

The spectrum is that of Ac-Phe-Asp-Ala-OMe with molecular ion at m/e 421.
OMe

of unreacted peptide (Rf 20). However, four ninhydrin-positive products were found in roughly equal amounts: Rfs 33,36,40 and 44. This was taken to show that extensive methanolysis of the tripeptide had occurred.

Use of 2.2'-dimethoxypropane:

Esterification according to G.C. Uhle (ref. 90) 2,2'-dimethoxypropane is added to the reaction mixture to act as a water scavenger.

Studies of the progress of the esterification were performed:

Exp. (17) To DL-β-phenylalanine (BDH)(5.5 mg) was added absolute methanol (1.5 ml), acetyl chloride (100 μl) and 2,2'-dimethoxypropane (1.5 ml). The acid dissolved at once. Aliquots (0.25 ml) were taken at the following times after addition to the reagents: 1 minute, 1 hour, 4½ hours, 24 hours and 77 hours. The aliquots were evaporated at room temperature (in a few seconds) dissolved in methanol and re-evaporated several times. The residues contained an increasing amount of red gum. The product from each aliquot was dissolved in methanol (50 μl). Analytical TLC (250μ silica gel G; solvent BEW plus 1% HAc: sprayed ninhydrin/$Cd(OAc)_2$) showed that no unreacted phenylalanine remained after 77 hours. More than one ninhydrin-positive product was formed. The red gum-like residue was produced in increasing amounts from 4½ hours to 77 hours. It was not positive to ninhydrin and a solution in dilute aqueous ammonia was light yellow, changing to red on acidification.

Exp. (18) To DL-Ala-Gly-Gly (Sigma)(5.6 mg) was added absolute methanol (1.5 ml) acetyl chloride (100 μl) and 2,2'-dimethoxypropane (1.5 ml). The peptide dissolved at once. Aliquots (0.25 ml) were taken at the following times after addition of the reagents: 1 minute, 1 hour, 4½ hours, 24 hours, 77 hours. The aliquots were evaporated at room temperature (in a few seconds) redissolved in methanol and re-evaporated several times. The residue contained an increasing amount of red gum. The product from each aliquot was dissolved in methanol (50 μl). Analytical TLC (250μ silica gel G; solvent BEW plus 1% H Ac: sprayed ninhydrin/$Cd(OAc)_2$) showed that no unreacted peptide remained

after 77 hours. The red gum-like residue had Rf 80-85, was produced in increasing amount from 4½ hours to 77 hours, was not positive to ninhydrin, was yellow in alkaline solution and red in acid. It was apparently identical with the red gum produced in the reaction with phenylalanine. A compound (Rf 22) was the first product. A second compound (Rf 28) became a significant product after 4½ hours when the reaction was still incomplete: the two were present in equal amounts after 77 hours when no free peptide remained.

Esterification with dry methanolic hydrogen chloride:

Two variations of this esterification method had been used in preparing peptide esters for acetylation and permethylation prior to mass spectrometry.

Generation of dry methanolic hydrogen chloride:

All-glass, oven-dried apparatus was used. Concentrated sulphuric acid was run onto lumps of sodium chloride. The gas evolved was dried with concentrated sulphuric acid, then bubbled into anhydrous, absolute methanol (65 ml) which was cooled on ice. After one hour the weight gained by the methanol solution was recorded (14.9 g). The solution of hydrogen chloride in methanol was 6.2N.

A. esterification after Geddes, et al (ref. 51):

"Dissolve the peptide (about 10 μmoles) in 1 ml of the reagent prepared by bubbling dry HCl(g) through methanol, seal and leave overnight at room temperature". The strength of the methanolic HCl was not specified; this was taken to mean that it was not considered critical.

Exp. (19) DL-Ala-Gly-Gly (Sigma)(2.0 mg) was dried under vacuum ($\leqslant$ 0.5 mm Hg; liquid-air vapour trap 30 minutes) and suba sealed under dry nitrogen. Anhydrous methanolic HCl (1.0 ml, 6.2N) was injected onto the peptide, which dissolved at once. The clear solution was kept at room temperature for 13 hours, evaporated at room temperature, redissolved in methanol and re-evaporated several times. The product was dissolved in methanol (100 μl) for analytical TLC (see over).

B. esterification after Agarwal, et al (ref. 52):

"0.1N methanolic HCl at 20°C for 12 hours".

Exp. (20) DL-Ala-Gly-Gly (Sigma)(2.2 mg) was dried under vacuum ($\leqslant$ 0.5 mm Hg; liquid air vapour trap; 30 minutes) and suba sealed under dry nitrogen. Anhydrous methanol (6.0 ml) and anhydrous methanolic HCl (100 µl, 6.2N) was injected onto the peptide, which dissolved at once. The solutions were kept at room temperature for 12 hours, evaporated at room temperature, redissolved in methanol an re-evaporated several times. The products were each dissolved in methanol (100 µl).

Analytical TLC (250µ silica gel G; solvent BEW plus 1% HAc, double-developed; sprayed ninhydrin/$Cd(OAc)_2$) (plate 22) was performed the same day. At least three ninhydrin positive products were found. The use of 6.2N methanolic HCl has led to more extensive methanolysis of the peptide than 0.1N HCl. In both cases there was virtually no unreacted peptide left.

Further analytical TLC (250µ silica gel G; solvent BEW plus 1% HAc; sprayed ninhydrin/$Cd(OAc)_2$, Cl_2-1% starch/1% KI) showed that as well as the ninhydrin-positive products (Rfs 18,23,29) there was one not positive to ninhydrin with Rf 47, in higher concentration in the products from reaction with 6.2N methanolic HCl.

C. Study of the progress of reaction, esterifying with dry methanolic HCl:

Exp. (21) DL-Ala-Gly-Gly (Sigma)(3.1 mg) was dried under vacuum ($\leqslant$ 0.5 mm Hg; liquid air trap: 20 minutes) and suba sealed under dry nitrogen. Anhydrous methanolic HCl (1.5 ml, 6.2N) was injected and the peptide dissolved at once. The solution was kept in a dessicator at room temperature. Aliquots (0.20 ml) were taken by dry syringe at the following times after addition of the reagent: 1 minute, 1 hour, 3 hours, 6 hours and 24 hours. The aliquots were thoroughly dried at room temperature and each aliquot was dissolved in methanol (50 µl).

Exp. (22) DL-Ala-Gly-Gly (6.1 mg) was dried under vacuum ($\leqslant$ 0.5 mm Hg, liquid-air trap, 20 minutes) and suba-sealed under nitrogen. Anhydrous methanol (6.0 ml) was injected, followed by anhydrous methanolic HCl (100 μl , 6.2N). The peptide dissolved at once. The solution was kept in a dessicator at room temperature. Aliquots (0.5 ml) were taken by dry syringe at the following times after addition of the reagent: 1 minute, 1 hour, 3 hours, 6 hours and 24 hours. The aliquots were thoroughly dried at room temperature and each aliquot was dissolved in methanol (50 μl).

Analytical TLC (250 μ silica gel G; solvent BEW plus 1% HAc; sprayed ninhydrin/$Cd(OAc)_2$)(see plates 23,24) was performed the same day. The pattern of products and their order of appearance is the same for both reagents. Three ninhydrin-positive products are formed. The compound first formed has Rf 18, the second Rf 15 and the third Rf 24. No unreacted peptide remained after 3 hours using 6.2N HCl in methanol, after 6 hours using 0.1N HCl in methanol. At this stage, extensive methanolysis has occurred with both reagents. None of the esterification procedures so far examined was considered satisfactory because of extensive methanolysis of the peptide.

Esterification with Diazomethane:

An ethereal solution of diazomethane was prepared as follows: Methyl cellosolve (35 ml) diethyl ether (25 ml) and 60% aqueous potassium hydroxide (10 ml) were chilled in ice. N-methyl-N-nitroso-toluene-p-sulphonamide (2.14 g) was added and the mixture chilled in ice for a further 15 minutes. On gentle warming an ethereal solution of diazomethane distills, recovery about 15 mls containing about 0.2 g (5 millimole) diazomethane.

Exp. (23) A small sample of Gly-DL-Val (N.B.Co) was introduced on the direct insertion probe for mass spectrometry. The spectrum obtained at 200°C showed the expected molecular ion of m/e 174 and no peaks of higher mass.

Gly-DL-Val (NBCo) (1 mg) was suspended in methanol (2 ml). An ethereal

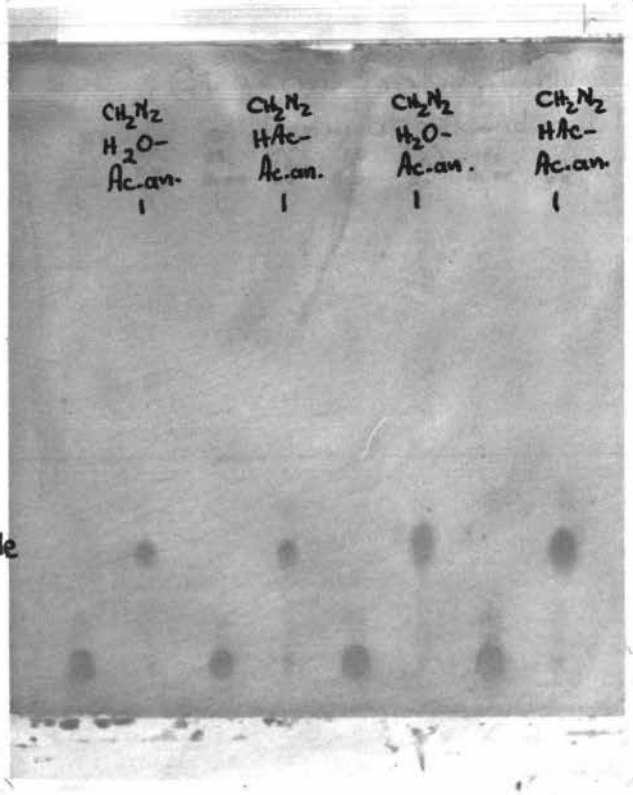


Plate 21. Check for chain cleavage in reaction of acetic anhydride + water with Ala-Gly-Gly. Reaction products treated with diazomethane (Exp. (13a)).

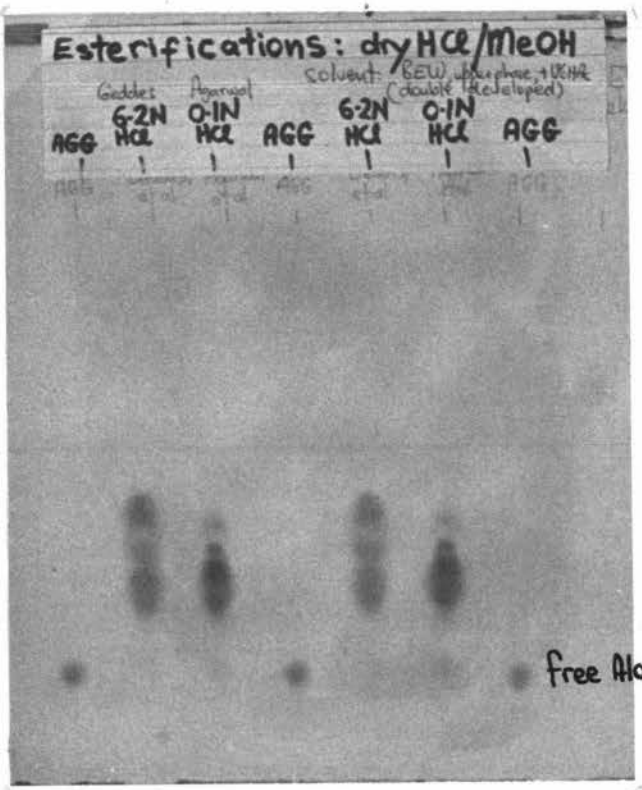


Plate 22. Esterification of Ala-Gly-Gly with 0.1N and 6.2N HCl in methanol. Sprayed with ninhydrin/$Cd(OAc)_2$ (Exps. (19)and (20)).

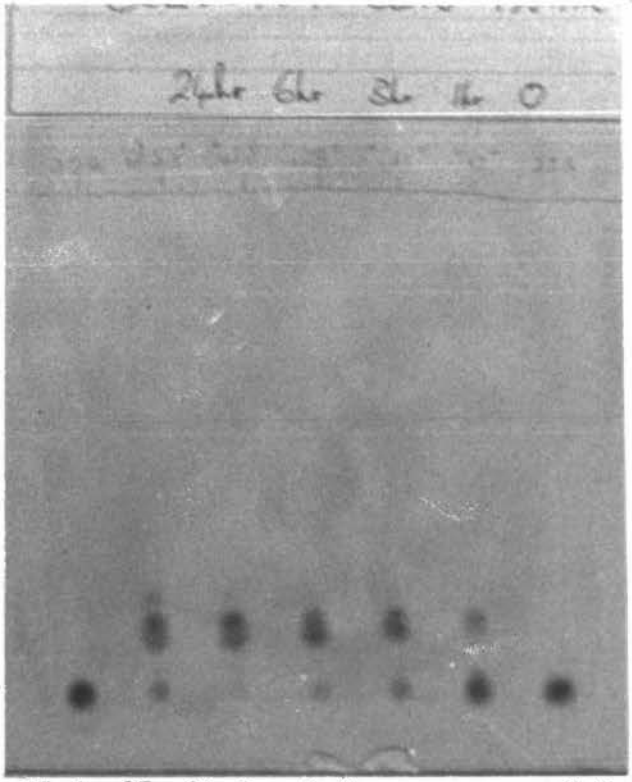


Plate 23. Study of the progress of the reaction, esterifying Ala-Gly-Gly with 0.1N HCl in methanol (Exp. (22)).

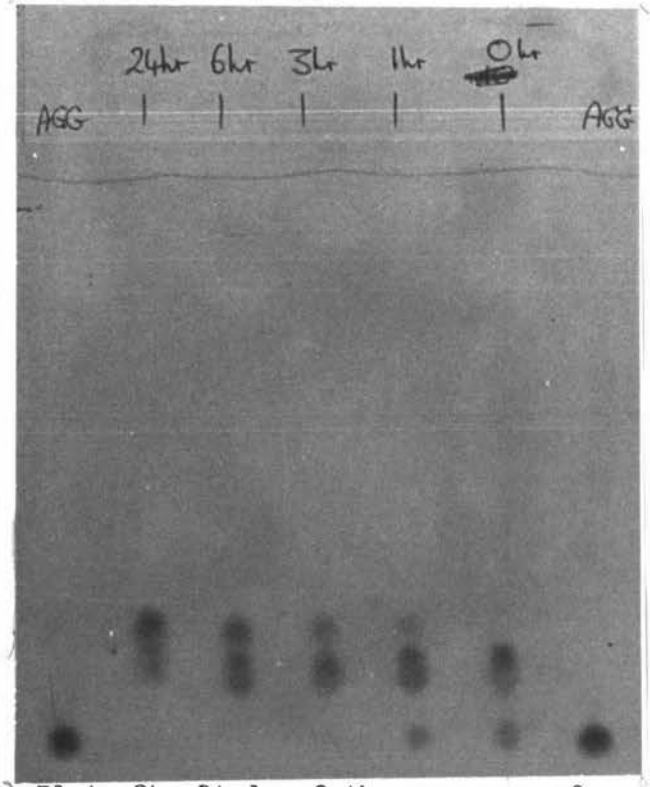


Plate 24. Study of the progress of the reaction, esterifying Ala-Gly--Gly with 6.2N HCl in methanol (Exp. (21)).

solution of diazomotane (2 ml) was added. The peptide at once dissolved with vigorous evolution of gas; excess diazomethane was added and the solution left in ice for 1 hour. Excess diazomethane was removed by gentle warming and the solution evaporated at room temperature. The product was applied in methanol to the direct insertion probe for mass spectrometry.

The mixed spectra observed contained the series of ions: the expected molecularion m/e 188, and ions at m/e 202, 216 and 230. Initially m/e 216 was the most intense of these, though after a short time the ratios of intensities settled at 10 to 2 to 1 to.05. The mass spectra did not indicate the location of the extra methyl groups. (Fig. 15).

Exp. (24) DL-Ala-Gly-Gly (Sigma)(3.0 mg) was suspended in methanol (2 ml). Excess ethereal diazomethane (5 ml, 1½ millimoles) was added and the yellow suspension stirred for 30 minutes at room temperature. Excess diazomethane was removed by gentle warming and the solution evaporated at room temperature. The residue was applied to the direct insertion probe for mass spectrometry.

The mixed spectra obtained at a sample temperature of 160°C showed the expected molecular ion at m/e 217 and equally intense homologous ions at m/e 231, m/e 245 (Fig. 16). The mass spectra did not indicate the location of the extra methyl groups. An intense ion at m/e 242 and its homologue at m/e 256 could not be explained.

Exp. (25) To DL-Ala-Gly-Gly (Sigma)(3.3 mg) was added distilled water (1.0 ml), acid (1.0 ml) and acetic anhydride (1.0 ml). The peptide dissolved at once. The solution was kept at room temperature. Aliquots (1.0 ml) were taken 2 hours, 4 hours and 6 hours after the addition of the reagents, evaporated at room temperature, redissolved in methanol (1 ml) and re-evaporated twice. The product from each aliquot was dissolved in methanol (1.1 mls) and an aliquot (100μl) removed for analytical TLC (250μ silica gel G; solvents <u>BEW plus 1% HAc</u>, <u>BAW 4:1:1 v/v</u>; sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2-1% starch/1% KI). Acetylation

Fig. (14) Mass spectrum of Ac-Phe-Asp-Ala-Ser-Val-OMe;
OMe OAc

see Fig. 4 between pages 49 and 50 .

High Resolution Data: Expected Elemental Compositions:

m/e649 = $C_{30}H_{43}N_5O_{11}$ $C_{30}H_{43}N_5O_{11}$

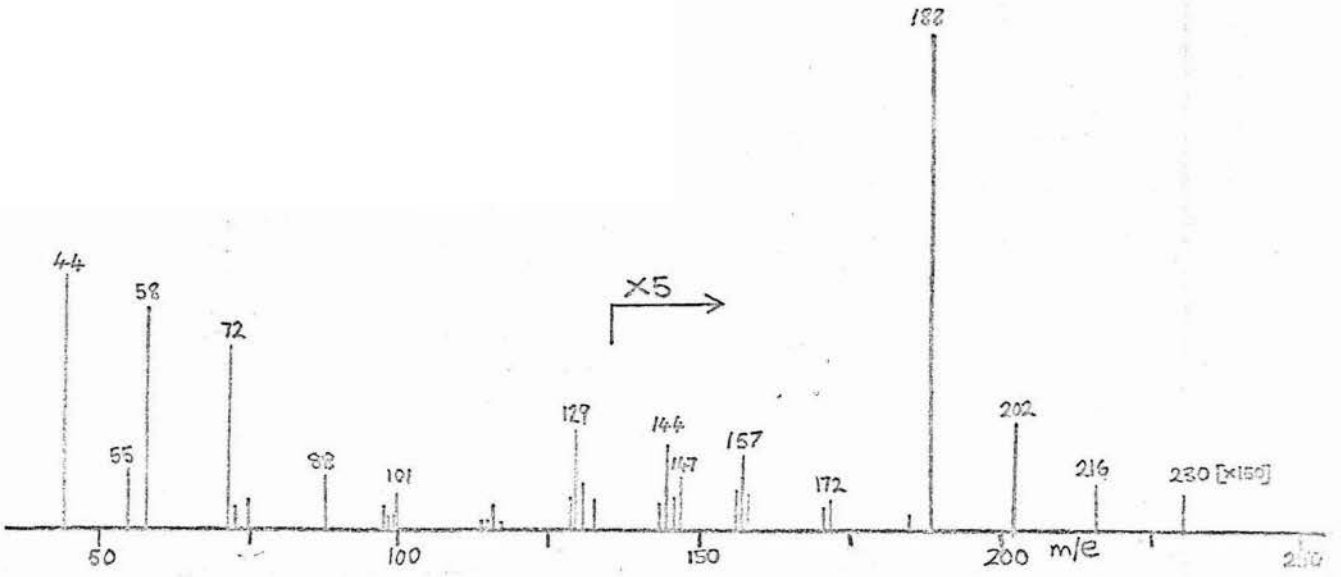


Fig. 15 Mass Spectrum of Gly-DL-Val treated with diazomethane (Exp. (23)). Theoretical molecular ion for Gly-DL-Val-OMe is at m/e188.

High Resolution Data:

m/e72 = $C_4H_{10}N$

The fragment $\underset{CH_3}{NH}-CH_2-C\equiv O^+$, m/e72 is not present.

was at least 95% complete in all three aliquots and no significant by-products were detected.

Excess ethereal diazomethane (5 ml, 1½ millimoles), generated as previously described, was added to each of the methanol solutions of the acetylation products. The yellow solutions were left at room temperature for 1 hour, excess diazomethane removed by gentle warming and the solutions evaporated at room temperature. The products were each dissolved in methanol (100µl). Analytical TLC (250µ silica gel G; solvents A, BEW plus 1% H Ac, BAW 4:1:1 v/v, Q: sprayed ninhydrin/$Cd(OAc)_2$ and Cl_2 -1% starch/1% KI) showed that the small amount of free peptide had given rise to at least two ninhydrin-positive products, while the acetyl peptide had given a single ninhydrin negative product.

PERMETHYLATION, USING THE HAKOMORI-VILKAS TECHNIQUE:

Exp. (26) Experimental conditions according to Thomas (refs. 46, 65).

To DL-Leu-Gly-Phe (NBCo.)(2 mg) was added methanol (2 ml) and acetic anhydride (0.5 ml). After 28 hours stirring at room temperature the solution was evaporated at 50°C, dried under vacuum ($\leqslant$ 0.3 m Hg) at 60°C and "Suba" sealed.

To NaH/oil dispersion (80 mg, 50% NaH) pre-rinsed several times with dry hexane and dried under vacuum, was added dry DMSO (0.8 ml) under nitrogen. The temperature of the reaction mixture was raised to 110°C, and quickly cooled to room temperature. A portion of the grey solution (0.2 ml) was added by syringe to well-dried Ac-DL-Leu-Gly-Phe (2 mg) under nitrogen. After 15 minutes stirring at room temperature, chilled I_2-free CH_3I(0.3 ml) was injected into the reaction mixture. After a further 1 hour 40 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed, evaporated at room temperature, and applied in chloroform to the direct insertion probe for mass spectrometry.

The initial spectrum contained a strong peak at m/e 448 but the relative intensity of this peak dropped and a fairly satisfactory spectrum was

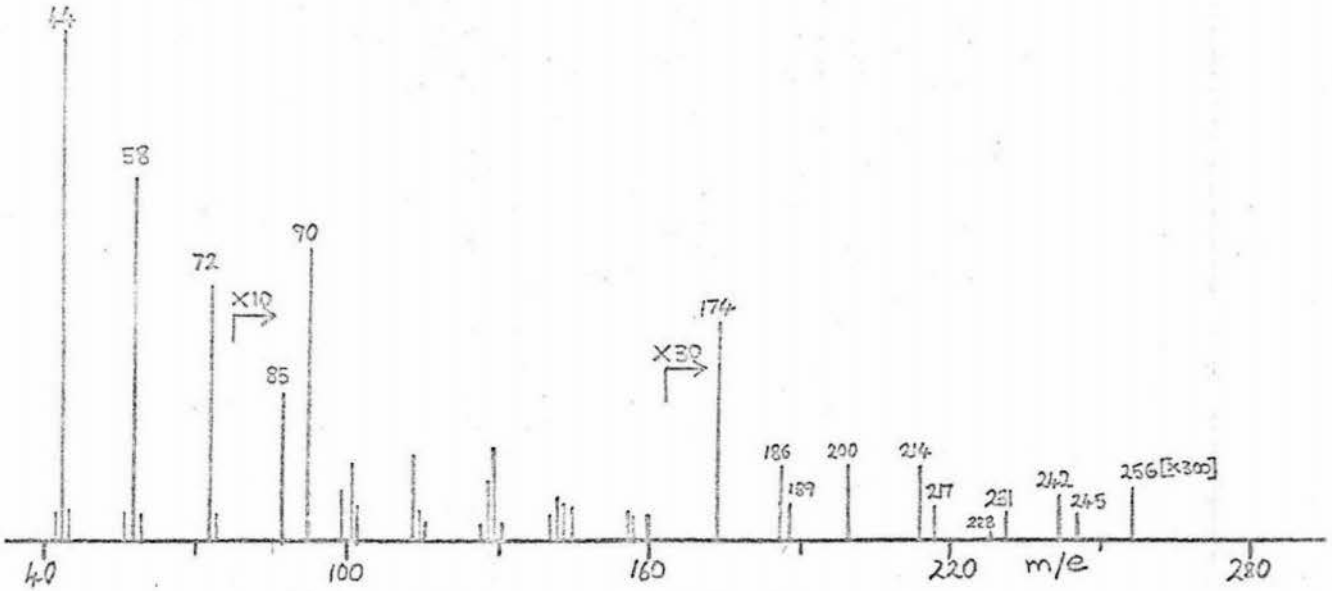


Fig. 16 Mass spectrum of DL-Ala-Gly-Gly treated with diazomethane. Theoretical molecular ion for DL-Ala-Gly-Gly-OMe is at m/e217.

High Resolution Data:

	Expected Elemental Compositions:
m/e217 = $C_8H_{15}N_3O_4$	$C_8H_{15}N_3O_4$
m/e231 = $C_9H_{17}N_3O_4$	-
m/e245 = $C_{10}H_{19}N_3O_4$	-
m/e174 = $C_7H_{14}N_2O_3$	-
m/e242 = $C_{10}H_{16}N_3O_4$	-

Metastable Transition:

m/e217 ⟶ m/e174 ($-CH_3NO$)

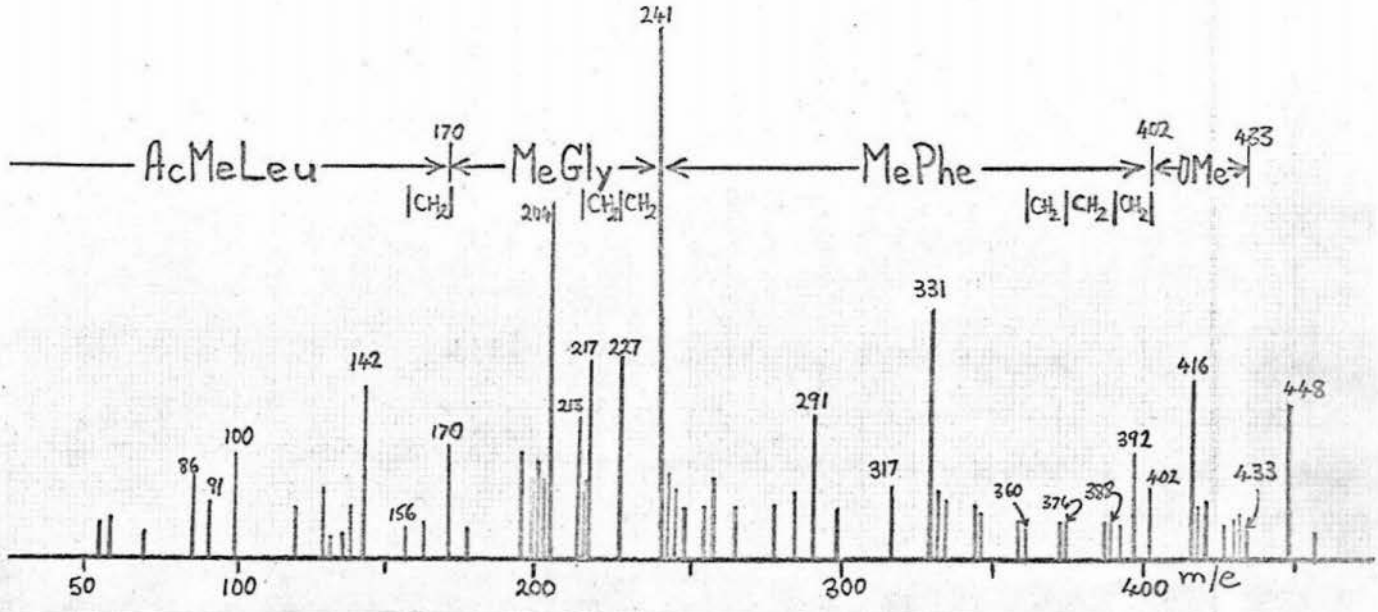


Fig. 17 Mass spectrum of permethylated Ac-Leu-Gly-Phe showing partial methylation (Exp. (26)).

obtained (Fig. 17) which showed the expected ions formed by fragmentation of the peptide bond. However, in the region of the expected molecular ion at m/e 436, which was absent, there were a number of other strong ions. There was no sign of peaks 14 m.u. higher than the expected sequencing peaks, but there was evidence of peaks 14 m.u. lower indicating that the compound was not fully methylated.

Examination of the spectrum of the acetylated peptide from above prior to permethylation showed no unesterified Ac-Leu-Gly-Phe. A spectrum corresponding to Ac-Leu-Gly-Phe-OMe was obtained, though the expected molecular ion at m/e 391 was accompanied by ions of almost equal intensity at m/e 401 and m/e 433 (Fig. 18).

Although Thomas' reaction conditions had not been exactly duplicated, especially in the acetylation which had gone on for longer time and been worked up at higher temperatures (50°-60°C), these poor results led to an examination of other methods of acetylation and the conditions for generating the carbanion.

Exp. (27) Large scale prepartion of dimethylsulfinyl carbanion: according to Corey and Chaykovsky (ref. 96).

NaH/oil dispersion (2.15 g, 50% NaH) was rinsed three times with dry hexane, decanting the supernatant. Final traces of hexane were removed under vacuum. Dry redistilled DMSO (20 ml) was injected under nitrogen. Magnetic stirring was maintained at 64°C - 68°C for 1½ hours, when evolution of gas had ceased. A liquid paraffin filled U-tube was used as a pressure-release valve. The product was stored under nitrogen at -20°C. The product was a dark-grey cloudy solution. In contrast to the description given by Corey, no yellow colour was observed, though traces of product in the reaction vessel went yellow on exposure to air or on overheating.

Permethylation Conditions:

To investigate conditions for permethylation pure acetylphenylanine methyl ester was used:

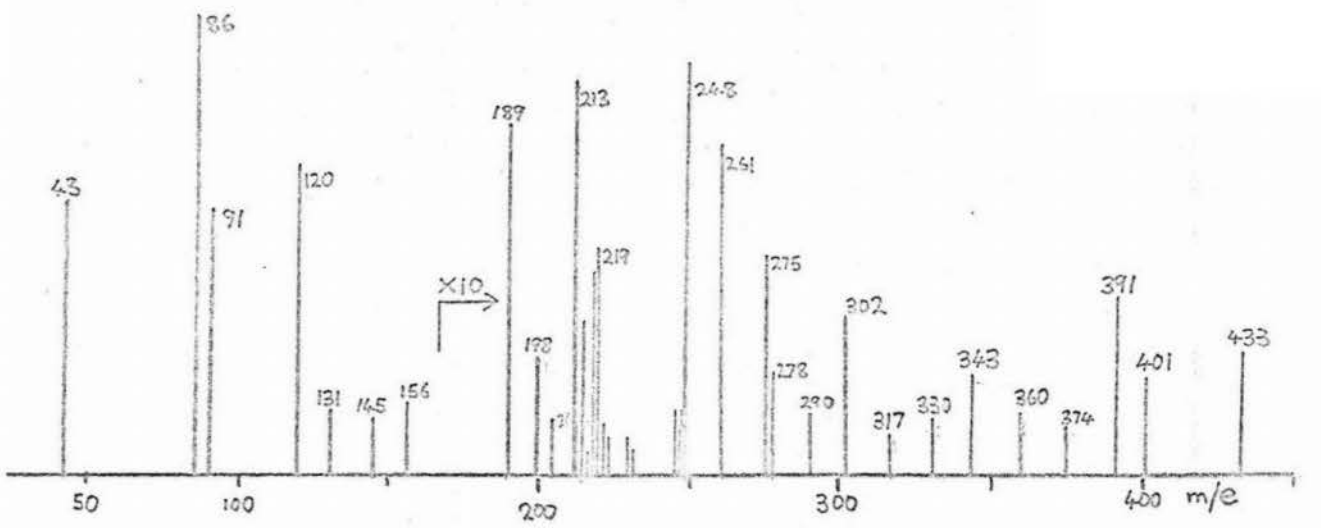


Fig. 18 Mass spectrum of DL-Leu-Gly-Phe treated with methanol + acetic anhydride (Exp. (1)).

High Resolution Data:

m/e433 = $C_{22}H_{31}N_3O_6$

m/e156 = $C_8H_{14}NO_2$

Expected Elemental Composition:

$C_{22}H_{31}N_3O_6$ (diacetyl)

$C_8H_{14}NO_2$ (monoacetyl)

Methanol (150 ml) was added to DL- β -phenylalanine (BDH)(5.0g). The mixture was chilled in ice and thionyl chloride (20 ml) was added dropwise with stirring. The clear solution formed was refluxed for 3 hours, then evaporated under vacuum. The pale yellow solid was recrystalised from ethanol-diethyl ether. The fine white crystals decomposed at about 150°C. Literature value 158°C, decomp.

A solution of the phenylalanine methyl ester hydrochloride was neutralised with sodium bicarbonate and extracted with diethyl ether. Evaporation of the ether gave a yellow oil which went solid on standing.

Methanol (10 ml) was added to this solid (1.0 g) followed by acetic anhydride (0.7 ml). After 3 hours stirring at room temperature, the solution was evaporated under vacuum. The white solid remaining was dissolved in chloroform and washed successively with 1N HCl, 5% $NaHCO_3$ and distilled water. After drying, the solution was evaporated.

The white solid product was analysed by TLC and mass spectrometry and shown to be pure acetylphenylalanine methyl ester.

Acetylphenylalanine methyl ester was permethylated under a variety of conditions, using the solution of dimsyl carbanion in DMSO previously generated (see Exp. 27) and stored at -20°C for eight weeks.

Exp. (28) Acetylphenylalanine methyl ester (2.3 mg) was dried under vacuum ($\leqslant$ 0.5 mm Hg, liquid-air trap, 30 minutes) then "Suba" sealed under nitrogen. Dimsyl sodium in DMSO (0.2 ml) was added by syringe. After stirring 30 minutes at room temperature, chilled I_2-free CH_3I (0.30 ml) was added dropwise by syringe. After a further 1 hour stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed with water, evaporated at room temperature and applied to the direct insertion probe for mass spectrometry.

The expected spectrum, molecular ion at m/e 235 was obtained briefly on insertion of the probe. This was quickly replaced by a similar spectrum in which m/e 235 and those fragments derived from it which still contained the ester

group (M-91, M-91-42, M-73) were missing (Fig. 19). Several new peaks appeared at mass up to m/e 281. High resolution measurements showed some of these to be sulphur containing. Apparently some form of substitution had occurred at the ester group.

Shorter equilibration time with dimsyl carbanion:

Exp. (29) Acetylphenylalanine methyl ester (3.5 mg) was dried under vacuum then "Suba" sealed under nitrogen. Dimsyl sodium in DMSO (0.20 ml) was added by syringe. The solution was stirred at room temperature and chilled, I_2-free CH_3I(0.30 ml) was at once added dropwise to the solution. After 1 hour stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed, evaporated at room temperature and applied to the direct insertion probe for mass spectrometry.

The expected molecular ion at m/e 235 came up at a sample temperature of 70°C, but was quickly replaced by ions at m/e 245, 259 (Fig. 20). The metastable detector showed that the principal fragment ion at m/e 176 was derived from m/e 204, 245 and 266. No metastables were detected for the ion at m/e 259. Dropping the energy of the ionising electron beam also indicated that m/e 259 was a molecular ion, as all other peaks in the spectrum decreased markedly in intensity with respect to it.

Short equilibration time with dimsyl sodium and reduced reaction time with methyl iodide:

Exp. (30) Acetylphenylalanine methyl ester (2.9 mg) was dried under vacuum and "Suba" sealed under nitrogen. Dimsyl sodium in DMSO (0.20 ml) was added by syringe. The solution was stirred at room temperature and chilled I_2-free CH_3I (0.30 ml) was at once added. After 4 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed, evaporated at room temperature and applied to the direct insertion probe for mass spectrometry.

A spectrum including the expected molecular ion at m/e 235 was observed

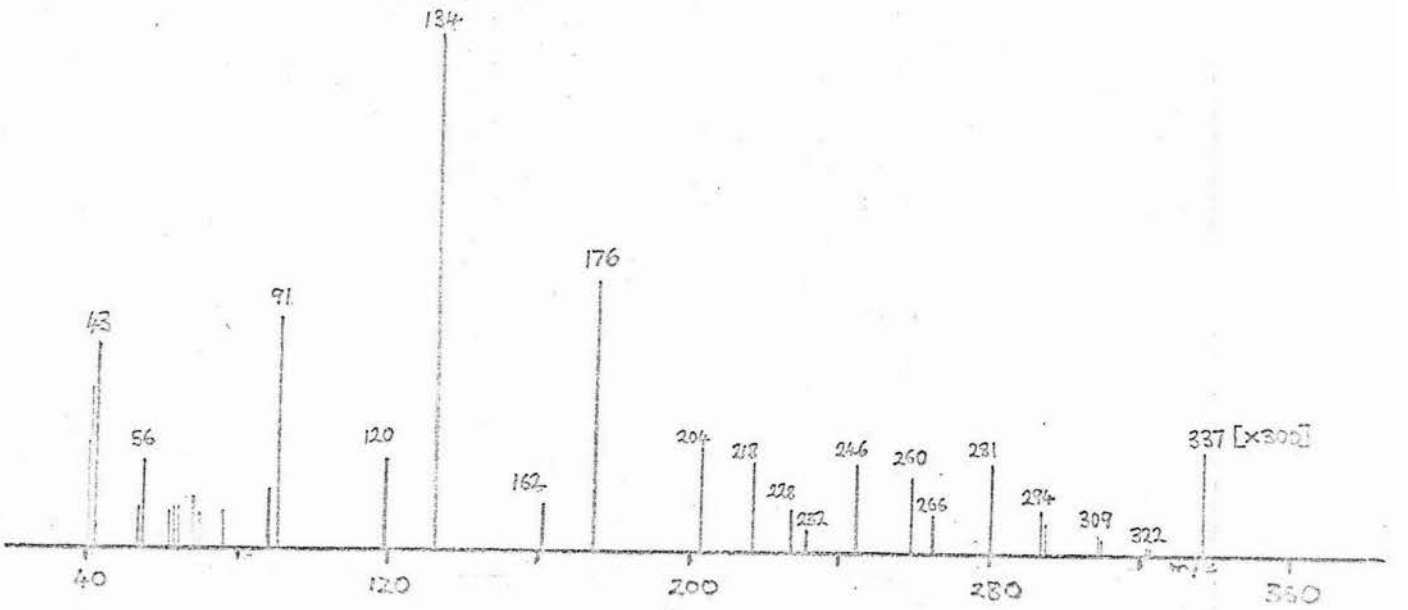


Fig. 19 Mass spectrum of permethylated Ac-Phe-OMe, 30 minutes with dimsyl sodium and 1 hour with methyl iodide (Exp. (28)).

High Resolution Data:

m/e281 = $C_{15}H_{23}NO_2S$ m/e260 = $C_{16}H_{22}NO_2$

m/e266 = $C_{14}H_{20}NO_2S$ m/e246 = $C_{15}H_{20}NO_2$

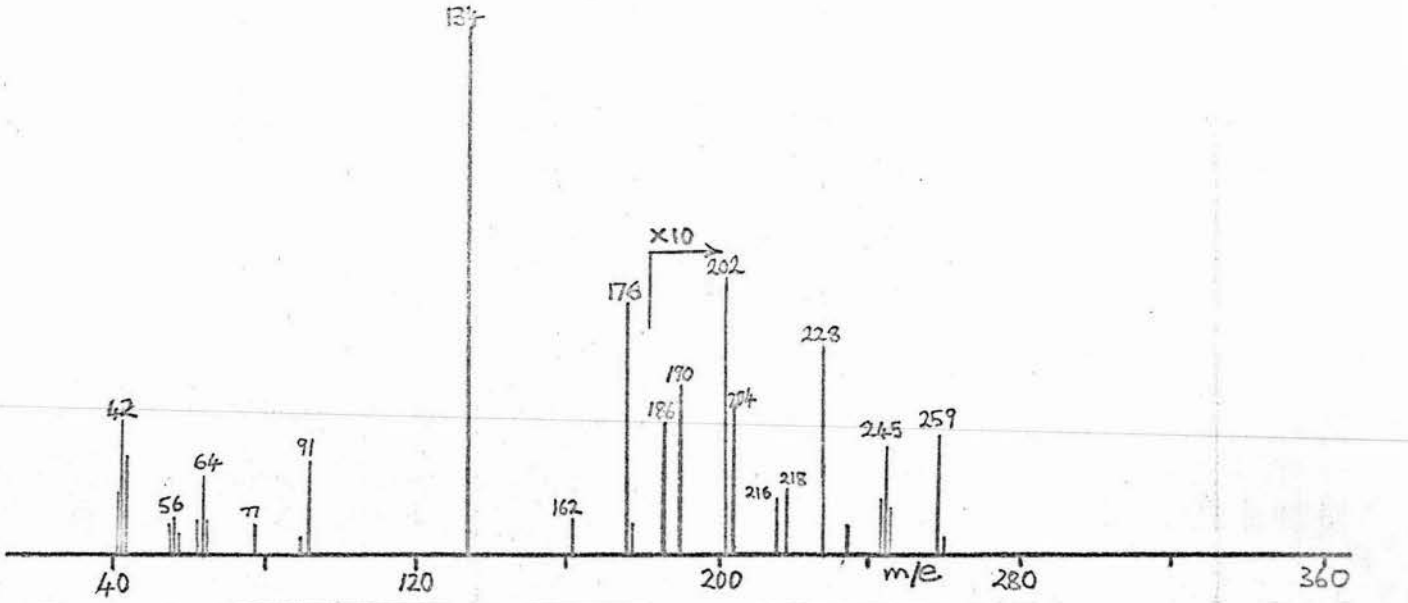


Fig. 20 Mass spectrum of permethylated Ac-Phe-OMe, 3 minutes with dimsyl sodium and 1 hour with methyl iodide (Exp. (29)).

High Resolution Data:

m/e259 = $C_{16}H_{21}NO_2$

m/e245 = $C_{15}H_{19}NO_2$

m/e235 = $C_{13}H_{17}NO_3$ and $C_{15}H_{23}O_2$

m/e176 = $C_{11}H_{14}NO$

m/e162 = $C_{10}H_{10}O_2$ - therefore not due to incomplete methylation.

Metastable Transition:

m/e245 ⟶ m/e176 ($-C_4H_5O$)

for a short time. The principal spectrum had ions up to m/e 337 (Fig. 21). In general the product was the same as from the previous two permethylations, despite the very short reaction times.

Use of freshly generated dimsyl solution:

Exp. (31) NaH/oil dispersion (20 mg, 50% NaH) was rinsed three times with dry petroleum ether (80° - 100°C fraction) decanting the liquid. Final traces of petroleum ether were removed under vacuum, and the flask "Suba" sealed under nitrogen. Dry DMSO (0.20 ml) was added by syringe. A liquid-paraffin filled U-tube was used as a pressure release valve. The mixture was stirred at 65° for 65 minutes, then cooled to room temperature. Acetylphenylalanine methyl ester (2.5 mg) was added. After five minutes stirring, chilled I_2-free CH_3I (0.30 ml) was added dropwise. Stirring was continued at room temperature for 1 hour. Water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

The expected spectrum was obtained "probe-cut" over a prolonged time. The molecular ion at m/e 235 was accompanied by an ion at m/e 249 about 25% as intense. The spectrum was similar to the "expected spectra" obtained from the three previous permethylations, although the higher homologous peaks were absent in the previous spectra.

On heating the sample at 120°C the molecular ions vanished and a new spectrum was obtained (Fig. 22). Some peaks were common to both spectra. The molecular ion appeared to be at m/e 323. Although it was difficult to interpret the spectrum exactly, it clearly arose from a compound substituted at the ester group of the acetylphenylalanine methyl ester.

Permethylation of Acetylphenylalanine:

Exp. (32) Methanol (10 ml) was added to DL-β-phenylalanine (BDH)(1.0g). Acetic anhydride (0.7 ml) was added to the stirred suspension. After 30 minutes stirring at room temperature, the solution was evaporated. The solid residue

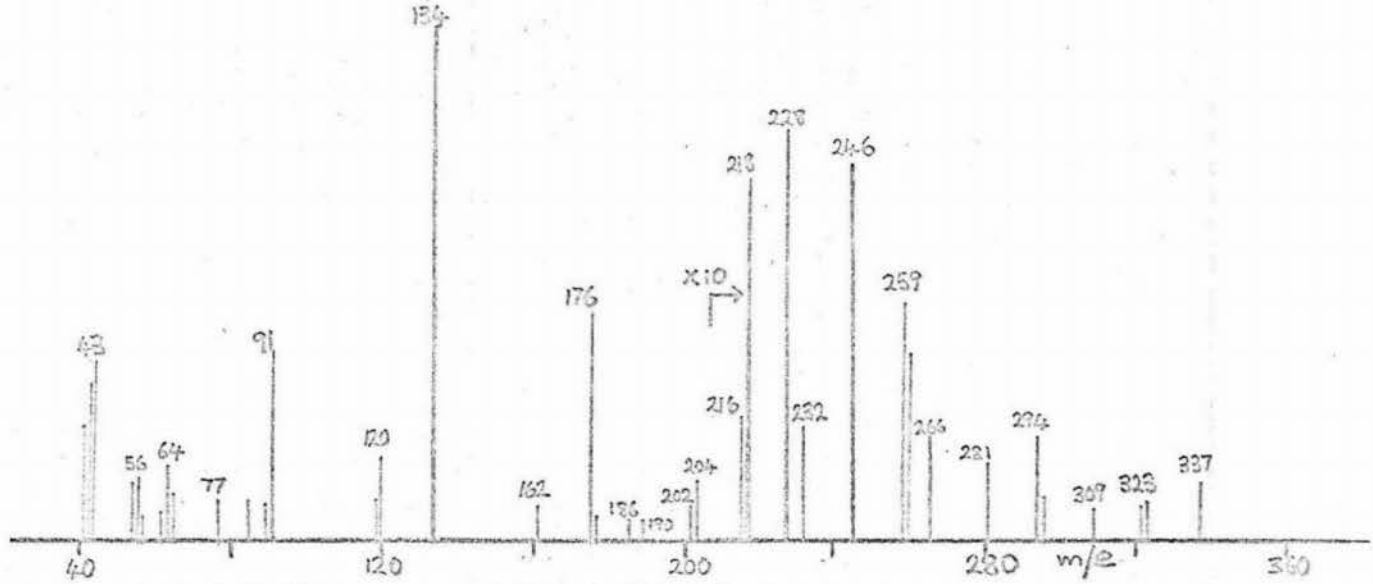


Fig. 21 Mass spectrum of permethylated Ac-Phe-OMe, 3 minutes with dimsyl sodium and 4 minutes with methyl iodide (Exp. (30)).

Metastable Transitions:

m/e323 ⟶ m/e260
m/e309 ⟶ m/e246
m/e294 ⟶ m/e246
m/e281 ⟶ m/e266 (strong)

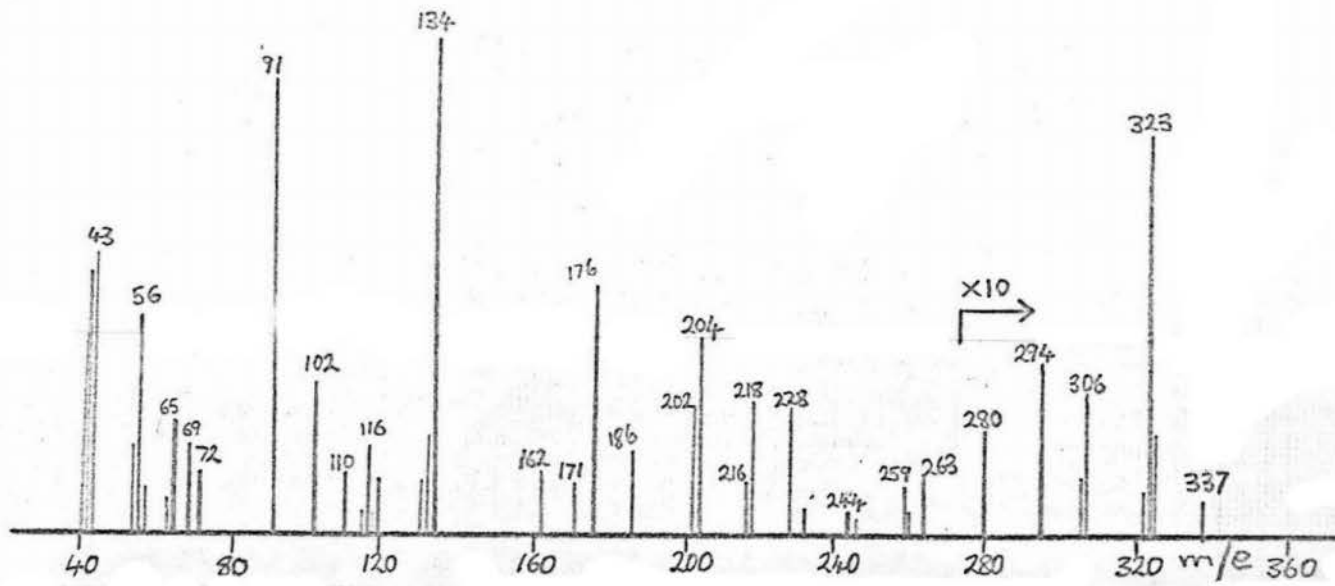


Fig. 22 Mass spectrum of permethylated Ac-Phe-OMe, 5 minutes with dimsyl sodium generated "in situ", 60 minutes with methyl iodide (Exp. (31)).

High Resolution Measurements:

m/e263 = $C_{14}H_{19}N_2O_3$
m/e260 = $C_{16}H_{22}NO_2$
m/e259 = $C_{16}H_{21}NO_2$
m/e244 = $C_{15}H_{18}NO_2$
m/e228 = $C_{15}H_{18}NO$
m/e218 = $C_{13}H_{16}NO_2$
m/e216 = $C_{14}H_{18}NO$
m/e176 = $C_{11}H_{14}NO$

was dissolved in 0.1N aqueous NaOH, washed with chloroform, acidified and extracted into ethyl acetate. The ethyl acetate solution was washed with dilute acid and water, dried and then evaporated. The white solid product was dried under vacuum at 50°C. Analytical TLC in four solvents revealed only one compound, chromatographing in the position expected for acetylphenylalanine.

NaH/oil dispersion (21 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml), as previously described. Acetylphenylalanine (3.2 mg) was added under nitrogen. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was added by syringe to the stirred solution. After 1 hour stirring at room temperature, water (1 ml) was injected to stop the reaction and the product was worked up and applied to the direct insertion probe for mass spectrometry.

A strong spectrum was obtained, "probe-out" at room temperature (Fig. 23). This was interpreted as due to a mixture of two homologous compounds with molecular ions at m/e 235, 249. The ratio of intensities was 3:1. On heating the ion source, probe in, only two weak ions with mass higher than m/e 249 were observed, at m/e 323, 263. Metastable detection indicated that these did not arise from the same compound. An ion at m/e 323 had been observed in the spectra of permethylated acetylphenylalanine methyl ester.

PERMETHYLATION OF ACYL-PEPTIDE ESTERS.

Permethylation of Ac-Phe-Asp-Ala-Ser-Val-OMe:
OMe

Exp. (33) Phe-Asp-Ala-Ser-Val (M.R.) was esterified with methanol + acetyl chloride and acetylated with acetic acid + acetic anhydride and a portion of the product was examined by mass spectrometry, as previously described (see Exp. (14)). This showed the expected spectrum of Ac-Phe-Asp-Ala-Ser-Val-OMe
OMe OAc
with molecular ion at m/e 649.

This product (about 2 mg) was dried under vacuum ($\leq$ 0.4 mm Hg, liquid-air trapped pump, 30 minutes), "Suba" sealed under nitrogen, and dissolved in DMSO.

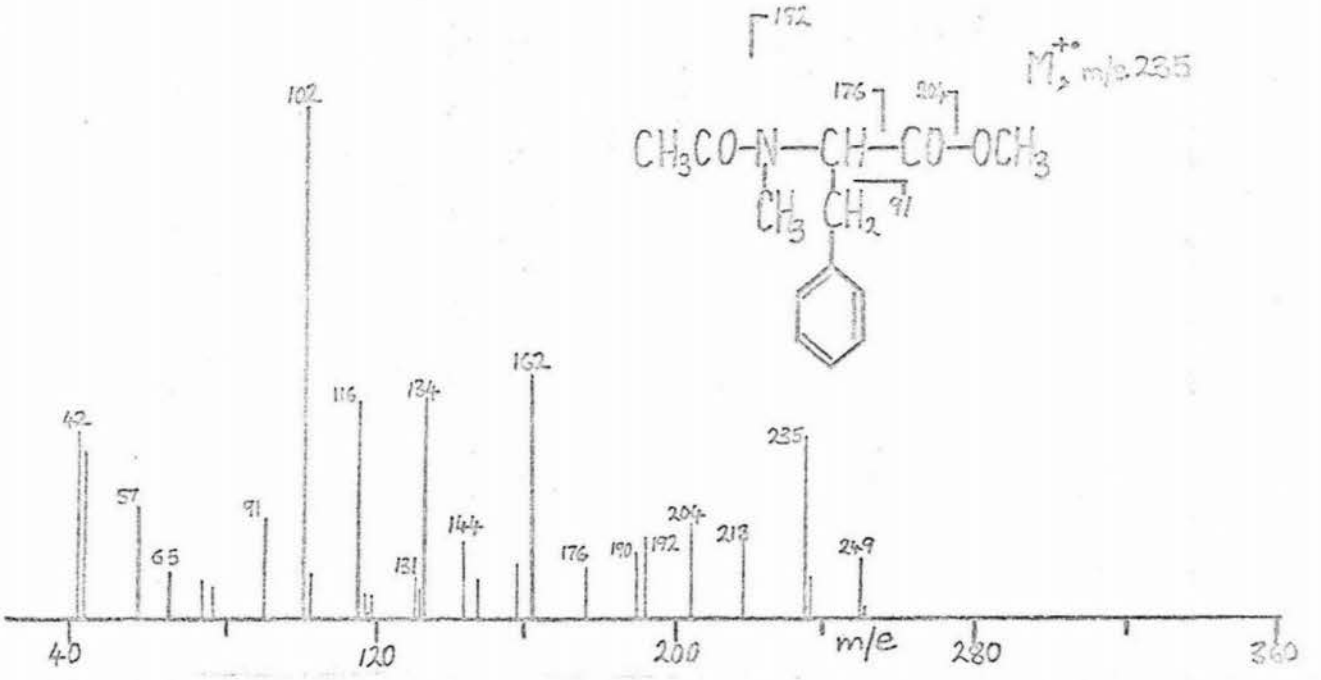


Fig. 23 Mass spectrum of permethylated Ac-Phe-OH, 4 minutes with dimsyl sodium generated "in situ", 60 minutes with methyl iodide (Exp. (32)).

On further heating of the sample, ions were observed at:

m/e323 = $C_{20}H_{21}NO_3$ and m/e263 = $C_{14}H_{19}N_2O_3$

Metastable transitions:

m/e305 --> m/e263;

There was no metastable corresponding to m/e323 giving rise to m/e263.

NaH/oil dispersion (21 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, heating for 66 minutes at 70° to 50°C. After cooling to room temperature, the dried diacetyl-peptide diester was added in DMSO (0.4 ml) by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was added dropwise to the vigorously stirred solution. After a further 20 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform and applied to the direct insertion probe for mass spectrometry.

As the temperature of the ion source was increased, there was some evidence of spectra at about 135°C and 185°C. At 220° - 230°C an intense spectrum was obtained with no obvious molecular ion (Fig. 24). This spectrum was very different from that of the permethylated acetyl-peptide (see Exp.(47), Fig. 41) although the normal sequencing peaks are present in both spectra up to and including the seryl residue. Obvious differences in this spectrum are the intense ions at m/e 428 and m/e 543 and the absence of the theoretical molecular ion(s).

Exp. (34) Phe-Asp-Ala-Ser-Val (M.R.)(2.7 mg) was suspended in methanol (3.0 ml) and acetyl chloride (40 µl) added. The peptide dissolved at once. After standing at room temperature for 18 hours, the solution was evaporated at room temperature. Acetic acid (1.25 ml), water (0.50 ml) and acetic anhydride (1.25 ml) were added, the solution stirred at room temperature for 2 hours, evaporated at room temperature, dissolved in water and re-evaporated. The product was dried under vacuum, "Suba" sealed under nitrogen and dissolved in DMSO (0.40 ml).

NaH/oil dispersion (20 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, heating at 74°-58°C for 1 hour. After cooling to room temperature, the dried acetyl peptide ester in DMSO was added by syringe. After 10 minutes, chilled I_2-free CD_3I (0.25 ml) was added dropwise to the vigorously stirred solution. After a further 12 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted

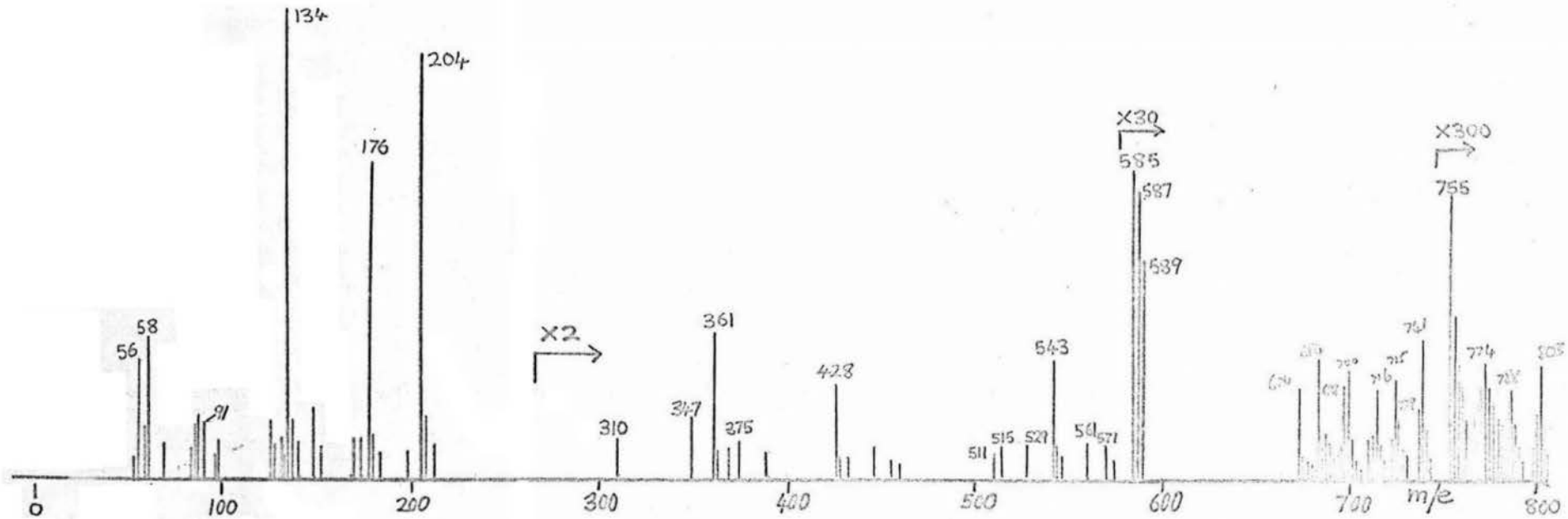


Fig. 24 Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val-OMe (Exp. (33)). The spectrum shows evidence of cyclisation at both ester groups. OMe OAc

High Resolution Data:

m/e347 =	$C_{18}H_{23}N_2O_5$	normal sequence ion
m/e361 =	$C_{19}H_{25}N_2O_5$	normal sequence ion
m/e428 =	$C_{23}H_{30}N_3O_5$	cyclised at aspartyl
m/e446 =	$C_{23}H_{32}N_3O_6$	normal sequence ion
m/e511 =	$C_{27}H_{35}N_4O_6$	cyclised at aspartyl
m/e543 =	$C_{28}H_{39}N_4O_7$	cyclised at aspartyl
m/e547 =	$C_{27}H_{39}N_4O_8$	normal sequence ion
m/e561 =	$C_{28}H_{41}N_4O_8$	normal sequence ion
m/e571 =	$C_{30}H_{43}N_4O_7$	cyclised at aspartyl
m/e725 =	$C_{39}H_{59}N_5O_8$	cyclised at both esters
m/e739=	$C_{40}H_{61}N_5O_8$	cyclised at both esters

Metastable Transitions:

m/e543 ⟶ m/e428
m/e543 ⟶ m/e511
m/e739 ⟶ m/e571

into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

As the temperature of the sample was raised, there was no evidence of "early" spectra. At 220°C a strong spectrum was obtained, which did not show the expected molecular ion, though there were several intense ions in this region (Fig. 25). A notable feature is the absence of any evidence of a methyl ester at the aspartyl residue, which should give a fragment ion at m/e 353. Although the expected sequencing peaks up to and including the seryl residue are present, there are many other stronger ions which cannot be readily explained.

Exp. (35) To Phe-Asp-Ala-Ser-Val (M.R.)(2.0 mg) was added water (2.5 ml) and acetic anhydride (2.5 ml). The heterogeneous mixture was stirred at room temperature for 2 hours, and the resulting clear solution was evaporated at room temperature, the residue redissolved in water and re-evaporated.

The product was dissolved in methanol (2.0 ml) and chilled at -20°C. Ethereal diazomethane (6 ml, .002 mole) generated from N-methyl-N-nitrosotoluene-p-sulphonamide as previously described, was added and the yellow solution left at room temperature for 4 hours, then evaporated, dried under vacuum ($\leq$ 0.5 mmHg, liquid-air trapped pump, 20 minutes) "Suba" sealed under nitrogen and dissolved in DMSO (0.45 ml).

NaH/oil dispersion (25 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, stirring at 67° to 60°C for 1 hour. After cooling to room temperature, the acetyl-peptide diester in DMSO was added by syringe. After 5 minutes, chilled I_2-free CH_3I(0.30 ml) was added rapidly to the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

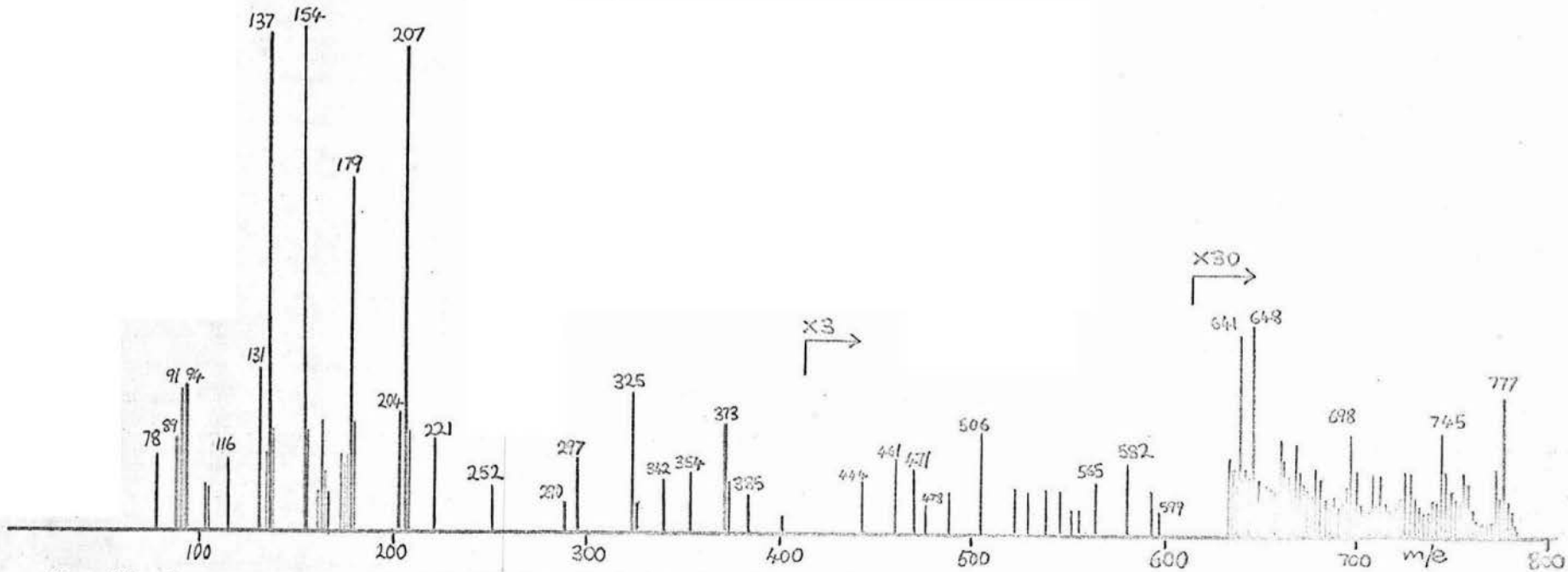


Fig. 25 Mass spectrum of perdeuteriomethylated Ac-Phe-Asp-Ala-Ser-Val-OMe (Exp. (34)).
OMe

Fig. 26 Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val-OMe (Exp. (35)).
OMe

See Fig. 2(b) between pages 35 and 36 .

Although the temperature of the ion source was raised slowly, there was no evidence of "early" spectra. At 200° to 230°C, a strong spectrum was obtained, which appeared to be a complex mixture of compounds (Fig. 26). The molecular ion region contained a series of peaks, that at m/e 741 having the highest mass.

Permethylation by addition of dimsyl sodium in DMSO to a mixture of CH_3I and Ac-Phe-Asp-Ala-Ser-Val-CMe:
OMe

Exp. (36) To Phe-Asp-Ala-Ser-Val (M.R.)(2.0 mg) was added methanol (2.0 ml) and acetyl chloride (40 µl). The peptide dissolved at once. After 17 hours at room temperature, the solution was evaporated. Acetic acid (1.25 ml), water (0.5 ml) and acetic anhydride (1.25 ml) were added to the residue. After stirring for 2 hours at room temperature, the solution was evaporated and dried under vacuum ($\leqslant$ 0.3 mmHg, liquid-air trap, 30 minutes), "Suba" sealed under nitrogen and dissolved in DMSO (0.40 ml).

NaH/oil dispersion (23 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, heating for 68 minutes at 72° to 65°C. The solution was cooled to room temperature and injected into a vigorously stirred solution of I_2-free CH_3I (0.30 ml) and the acetyl-peptide diester in DMSO (0.30 ml). After a further 10 minutes stirring at room temperature, water (1 ml) was injected, the product extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

As the temperature of the ion source was raised, two distinct, very strong spectra were observed. These made up the bulk of the sample. At about 220°C a third strong spectrum was obtained (Fig. 27). This was relatively clean, though due to a mixture of several compounds. Eight molecular ions were observed, at m/e 687, 691, 701, 705, 715, 729, 745, 759. The rest of the spectrum was very complex, although there is evidence of a reduction in the extent of extra-methylation compared with Exps. (33), (34), (35) and (47).

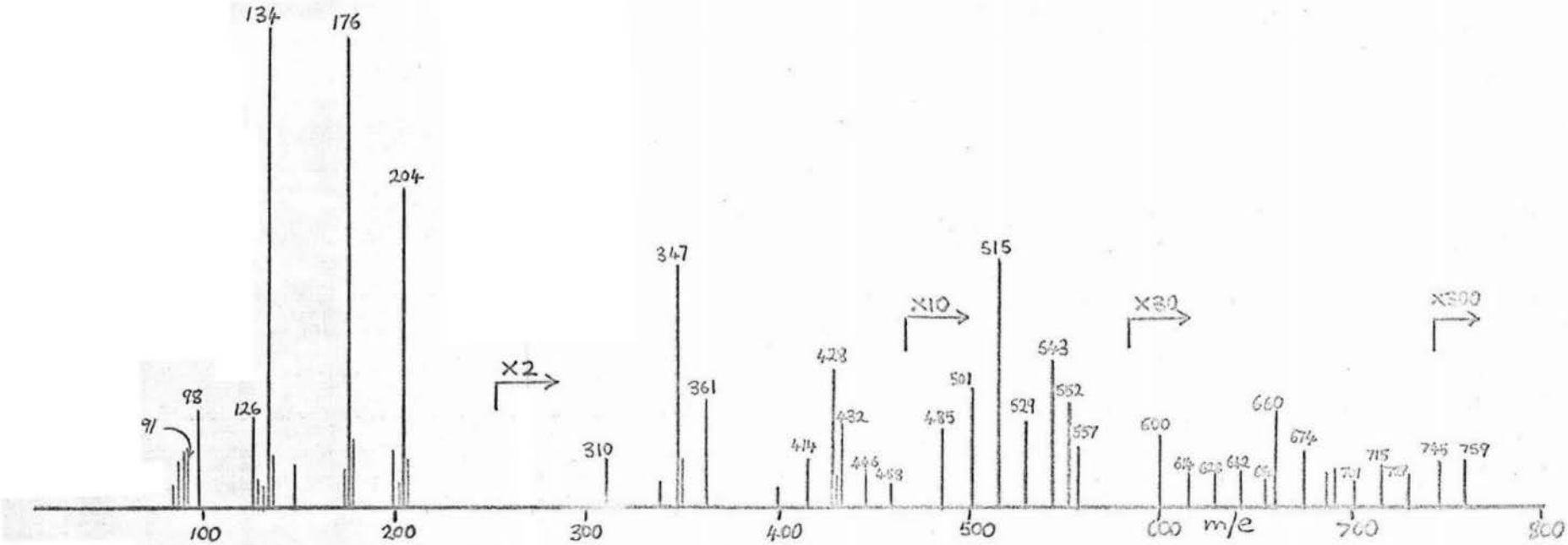


Fig. 27 Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val-OMe, adding dimsyl sodium to a mixture of methyl iodide and peptide; eight OMe molecular ions are apparent (Exp. (36)).

High Resolution Measurements:

m/e729 = $C_{37}H_{55}N_5O_{10}$ cyclised at aspartyl + 4 x CH_2, also m/e557 = $C_{29}H_{41}N_4O_7$

m/e715 = $C_{36}H_{53}N_5O_{10}$ cyclised at aspartyl + 3 x CH_2, also m/e543 = $C_{28}H_{39}N_4O_7$

m/e701 = $C_{35}H_{51}N_5O_{10}$ cyclised at aspartyl + 2 x CH_2, also m/e529 = $C_{27}H_{37}N_4O_7$

m/e705 = $C_{35}H_{55}N_5O_{10}$ expected uncyclised molecular ion + 1 x CH_2, also m/e446 = $C_{23}H_{32}N_3O_6$

m/e691 = $C_{34}H_{53}N_5O_{10}$ expected uncyclised molecular ion, also m/e432 = $C_{22}H_{30}N_3O_6$

Metastable Transitions:

m/e745 ⟶ m/e543

m/e729 ⟶ m/e557

m/e715 ⟶ m/e543

m/e701 ⟶ m/e529

m/e705 ⟶ m/e446

m/e691 ⟶ m/e432 and m/e347 (expected sequencing ions)

m/e687 ⟶ m/e428

Permethylation of Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OMe:

Exp. (37) Cbz-Gly-Pro-Gly-Gly-Pro-Ala (M.R.)(2.0 mg) was dissolved in methanol (2 ml) and chilled at -20°C. Ethereal diazomethane (6 ml, .002 mole), generated from N-methyl-N-nitrosotoluene-p-sulphonamide as previously described, was added and the yellow solution left at room temperature for 4 hours, then evaporated, dried under vacuum ($\leqslant$ 0.5mm Hg, liquid-air trap, 30 minutes), "Suba" sealed under nitrogen and dissolved in DMSO (0.45 ml).

NaH/oil dispersion (21 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.25 ml) as previously described, stirring at 69° to 62°C for 1 hour. After cooling to room temperature, the Cbz-peptide ester in DMSO was added by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated at room temperature and applied to the direct insertion probe for mass spectrometry.

The spectrum obtained at 210°C contained the expected sequencing ions up to and including the prolyl residue in position 5 (Fig. 28). The expected molecular ions at m/e 686 and m/e 700 were also present, but strong ions at m/e 710, 696, 679, 644 and 613 were present that did not occur in the spectrum of the permethylated Cbz-peptide (cf. experiment (43); Fig 36).

Permethylation of Ac-Ala-Gly-Gly-OH:

Exp. (38) To DL-Ala-Gly-Gly (Sigma)(5.0 mg) was added water (2.5 ml) and acetic anhydride (2.5 ml). The heterogeneous mixture was stirred at room temperature for 3½ hours, the clear solution evaporated at room temperature and the product dried under vacuum ($\leqslant$ 0.3mm Hg, liquid-air trap, 20 minutes) and a portion applied to the direct insertion probe for mass spectrometry. The spectrum obtained at a sample temperature of 170°C showed only the expected product with molecular ion at m/e 245; there was a strong M + 1 peak (Fig. 29). The remainder of the product (4 mg) was "Suba" sealed under nitrogen and dissolved in DMSO (0.4 ml).

Fig. 28 Mass spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OMe (Exp. (37)).
See Fig. 1(b) between pages 35 and 36 .

High Resolution Data:

m/e710 =	$C_{37}H_{54}N_6O_8$	molecular ion
m/e700 =	$C_{35}H_{52}N_6O_9$	expected molecular ion
m/e686 =	$C_{34}H_{50}N_6O_9$	expected molecular ion
m/e679 =	$C_{36}H_{51}N_6O_7$	m/e710 − OMe
m/e644 =	$C_{33}H_{52}N_6O_7$	molecular ion
m/e613 =	$C_{32}H_{49}N_6O_6$	m/e644 − OMe

Metastable Transitions:

m/e710 ⟶ m/e679
m/e710 ⟶ m/e570
m/e710 ⟶ m/e473
m/e686 ⟶ m/e570
m/e686 ⟶ m/e473

Metastables for m/e686, 644 were not detected.

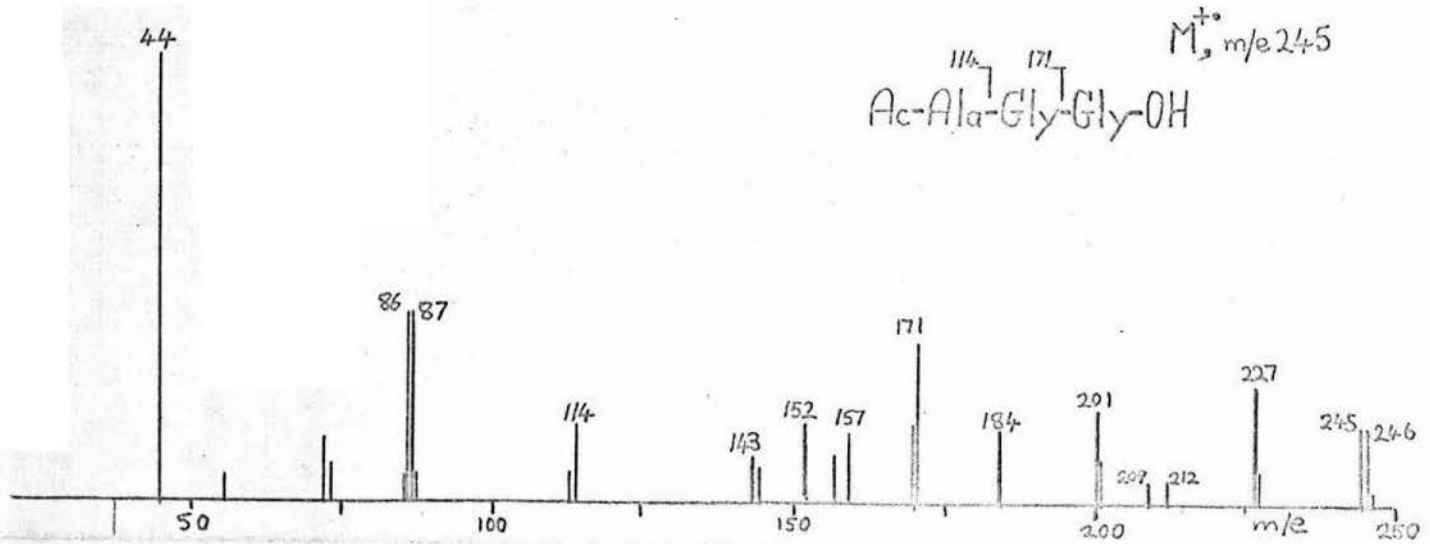


Fig. 29 Mass spectrum of Ac-Ala-Gly-Gly-OH (Exp. (38)).

NaH/oil dispersion (26 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.25 ml) as previously described, stirring at 60°C for 65 minutes. After cooling to room temperature, the dried acetyl peptide in DMSO (0.45 ml) was added by syringe. After 5 minutes at room temperature, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and a portion applied to the direct insertion probe for mass spectrometry.

The spectrum obtained had the molecular ion at m/e 329, 28 m.u. higher than the theoretical product (Fig. 30). Examination of the spectrum showed that extra-methylation had occurred only in the C-terminal glycine residue. Several unexpected ions occurred in the spectrum, at m/e 311, 185, 184, 170 and 169.

Permethylation of Ac-Ala-Gly-Gly-OMe:

Exp. (39) To DL-Ala-Gly-Gly (Sigma)(5.1 mg) was added water (2.5 ml) and acetic anhydride (2.5 ml). The heterogeneous mixture was stirred at room temperature for 3 hours, the clear solution evaporated at room temperature and the product re-dissolved in water and re-evaporated. The residue, Ac-Ala-Gly-Gly, was dissolved in methanol (2 ml) and chilled at -20°C. Ethereal diazomethane (6 ml, .002 mole) generated from N-methyl-N-nitrosotoluene-p-sulphonamide as previously described, was added and the yellow solution left at room temperature for 3 hours, then evaporated, dried under vacuum ($\leqslant$ 0.5 mm Hg, liquid-air trapped pump 20 minutes) and a portion of the product applied to the direct insertion probe for mass spectrometry. The spectrum obtained at a sample temperature of 160°C showed the expected molecular ion at m/e 259 accompanied by a small amount (estimated $\leqslant$ 2%) of the homologous compound with molecular ion at m/e 273. A similarly weak ion at m/e 303 could not be accounted for (Fig. 31). The remainder of the product (4 mg) was "Suba" sealed under nitrogen and dissolved in DMSO (0.45 ml).

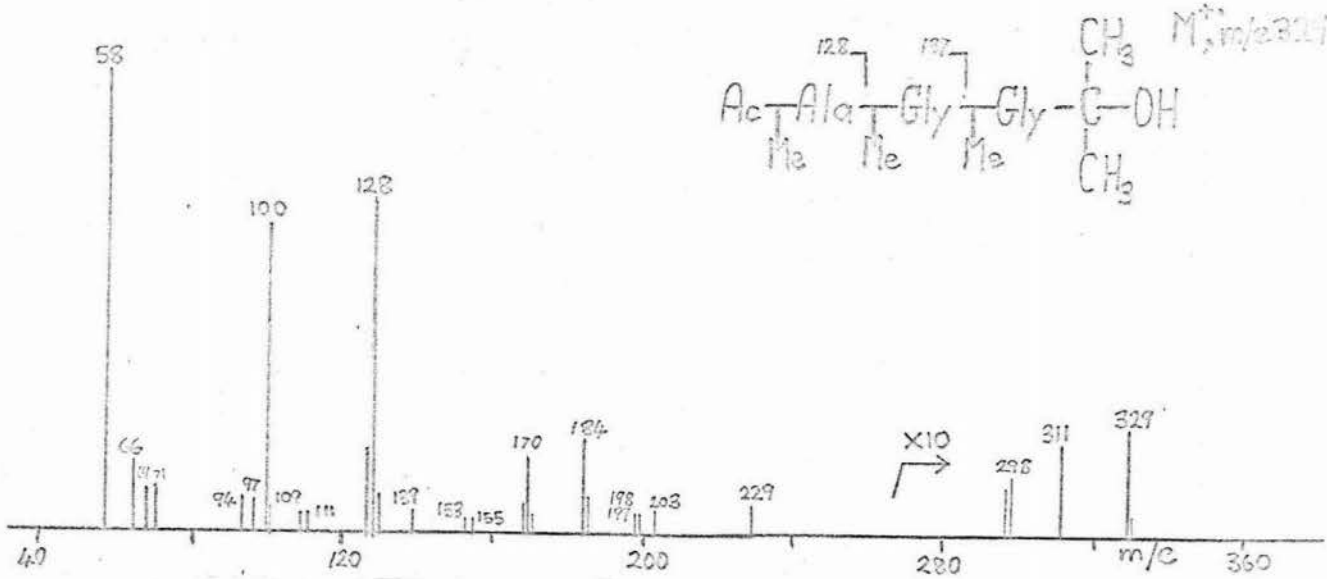


Fig. 30 Mass spectrum of permethylated Ac-Ala-Gly-Gly-OH (Exp. (38)).

High Resolution Data:

m/e329 = $C_{15}H_{27}N_3O_5$ m/e311 = $C_{15}H_{25}N_3O_4$

m/e229 = $C_{10}H_{17}N_2O_4$

Metastable Transitions:

m/e329 ⟶ m/e311 ⟶ m/e184 ⟶ m/e169
m/e329 ⟶ m/e298 ⟶ m/e170
m/e329 ⟶ m/e199; m/e329 ⟶ m/e203
m/e329 ⟶ m/e229; m/e329 ⟶ m/e198

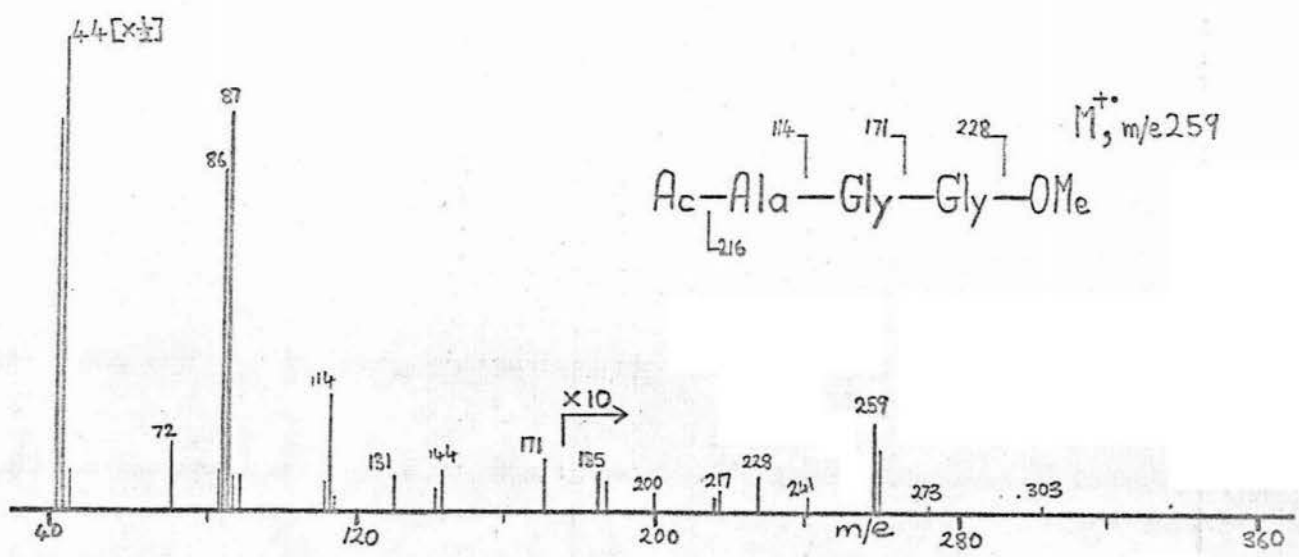


Fig. 31 Mass spectrum of Ac-Ala-Gly-Gly-OMe (Exp. (39)).

High Resolution Data: m/e303 = $C_{12}H_{21}N_3O_6$

NaH/oil dispersion (25 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.25 ml) as previously described, stirring at 60°C for 1 hour. After cooling to room temperature, the acetyl-peptide ester in DMSO was added by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and a portion applied to the direct insertion probe for mass spectrometry. The spectrum obtained at 140°C was the same as that from permethylated Ac-Ala-Gly-Gly, with the molecular ion at m/e 329. On further heating of the ion source the ions at m/e 311, 185, 184, 171, 170 vanished. At the same time, new ions appeared at m/e 339, 308, 284, 213. Although this spectrum (see Fig. 32) was in many respects similar to that obtained from permethylated Ac-Ala-Gly-Gly, metastable detection showed that the ions had different origins.

Hydrolysis and Analysis of Permethylated Ac-Ala-Gly-Gly-OH and Ac-Ala-Gly-Gly-OMe:

Exp. (40) After mass spectrometry, permethylated Ac-Ala-Gly-Gly (about 4 mg) and Ac-Ala-Gly-Gly-OMe (about 4 mg) were each hydrolysed in HCl (20.24%; 25 hours at 125°C), filtered, evaporated under vacuum at 50°C, redissolved in water and re-evaporated several times. The residues were each dissolved in water (100 µl) and 4 µl aliquots taken for analysis.

Analytical TLC on cellulose layers according to Haworth and Heathcote (ref. 121) was performed. One-dimensional TLC of the hydrolysates (400 µ cellulose MN300; solvent 2-propanol/butanone/1N HCl 65:15:25 v/v; sprayed ninhydrin/$Cd(OAc)_2$ and heated at 120°C) (plate 25) showed the presence of free alanine and glycine as well as N-methylalanine and N-methylglycine, in both cases. Two-dimensional TLC (solvents: according to Haworth and Heathcote; sprayed ninhydrin/$Cd(OAc)_2$ and heated at 120°C)(plates 26 and 27) was performed, with the plates developed concurrently. The pattern of products was identical in both hydrolysates, although that from permethylated Ac-Ala-Gly-Gly-OMe had higher

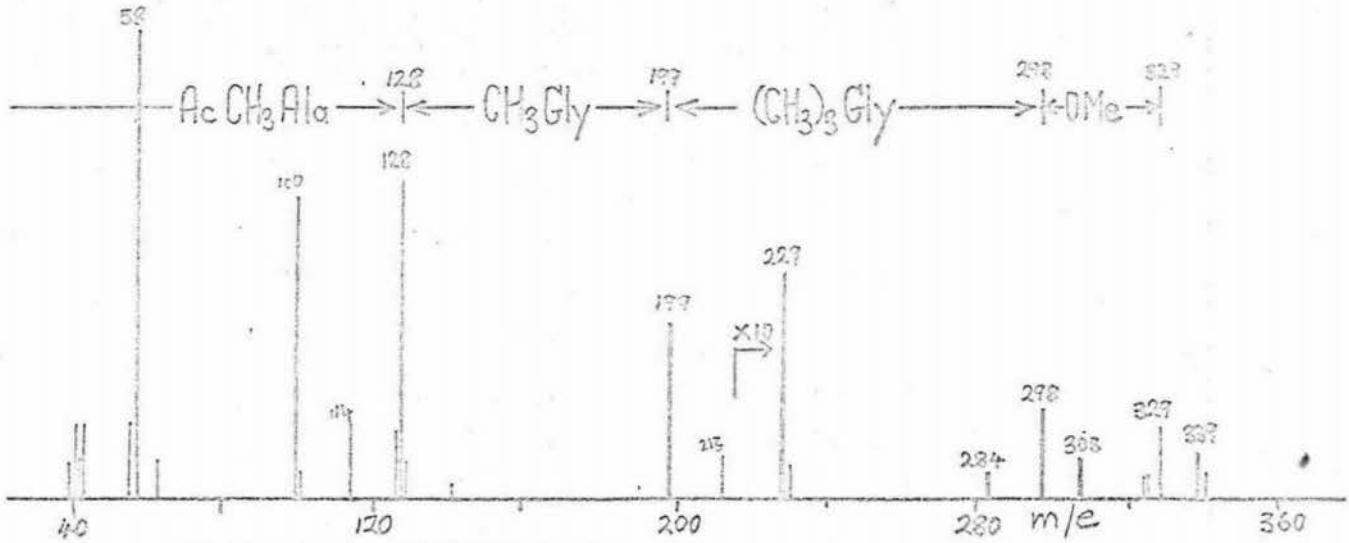


Fig. 32 Mass spectrum of permethylated Ac-Ala-Gly-Gly-OMe (Exp. (39)).

High Resolution Data:

m/e339 = $C_{17}H_{29}N_3O_4$

m/e308 = $C_{16}H_{26}N_3O_3$

m/e298 = $C_{14}H_{24}N_3O_4$

m/e213 = $C_{10}H_{17}N_2O_3$

m/e199 = $C_9H_{15}N_2O_3$

Metastable Transitions:

m/e339 ⟶ m/e308

m/e339 ⟶ m/e213 ⟶ m/e128 ⟶ m/e100

m/e339 ⟶ m/e199 ⟶ m/e128 ⟶ m/e100

m/e339 ⟶ m/e199 ⟶ m/e114 ⟶ m/e100

m/e329 ⟶ m/e229 ⟶ m/e128 ⟶ m/e100

No metastables were detected for m/e339 or m/e329

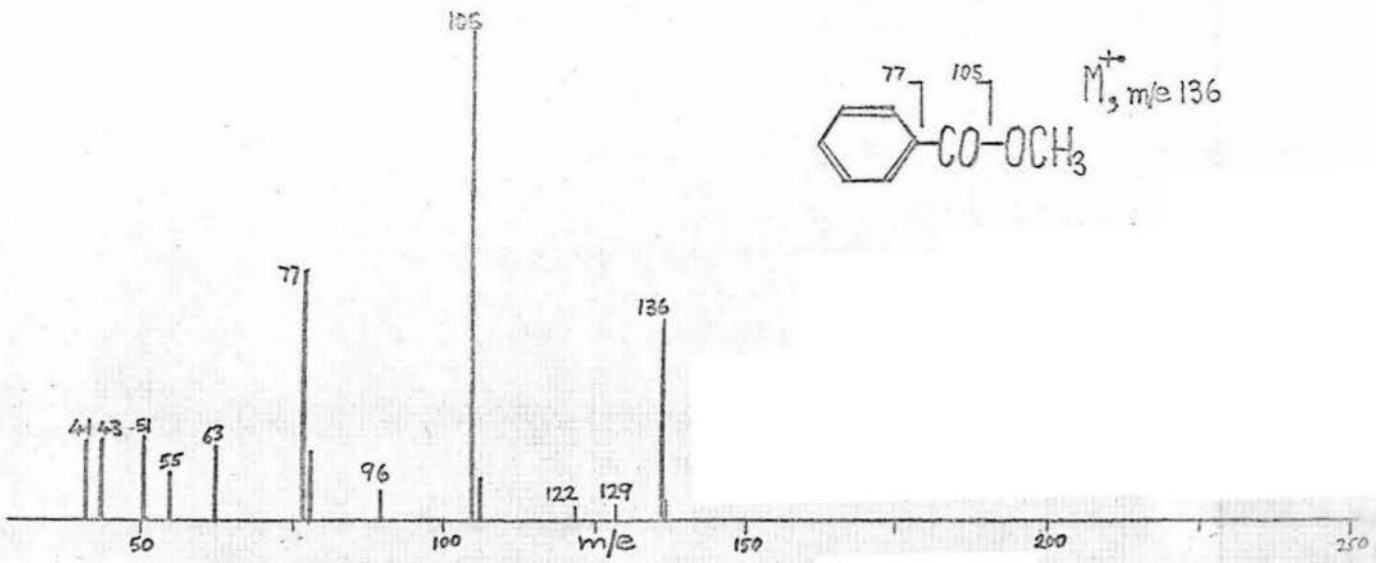


Fig. 33 Mass spectrum of permethylated benzoic acid (Exp. (41)).

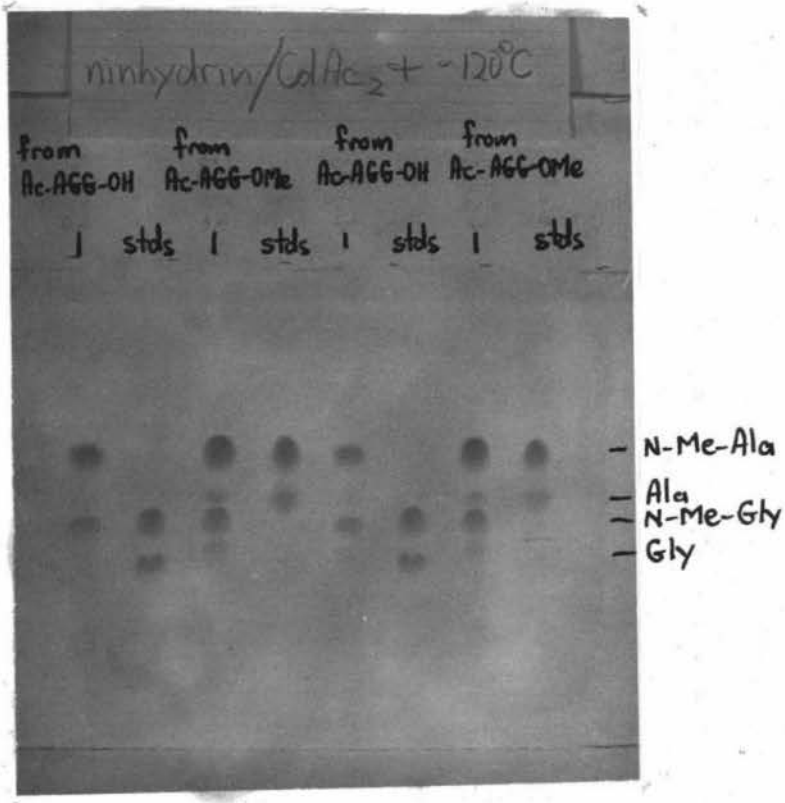


Plate 25. Hydrolysed permethylated Ac-Ala-Gly-Gly-OMe and Ac-Ala-Gly-Gly-OH. Solvent 2-propanol/butanone/HCl (Exp. (40)).

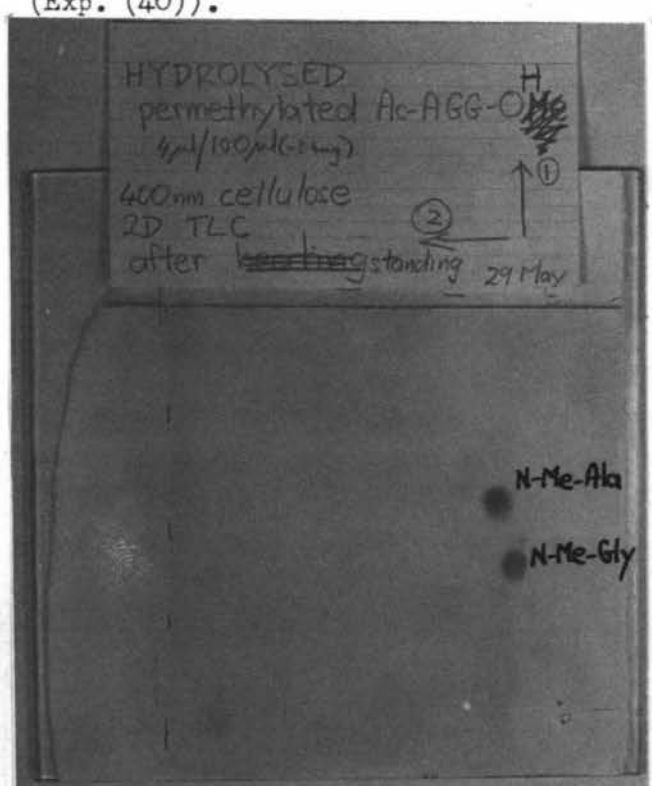


Plate 26. Hydrolysed permethylated Ac-Ala-Gly-Gly-OH (Exp. (40)).

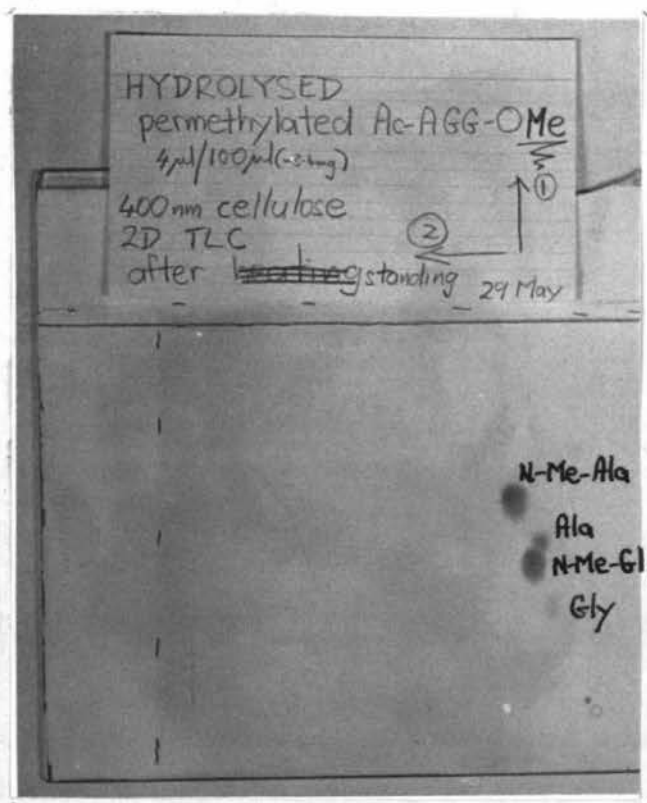


Plate 27. Hydrolysed permethylated Ac-Ala-Gly-Gly-OMe (Exp. (40)).

levels of free alanine and glycine. There was a significant amount of ninhydrin-positive compound with Rfs similar to Taurine in the hydrolysate from Ac-Ala-Gly-Gly-OH.

Permethylation of Benzoic Acid:

Exp. (41) Benzoic acid (BDH)(4.8 mg) was dried under vacuum ($\leq$ 0.4 mm Hg, liquid-air trap, 20 minutes), "Suba" sealed under nitrogen and dissolved in DMSO (0.45 ml).

NaH/oil dispersion (28 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.25 ml), stirring at 64°C for 1 hour. After cooling to room temperature, the benzoic acid in DMSO was added by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and applied to the direct probe for mass spectrometry.

A spectrum was obtained "probe-out" (Fig. 33) showing the expected molecular ion at m/e 136. No other compound was detected on inserting the probe and heating the sample to 160°C.

Permethylation of Methyl Benzoate:

Exp. (42) Methyl benzoate (3.7 mg) was dried over concentrated sulphuric acid then dissolved in DMSO (0.45 ml) under nitrogen.

NaH/oil dispersion (25 mg, 50% NaH)was used to generate dimsyl sodium in DMSO (0.25 ml) as previously described, stirring at 72° to 65°C for 1 hour. After cooling to room temperature, the methyl benzoate in DMSO was added by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated at room temperature and examined by mass spectrometry.

Using the direct insertion probe, a series of spectra were obtained

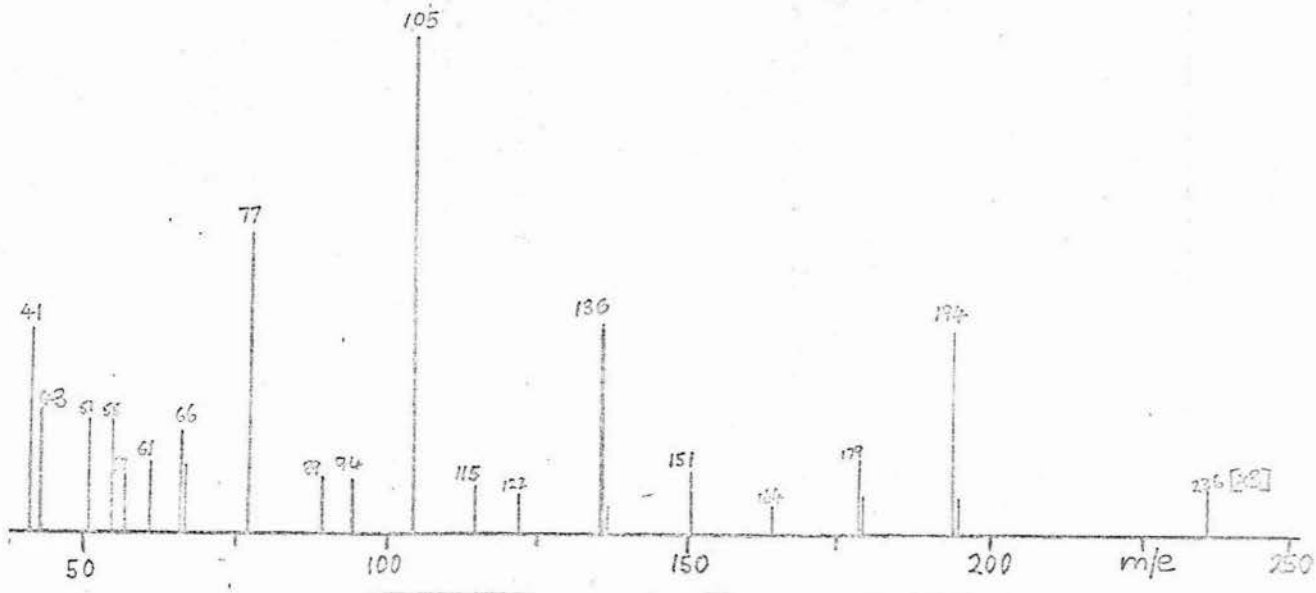


Fig. 34 Permethylated methyl benzoate, "probe out" (Exp. (42)).

High Resolution Data:

m/e236 = $C_{18}H_{20}$

m/e194 = $C_{11}H_{14}OS$

m/e179 = $C_{10}H_{11}OS$

m/e164 = C_9H_8OS

m/e151 = $C_9H_{11}S$

Metastable Transitions:

m/e194 ⟶ m/e179 ⟶ m/e151

No metastables were detected for m/e 194.

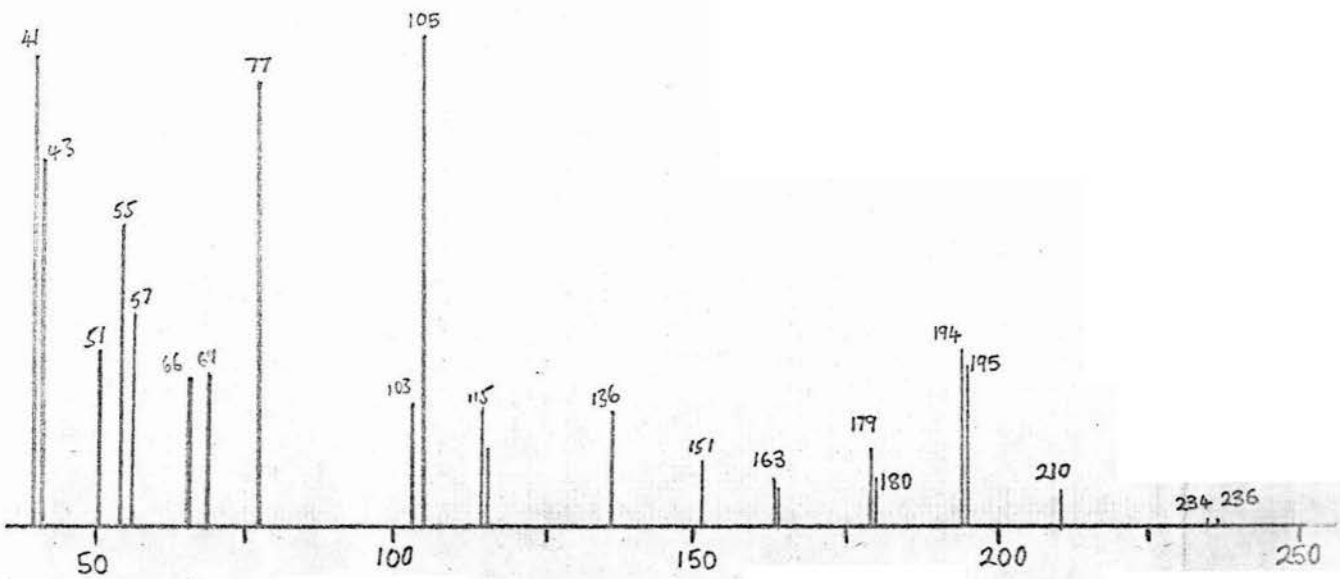
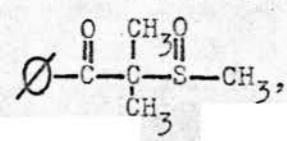


Fig. 35 Permethylated methyl benzoate, at 130°C (Exp. (42)).

High Resolution Data:

m/e210 = $C_{11}H_{14}O_2S$ - probably



m/e195 = $C_{10}H_{11}O_2S$

m/e115 = C_9H_7

(see Figs. 34, and 35). High resolution measurements and metastable detection were performed using the direct insertion probe and the gallium-inlet system. As well as the spectrum of methyl benzoate, spectra of several other compounds were evident. These included a dimethylated β-keto-sulfoxide, compounds with a sulphide group replacing the ester and a compound with only a single oxygen and no sulphur. It was not possible to estimate the ratios of these products as their volatilities differed.

EXTRA-METHYLATION.

Permethylation of Cbz-Gly-Pro-Gly-Gly-Pro-Ala:

Exp. (43) NaH/oil dispersion (20 mg, 50% NaH) was pre-rinsed three times with dry petroleum ether, dried under vacuum and "Suba" sealed under nitrogen. Dry DMSO (0.20 ml) was added by syringe, the mixture stirred at 65°C for 90 minutes, then cooled to room temperature. Cbz-Gly-Pro-Gly-Gly-Pro-Ala (M.R.) (1.6 mg) was added. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was injected dropwise into the vigorously stirred solution. After a further 60 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction and the product extracted into chloroform, washed, evaporated and the residue applied to the direct insertion probe for mass spectrometry.

A spectrum was obtained at 200°C showing a series of homologous peaks, with molecular ions at m/e 672, 686, 700 and 714 (see Fig. 36). The principal fragment ions due to rupture of the peptide bond also showed homologous peaks 14 m.u. apart. There was apparently extensive non-specific extra-methylation.

Perdeuteriomethylation of Cbz-Gly-Pro-Gly-Gly-Pro-Ala:

Exp. (44) NaH/oil dispersion (20 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described. Stirring was maintained for 80 minutes at 68° to 57°C. After cooling to room temperature, Cbz-Gly-Pro-Gly-Gly-Pro-Ala (M.R.)(1 mg) was added. After 5 minutes, chilled CD_3I (0.30 ml) was injected dropwise into the vigorously stirred solution. After a further 63 minutes stirring at room temperature, water (1 ml) was injected to stop the

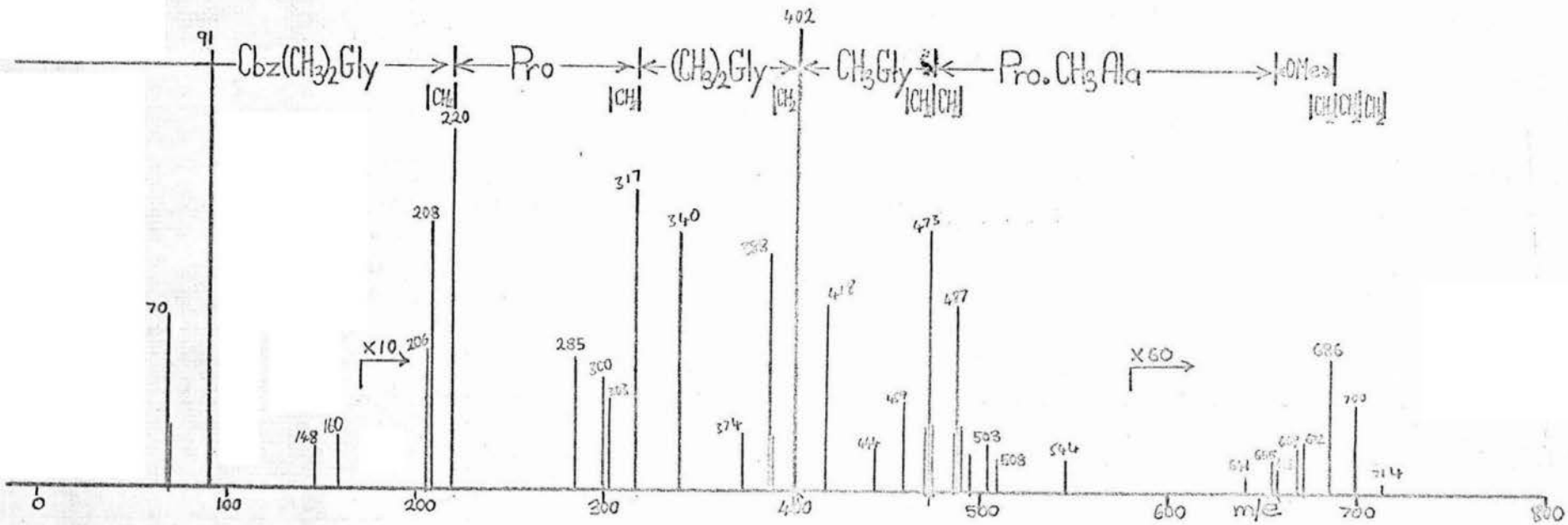


Fig. 36 Mass Spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala (Exp. (43)).

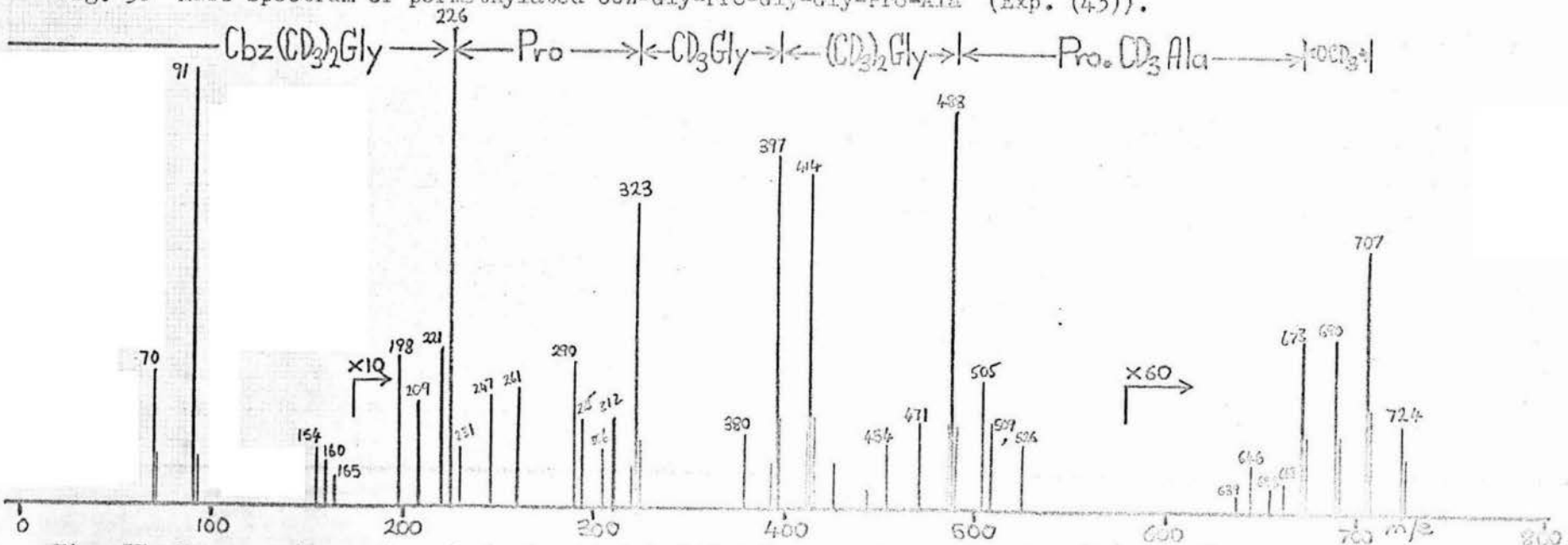


Fig. 37 Mass spectrum of perdeuteriomethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala (Exp. (44)).

Fig. 36 Mass spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala (see previous page).

High Resolution Data:

m/e220 = $C_{12}H_{14}NO_3$

m/e317 = $C_{17}H_{21}N_2O_4$

m/e402 = $C_{21}H_{28}N_3O_5$

m/e473 = $C_{24}H_{33}N_4O_6$

m/e686 = $C_{34}H_{50}N_6O_9$

m/e700 = $C_{35}H_{52}N_6O_9$

These elemental compositions fit for the sequencing peaks as marked on the mass spectrum.

Metastable Transitions:

m/e686 ⟶ m/e570 ⟶ m/e473 ⟶ m/e402 ⟶ m/e317 ⟶ m/e220

m/e700 ⟶ m/e584 ⟶ m/e487

m/e686 ⟶ m/e473; m/e686 ⟶ m/e402;

m/e473 ⟶ m/e317: m/e402 ⟶ m/e220;

reaction and the product extracted into chloroform, washed, evaporated and the residue applied to the direct insertion probe for mass spectrometry.

A spectrum was obtained at 200° to 220°C, with molecular ions at m/e 690, 707, 724 and 741 (see Fig. 37). The principal fragment ions due to rupture of the peptide bond also showed peaks 17 m.u. apart. Extra-methylation had occurred in the permethylation procedure.

Exposure to dilute acid:

Exp. (45) The permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala prepared above was dissolved in chloroform (5 ml) and shaken briefly with two small amounts (5 ml) of 1N aqueous HCl. After evaporation at room temperature, the product was applied to the direct insertion probe for mass spectrometry.

A spectrum was obtained at 200°C, identical with that of the permethylated compound that had not been exposed to acid (see Fig. 36, above).

Inverse permethylation of Cbz-Gly-Pro-Gly-Gly-Pro-Ala:

Exp. (46) NaH/oil dispersion (22 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, stirring for 58 minutes at 66°C. After cooling to room temperature, Cbz-Gly-Pro-Gly-Gly-Pro-Ala (M.R.)(2.4 mg) dissolved in DMSO (0.40 ml) was added by syringe. The solution of dimsyl/peptide in DMSO was stirred at room temperature for 20 minutes, then slowly injected (over 10 minutes) into vigorously stirred I_2-free CH_3I (0.40 ml) using a micrometer syringe. After a further 25 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction. The product was extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

A spectrum was obtained at 220°C with molecular ions at m/e 672, 686, 700 (see Fig. 38). Examination of the spectrum showed that one extra methyl group had been introduced into each of the glycine residues in positions 1 and 4. Extra-methylation at these sites was about 90% complete. Introduction of extra methyl groups at other sites was not significant.

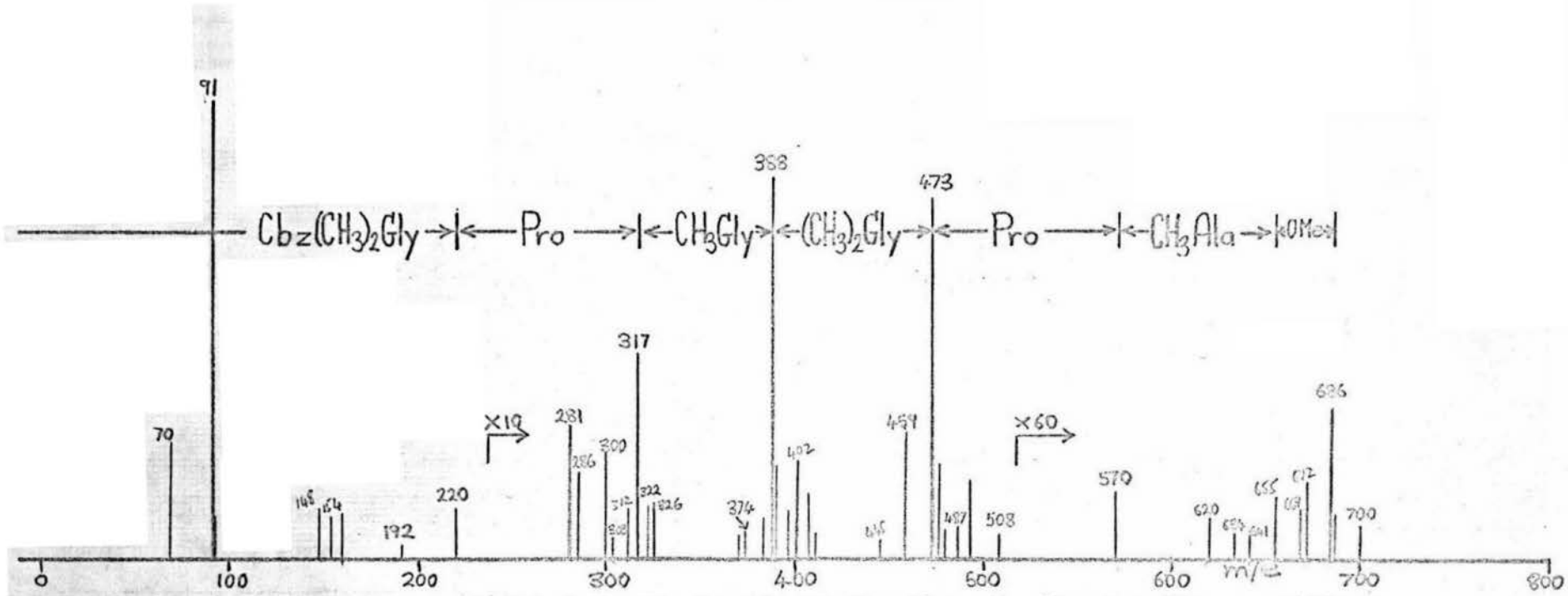


Fig. 38 Mass spectrum of permethylated Cbz-Gly-Pro-Gly-Gly-Pro-Ala; the dimsyl sodium + peptide was added to excess methyl iodide (Exp. (46)).
The spectrum shows specific extramethylation of the glycyl residues in positions 1 and 4.

High Resolution Data:

m/e192 = $C_{11}H_{14}NO_2$

Metastable Transitions:

m/e220 ⟶ m/e192 (strong)

which is consistent with extramethylation of the α- C atom of the N-terminal glycyl residue.

Permethylation of Ac-Phe-Asp-Ala-Ser-Val:

Exp. (47) To Phe-Asp-Ala-Ser-Val (M.R.)(2.4 mg) was added acetic acid (1.25 ml) and acetic anhydride (1.25 ml). After 65 minutes stirring at room temperature, the peptide had not dissolved; distilled water (0.5 ml) was added and the peptide dissolved at once. After a further 65 minutes stirring at room temperature, the solution was evaporated at room temperature, redissolved in water and re-evaporated.

NaH/oil dispersion (20 mg 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as described above, stirring for 85 minutes at 54° to 68°C. After cooling to room temperature, the dried acetylated peptide dissolved in DMSO (0.40 ml) was added by syringe. After 5 minutes, chilled I_2-free CH_3I (0.30 ml) was added dropwise to the vigorously stirred solution. After a further 20 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction and the product extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

Three distinct spectra were obtained as the sample was slowly warmed, at 90°-100°C, 150°-160°C and 180°-220°C (see Figs. 39,40 and 41). The last of these had molecular ions at m/e 691, 705 and was due to the expected product, molecular ion at m/e 691, and a homologue. The principal fragment ions due to rupture of the peptide bonds also showed an homologous peak 14 m.u. higher.

Examination of the spectrum showed than an extra methyl group had been introduced principally in the aspartyl residue to the extent of about 80%. Extra-methylation occurred only to a minor extent in the other residues.

Inverse Permethylation of Ac-Phe-Asp-Ala-Ser-Val:

Exp. (48) To Phe-Asp-Ala-Ser-Val (M.R.)(3.0 mg) was added acetic acid (1.25 ml) acetic anhydride (1.25 ml) and distilled water (0.50 ml). The peptide dissolved at once. After 55 minutes stirring at room temperature, the solution was evaporated at room temperature, redissolved in water (1 ml) and re-evaporated twice,

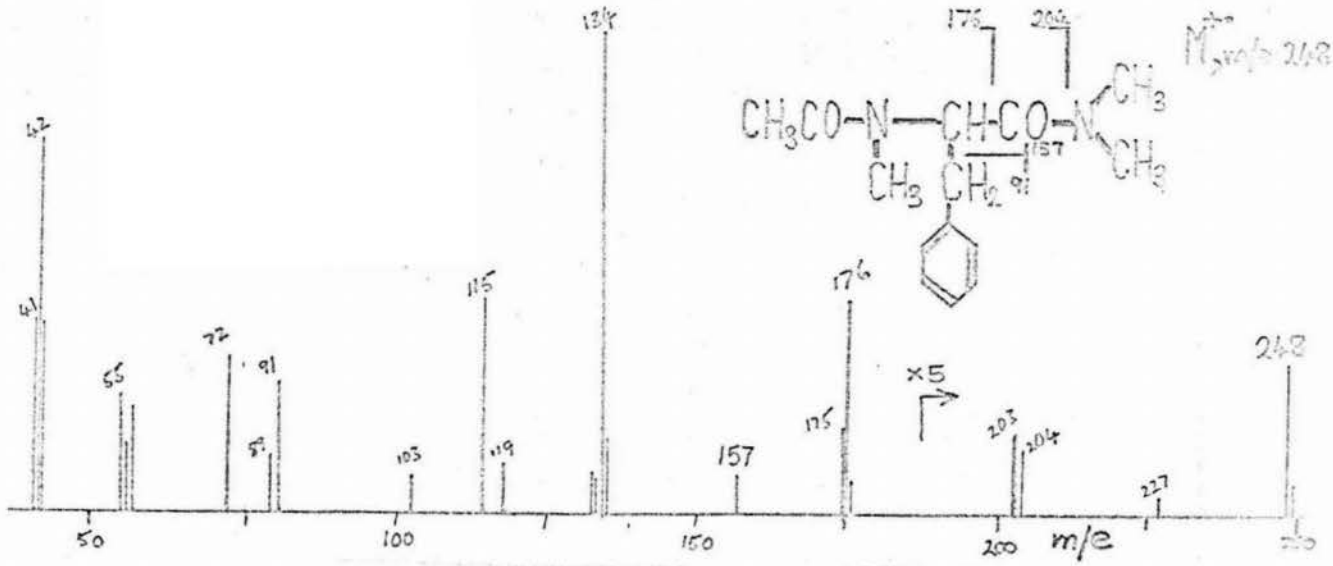


Fig. 39 Spectrum obtained from permethylated Ac-Phe-Asp-Ala-Ser-Val at a sample temperature of 95°C (Exp. (47)).

High Resolution Data:

m/e248 = $C_{14}H_{20}N_2O_2$, the molecular ion, as shown.

m/e176 = $C_{11}H_{14}NO$, M- $CO.N(CH_3)_2$ as shown.

m/e134 = $C_9H_{12}N$, m/e176-$CH_2{=}CO$.

m/e115 = $C_5H_{11}N_2O$, M-91-$CH_2{=}CO$

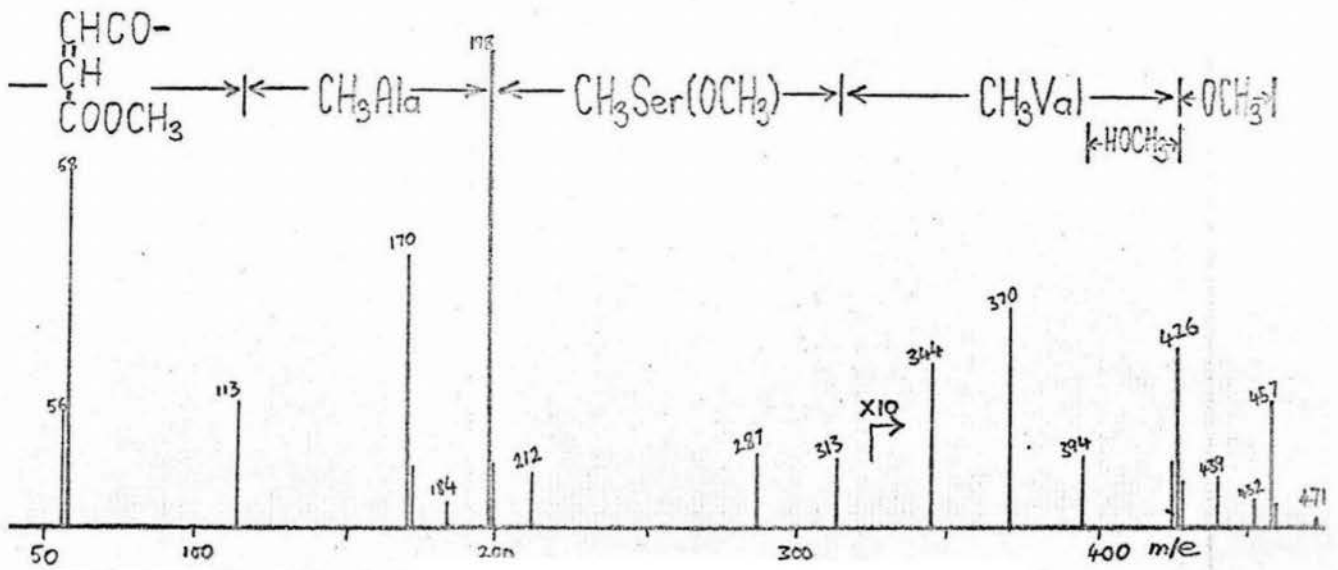


Fig. 40 Spectrum obtained from permethylated Ac-Phe-Asp-Ala-Ser-Val at a sample temperature of 155°C (Exp. (47)).

High Resolution Data:

m/e198 = $C_9H_{12}NO_4$ sequencing peak as shown.

m/e426 = $C_{20}H_{32}N_3O_7$ M - OCH_3

m/e457 = $C_{21}H_{35}N_3O_8$, the molecular ion, as shown.

then "Suba" sealed under nitrogen.

NaH/oil dispersion (21 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, stirring for 65 minutes at 67°C. After cooling to room temperature, the dried acetylated peptide dissolved in DMSO (0.40 ml) was added by syringe and the solution stirred at room temperature for 20 minutes. The DMSO solution of dimsyl-peptide was then slowly injected into vigorously stirred I_2-free CH_3I (0.30 ml) using a micrometer syringe. Addition of the 0.6 ml of solution took 12 minutes. After a further 7 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product was extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

Three distinct spectra were obtained as the temperature of the ion source was increased. The last of these was obtained at 220°C and had molecular ions at m/e 691 and 705; i.e. the theoretical product and a higher homologue (see Fig. 42). The principal fragment ions due to rupture of the peptide bonds also showed homologous peaks 14 a.m.u. higher. Examination of the spectrum showed that one extra methyl group had been introduced exclusively in the aspartyl residue to the extent of about 50%.

Permethylated Ac-Gly-Gly-OH:

Exp. (49) To Gly-Gly (BDH)(2.0g) was added acetic acid (5ml) and acetic anhydride (5 ml). The suspension was stirred overnight at room temperature, filtered and washed with concentrated HCl and acetone. The white solid product, dried over P_2O_5, had a melting point 178°-180°C. Literature value for Ac-Gly-Gly, 187°-189°C. Analytical TLC showed only one ninhydrin-negative component. Mass spectrometry of the solid product, introduced via the direct insertion probe, showed the expected spectrum with molecular ion at m/e 174. There was no evidence of any other compound.

Permethylation: NaH/oil dispersion (20.5 mg, 50% NaH) was pre-rinsed three times with dry petroleum ether, dried under vacuum and "Suba" sealed under nitrogen.

Fig. 41 Mass spectrum obtained from permethylated Ac-Phe-Asp-Ala-Ser-Val at 220°C (Exp. (47)).
See Fig. 4(b) between pages 49 and 50.

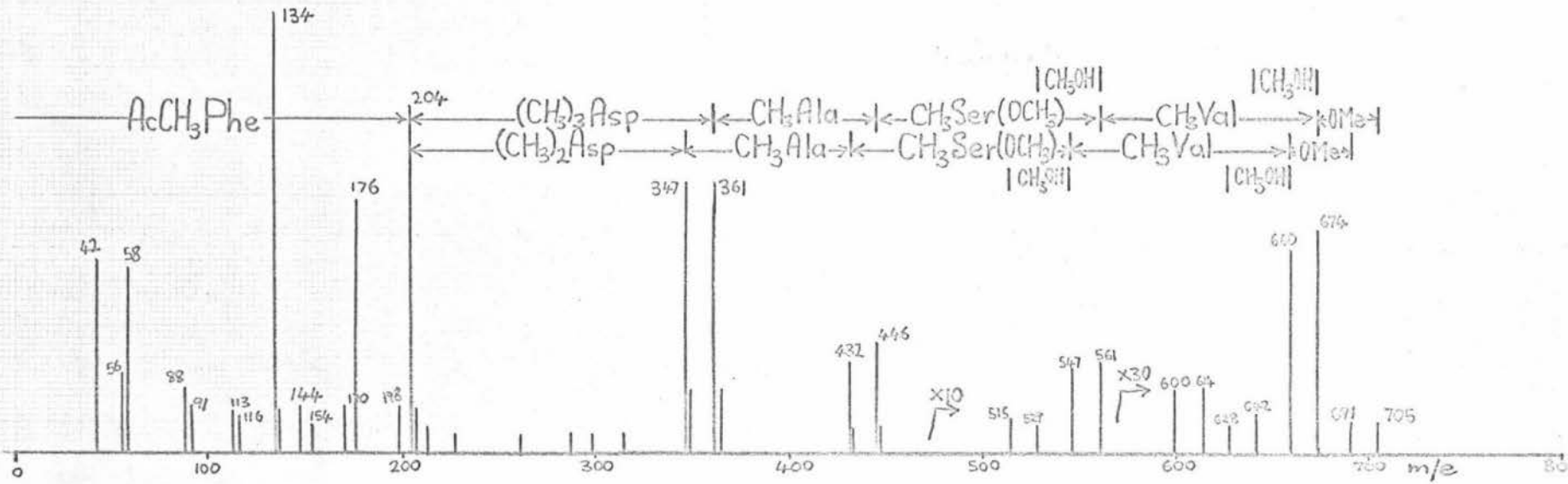


Fig. 42 Mass spectrum of permethylated Ac-Phe-Asp-Ala-Ser-Val at 220°C (Exp. (48)).
Dimsyl sodium + peptide was added to excess methyl iodide.

Metastable Transitions:

m/e446 ⟶ m/e361 ⟶ m/e204 ⟶ m/e176 ⟶ m/e132
m/e432 ⟶ m/e347 ⟶ m/e204 ⟶ m/e176 ⟶ m/e132
m/e674 ⟶ m/e642; m/e660 ⟶ m/e628; loss of methanol from seryl side chain.
m/e561 ⟶ m/e529; m/e547 ⟶ m/e515; loss of methanol from seryl side chain.

Dry DMSO (0.20 ml) was added by syringe and the mixture stirred for 80 minutes at 72°C to 60°C. After cooling to room temperature, Ac-Gly-Gly (5.0 mg) was added. After stirring 5 minutes, chilled I_2-free CH_3I (0.30 ml) was added dropwise. The stirring was continued at room temperature for 80 minutes, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and a portion applied to the direct insertion probe for mass spectrometry. A spectrum was obtained at 70°C to 80°C (see Fig. 43). The expected molecular ion at m/e 216 was accompanied by an ion at m/e 230. Examination of the spectrum showed that extra-methylation was confined to the C-terminal glycine residue and had occurred to the extent of about 30%.

Hydrolysis: The remainder of the permethylated Ac-Gly-Gly (about 3 mg) was refluxed with 6N HCl for 18 hours, evaporated under vacuum at 60°C, re-dissolved in water and re-evaporated several times. The product was dissolved in water (100 µl) for analytical TLC.

Ac-Gly-Gly (2 mg), the same as was used for the permethylation, was hydrolysed under identical conditions and dissolved in water (100 µl) for analytical TLC.

Analytical TLC: Two-dimensional TLC on cellulose layers, according to Haworth and Heathcote (ref. 121). With some modification, this was found to be satisfactory for the analysis of N-methyl amino acids. Analysis of hydrolysed Ac-Gly-Gly showed only glycine, Rfs 37 and 8 for 1st dimension, 2nd dimension respectively.

Duplicate analyses of hydrolysed permethylated Ac-Gly-Gly showed two major components in the ratio of 2-3:1. The more intense of these had Rfs 46 and 14, was positive to ninhydrin/$Cd(OAc)_2$ both with and without heating and also positive to the N-Alkyl-amino acid spray of Sheenan (ref. 122). The other had Rfs 62 and 20, was positive to ninhydrin/$Cd(OAc)_2$ only on prolonged heating and was positive to the N-alkyl-amino acid spray of Sheehan. Comparison with standards identified these as N-methylglycine and N-methylalanine

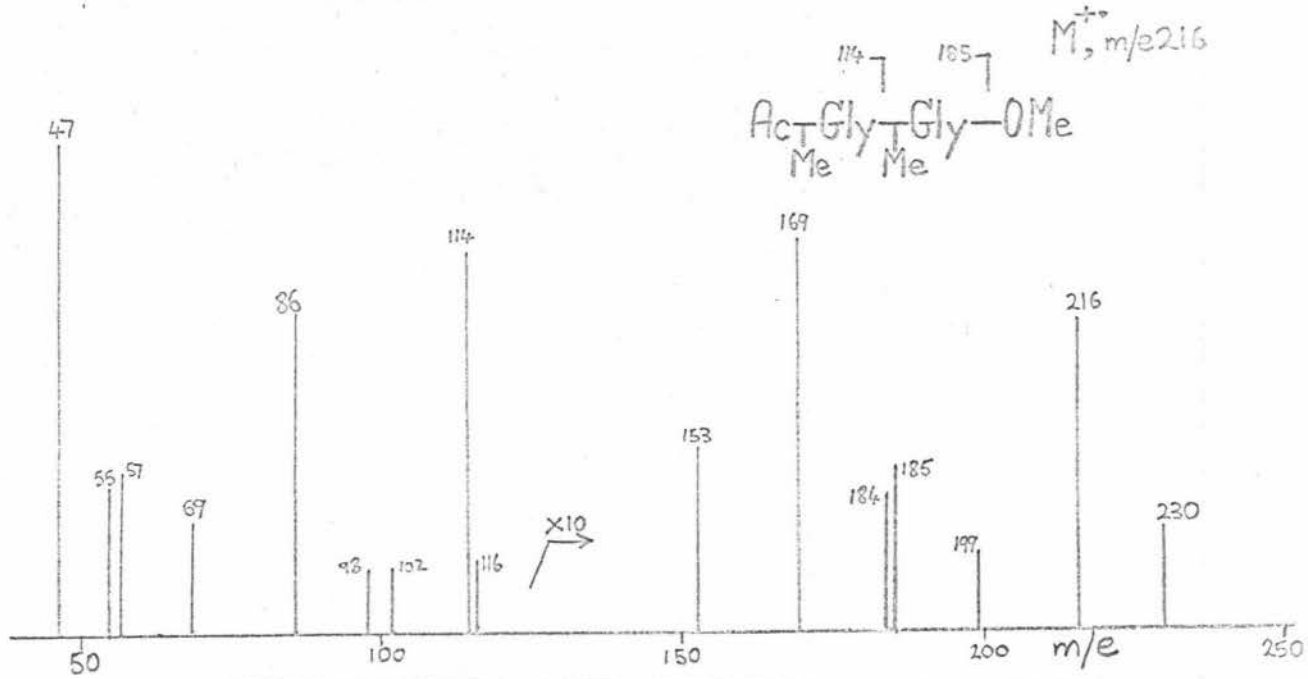


Fig. 43 Mass spectrum of permethylated Ac-Gly-Gly-OH (Exp. (49)).

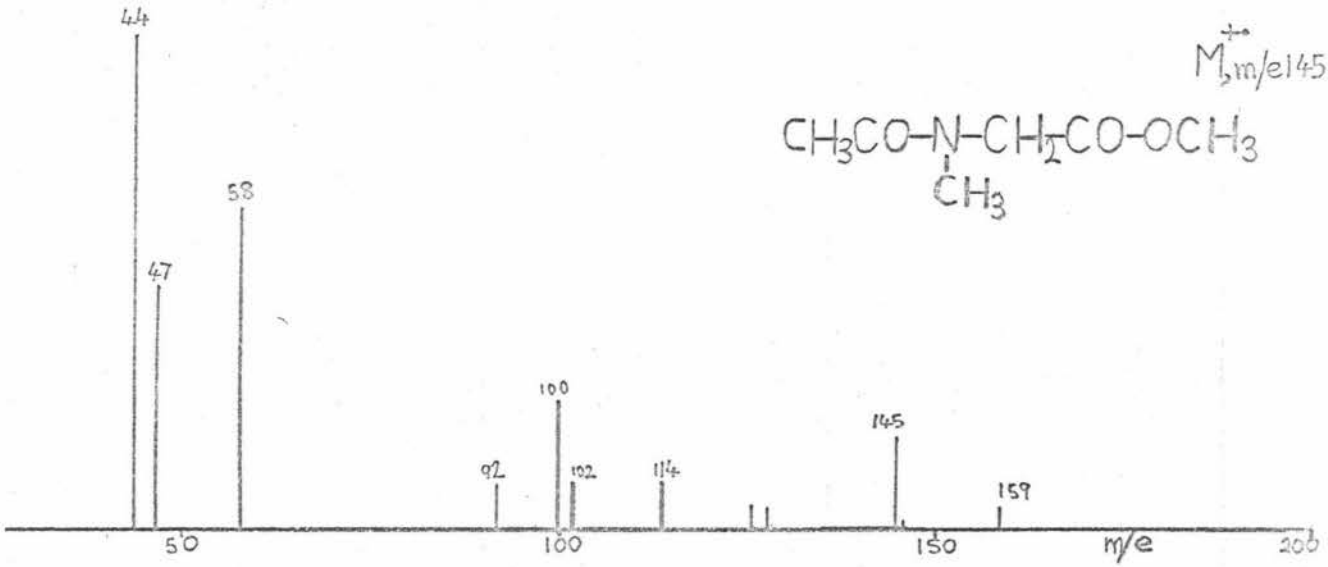


Fig. 44 Mass spectrum of permethylated Ac-Gly-OH (Exp. (50)).

High Resolution Data:

m/e159 =	$C_7H_{13}NO_3$	
m/e145 =	$C_6H_{11}NO_3$	expected molecular ion
m/e100 =	$C_5H_{10}NO$	
m/e 86 =	C_4H_8NO	$M-COOCH_3$
m/e 58 =	C_3H_8N	
m/e 44 =	C_2H_6N	$m/e86-CH_2{=}{=}CO$

Metastable Transitions:

m/e159 ⟶ m/e128 ⟶ m/e100 ⟶ m/e58 (all strong)
m/e145 ⟶ m/e114 ⟶ m/e86 ⟶ m/e44

respectively. A very small amount of free glycine was also detected.

Permethylated Ac-Gly-OH:

Exp. (50) To glycine (AR)(1.0g) was added acetic acid (5 ml) and acetic anhydride (5 ml). The suspension was stirred overnight at room temperature, filtered and the filtrate evaporated at room temperature. The residue was dissolved in the minimum amount of hot water. On cooling, clear crystals were deposited. These were dried over P_2O_5. Melting point 210°-211°C. Literature value for Ac-Gly, 206°-208°C. Analytical TLC showed only one ninhydrin-negative component.

Permethylation: NaH/oil dispersion (85 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (1.0 ml) as previously described, heating at 65°C for 1 hour under nitrogen. After cooling to room temperature, the dimsyl sodium in DMSO solution was added by syringe to a solution of Ac-Gly (104 mg) in DMSO (1.0 ml) under nitrogen. After stirring for a further 10 minutes to 75 minutes at room temperature, an equivalent amount of chilled, I_2-free CH_3I (0.218 ml) was injected and the stirring continued for 2 hours to 17 hours at room temperature. This procedure was repeated four times. On the last occasion, excess CH_3I (0.40 ml) was used and water (1 ml) injected to stop the reaction. The solution was saturated with NaCl when a brown oil came down. This was extracted into chloroform, washed, dried and evaporated at room temperature. The product was a yellow liquid.

A portion of the product was used for mass spectrometry. The sample was introduced as a vapour through the gallium inlet system. The spectrum obtained (see Fig. 44) showed the theoretical molecular ion at m/e 145 accompanied by an ion at m/e 159. Examination of the spectrum indicated that an extra methyl group had been introduced on the α-C atom of the glycine residue to the extent of about 20% to 40%.

Hydrolysis: The remainder of the permethylated Ac-Gly (about 100 mg) was hydrolysed with HCl (20.24%, 3 ml) in a sealed glass ampoule at 108°C for

66 hours. The solution was filtered, evaporated under vacuum at 50°C, re-dissolved in water and re-evaporated several times. The product, a yellow gum, was dissolved in water (2.0 ml) for analytical TLC.

Analytical TLC: TLC on cellulose layers, according to Haworth and Heathcote. Mixtures of glycine and N-methylglycine, alanine and N-methylalanine were used as standards. One dimensional TLC of the hydrolysed, permethylated Ac-Gly (400μ cellulose MN300; solvent 2-propanol/butanone/1N HCl 65:15:25 v/v; sprayed ninhydrin/$Cd(OAc)_2$ and heated at 120°C)(plate 28) showed the presence of N-methylglycine and N-methylalanine in the ratio of about 2 to 1. Traces of alanine and glycine were also detected.

Two dimensional TLC (solvents: according to Haworth and Heathcote; sprayed ninhydrin/$Cd(OAc)_2$ and heated at 120°C)(plate 29) confirmed the presence of these compounds only. Standard chromatograms using authentic compounds were run at the same time (see plates 30 and 31).

The ninhydrin/$Cd(OAc)_2$ spray gives slightly different colours with each of these compounds when examined immediately after heating. This aids in their identification.

SYNTHESIS OF N-METHYLALANINE: according to Fischer and Bergmann (1913) *

Tosylation: DL-alanine (BDH)(0.15 mole) was dissolved in 3N aqueous NaOH(100 ml) and p-toluenesulfonylchloride (0.20 mole) added. Stirred for 1 hour at 65°C. A clear solution formed. The product was precipitated with HCl (concentrated, 30 ml) and recrystalised from hot water. The fine white crystals had melting point 136°-139°C. Literature value for p-tosylalanine, 138°-139°C.

N-methylation: The p-tosylalanine (14.5g, .06 mole) was dissolved in 3N aqueous NaOH (65 mls, 0.18 mole). Dimethyl sulphate (12.5 mls, 0.13 mole) was added dropwise with vigorous stirring. After a further 25 minutes stirring, concentrated HCl (8 ml) was added.

* see "Chemistry of the Amino Acids". Greenstein and Winitz. Vol 3 pp 2756-57 (Wiley & Sons 1961).

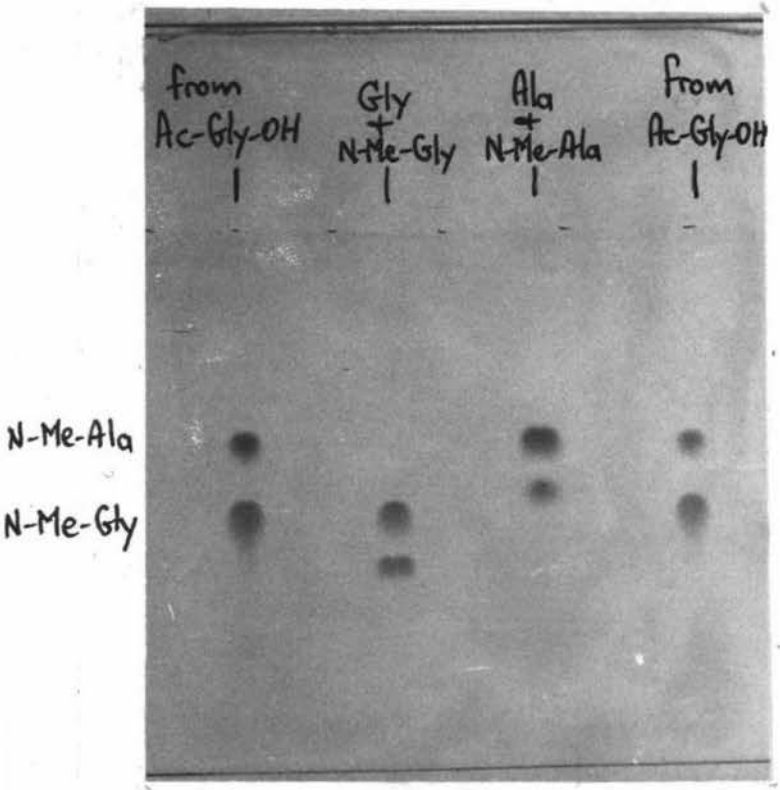


Plate 28. Hydrolysed permethylated Ac-Gly-OH. Solvent 2-propanol/butanone/ HCl (Exp. (50)).

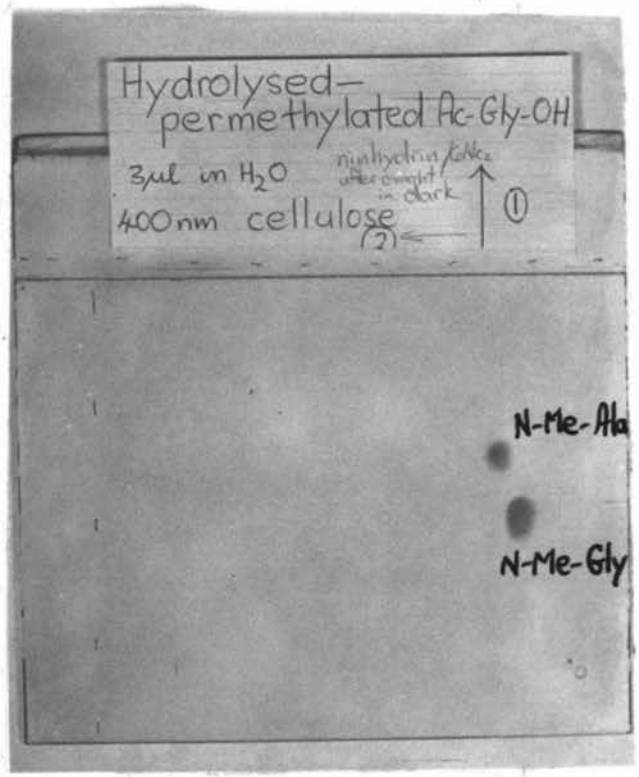


Plate 29. Hydrolysed permethylated Ac-Gly-OH (Exp. (50)).

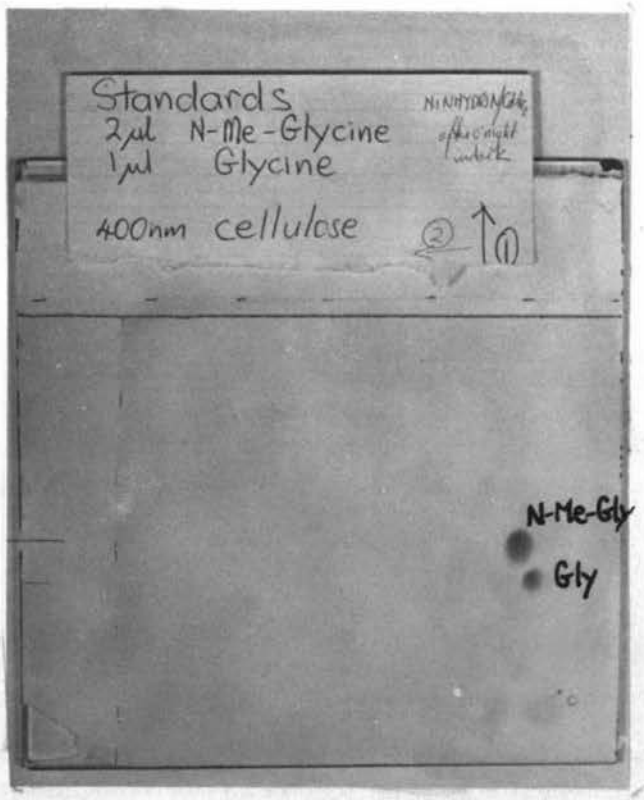


Plate 30.

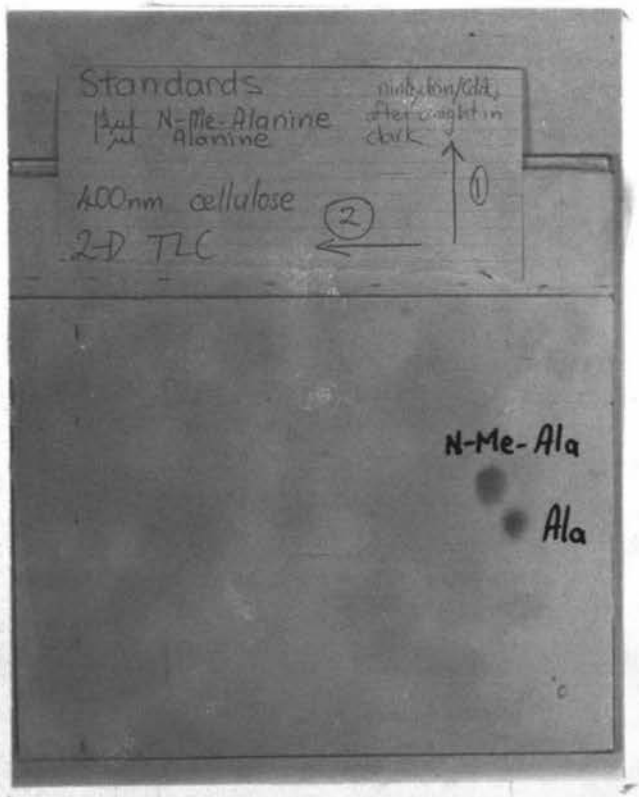


Plate 31.

Standard chromatograms run concurrently with Plate 29. Sprayed ninhydrin/$Cd(OAc)_2$ and heated at 120°C (Exp. (50)).

The white precipitate was dissolved in 10% aqueous K_2CO_3 (100 mls) and acidified with concentrated HCl. The solid white crystalline product was dried over P_2O_5 and had melting point 95° to 100°C. Literature value for p-tosyl-N-methylalanine, 122°C.

Recrystalisation from petroleum ether/acetone gave a product of melting point 95° to 100°C. Examination of a sample by mass spectrometry showed this to be a mixture of N-methylated and unmethylated p-tosylalanine.

Hydrolysis: The product (5.9 g) was hydrolysed with concentrated HCl (23 ml) in a sealed glass ampoule at 108°C for 40 hours. On cooling, a solid separated out and was removed by filtration. The filtrate was evaporated and recrystalised several times from ethanol/diethyl ether. The product decomposed at 135°-140°C. Theoretical for N-methylalanine hydrochloride, 165°C (decomposition). Analytical TLC showed the product to be contaminated with a small amount of free alanine.

CHAIN CLEAVAGE.

Perdeuteriomethylation of Ac-Phe-Asp-Ala-Ser-Val:

Exp. (51) To Phe-Asp-Ala-Ser-Val (M.R.)(2.5 mg) was added acetic acid (1.5 ml), water (0.5 ml) and acetic anhydride (1.5 ml). The solution was stirred at room temperature for 1 hour 40 minutes, evaporated under vacuum at room temperature, redissolved in water and re-evaporated. The acetyl peptide product was dried under vacuum ($\leqslant$ 0.3mm Hg, liquid-air trap 20 minutes), "Suba" sealed under nitrogen and dissolved in DMSO (0.4 ml).

NaH/oil dispersion (25 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, stirring at 65° for 1 hour. After cooling to room temperature, the dried acetyl-peptide in DMSO was added by syringe. After five minutes, chilled I_2-free CD_3I (0.28 ml) was injected dropwise into the vigorously stirred solution. After a further 20 minutes stirring at room temperature, water (1 ml) was injected st stop the reaction, the product extracted

into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

Three distinct spectra were obtained as the ion source was gradually heated. The first was obtained at room temperature "probe out" and had molecular ion at m/e 257 (see Fig. 45). At 150°C, probe-in , a second spectrum with molecular ion at m/e 475 was obtained (see Fig. 46). High resolution mass measurements and metastable detection were in agreement with structures based on the spectra obtained from the permethylated compound. All ions had the expected deuterium content.

On further heating, the spectrum of perdeuteriomethylated Ac-Phe-Asp Ala-Ser-Val was obtained.

UNKNOWN PEPTIDES.

Exp. (52) an aqueous solution (1 ml) of the unknown peptide (1 mg) was evaporated to dryness at room temperature and thoroughly dried at $\leq$ 0.5mm Hg on a liquid-air trapped pump for 40 minutes. Water (1.25 ml) was added and the residue dissolved; acetic anhydride (1.25 ml) was added. The solution was swirled at room temperature for 3 hours, then evaporated under vacuum at room temperature; water (1 ml) was added and the solution re-evaporated several times. The residue was thoroughly dried under vacuum ($\leq$ 0.3 mm Hg, liquid-air trap, 30 minutes) at room temperature, the "Suba" sealed under nitrogen.

NaH/oil dispersion (23 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.2 ml) as previously decsribed, stirring at 65°C for 1 hour and the solution cooled by stirring ten minutes at room temperature. The dried acetylated peptide was dissolved in DMSO (0.4 ml ; an insoluble residue remained) and added by syringe; the carbanion/peptide solution turned emerald green. Chilled, I_2-free CD_3I(0.3 ml) was at once injected rapidly into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

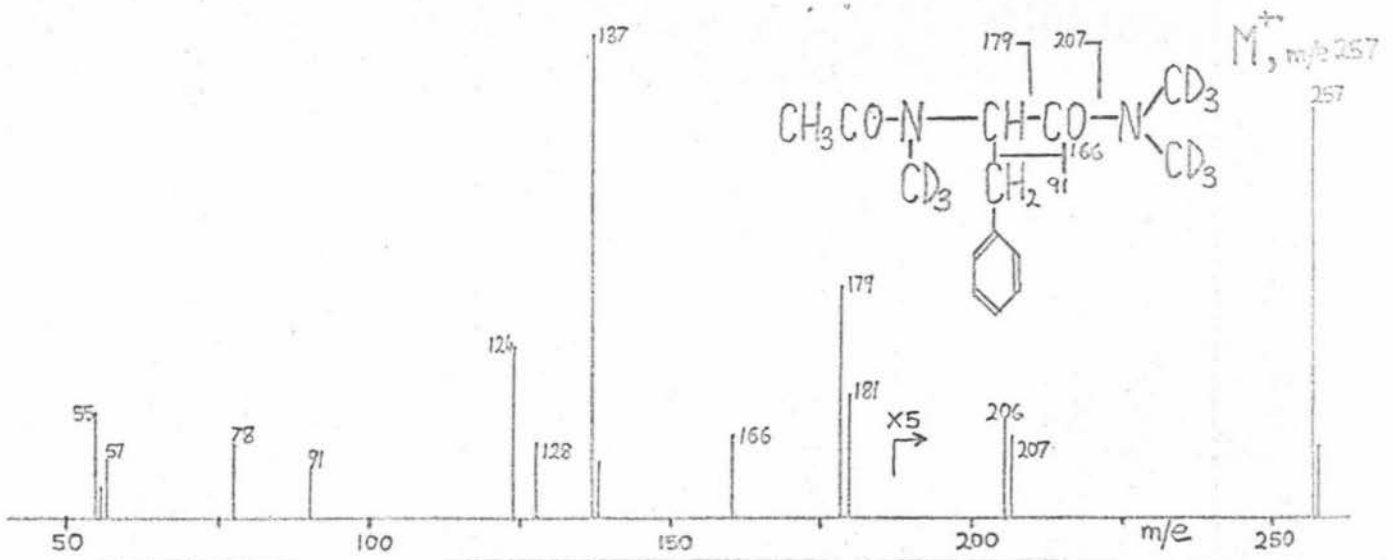


Fig. 45 Mass spectrum obtained from perdeuteriomethylated Ac-Phe-Asp-Ala-Ser-Val, sample "probe out" (Exp. (51)).

High Resolution Data:

m/e257 = $C_{14}H_{11}N_2O_2D_9$	m/e166 = $C_7H_4N_2O_2D_9$
m/e207 = $C_{12}H_{11}NO_2D_3$	m/e137 = $C_9H_9ND_3$
m/e181 = $C_{11}H_7NOD_6$	m/e124 = $C_5H_2N_2OD_9$
m/e179 = $C_{11}H_{11}NOD_3$	

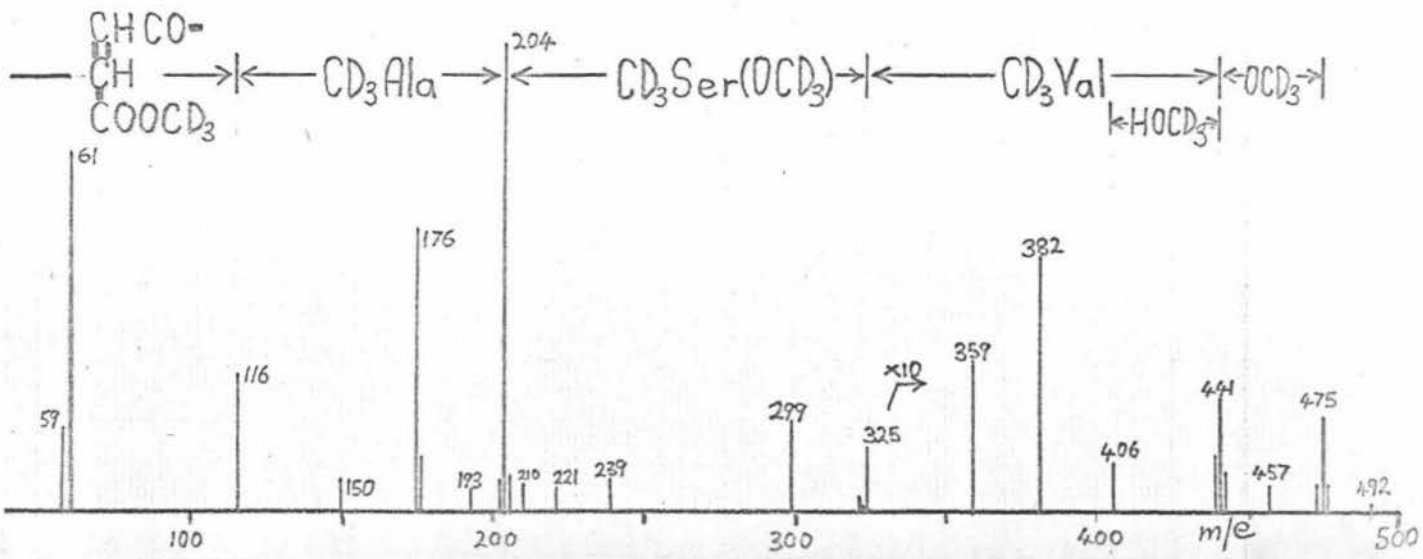


Fig. 46 Mass spectrum obtained from perdeuteriomethylated Ac-Phe-Asp-Ala-Ser-Val, at a sample temperature of 150°C (exp. (51)).

High Resolution Data:

m/e475 = $C_{21}H_{17}N_3O_8D_{18}$	m/e406 = $C_{19}H_{16}N_3O_6D_{12}$
m/e441 = $C_{20}H_{17}N_3O_7D_{15}$	m/e325 = $C_{14}H_9N_2O_6D_{12}$
m/e382 = $C_{17}H_{14}N_2O_7D_{12}$	m/e239 = $C_{11}H_{11}NO_4D_9$

All elemental compositions were in agreement with the proposed structures.

As the sample temperature was raised, a series of mixed spectra were obtained between 180° and 260°C (Figs. 47,48 and 49). Repeated scanning of the spectrum showed that three peptides were present and enabled their spectra to be separated by inspection (Figs. 5,6 and 7 , between pages 53 and 54). The sequence-determining fragment ions were obvious as the most intense ions in the spectra; the molecular ions were weak, but were detected with reasonable confidence. The final spectrum showed molecular ion at m/e 966 and contained intense anomalous peaks at m/e 836 and m/e 863. The deduced sequences were (in order of appearance):

I Glu-Val-Leu-Asn

II Asn-Glu-Asn-Leu-Leu

III Ala-Pro-Phe-Pro-Glu-Val-Phe

The experient was repeated under identical conditions using a second sample (2 mg) of the same unknown, permethylating with CH_3I. The product was applied to the direct insertion probe for mass spectrometry.

A series of mixed spectra were again obtained at sample temperatures of 180° to 260°C. Repeated scanning of the spectrum allowed the three separate spectra to be identified (Figs. 5,6 and 7 , between pages 53 and 54). These were more intense and the molecular ions were readily located.

All the sequencing peaks showed the expected mass shifts when compared to the spectra using deuteriomethylated derivatives. This confirmed the proposed sequences. The anomalous peaks in the third spectrum were found to contain eighteen deuterium atoms and were identified (see p. 54).

In both experiments, high resolution mass measurements and metastable detection were used to confirm the deduced structures. These are marked on the appropriate spectra.

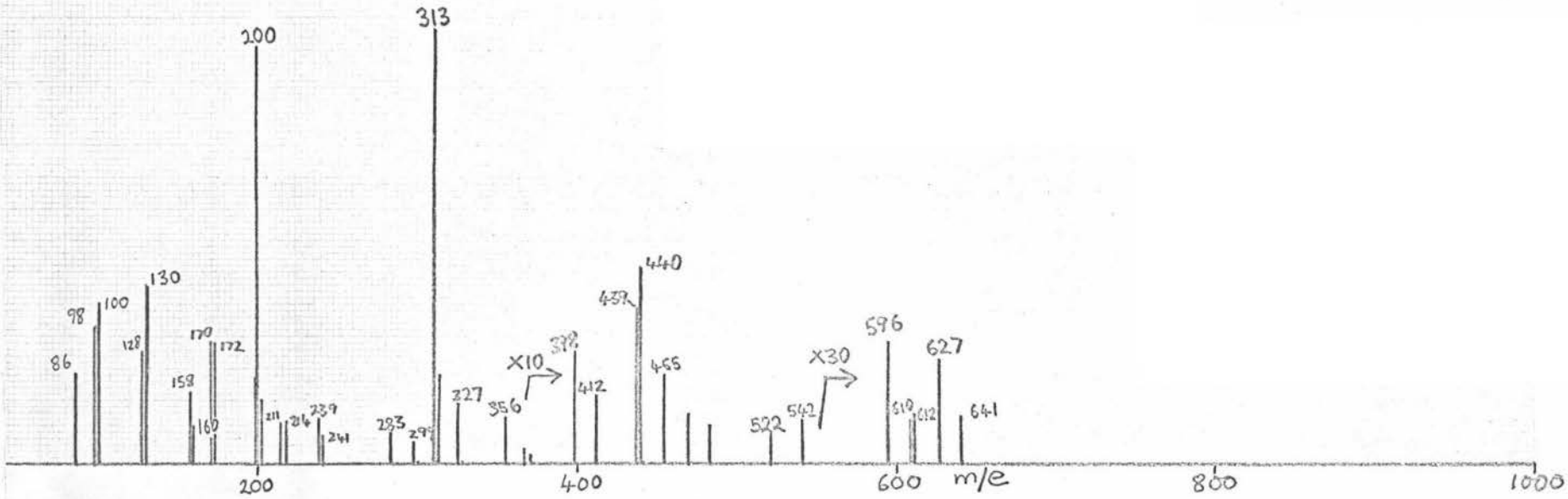


Fig. 47 Mixed mass spectra obtained from the permethylated cheese peptides at a sample temperature of 155°C (Exp. (52)). Predominantly peptide (I) with molecular ion at m/e627; see Fig. 5(a) between pages 53 and 54.

High Resolution Data:

m/e200 = $C_9H_{14}NO_4$ which is Ac(Me)–Glu(OMe)–

m/e313 = $C_{15}H_{25}N_2O_4$ which is Ac(Me)–Glu(OMe)–Val(Me)–

The corresponding spectrum from the perdeuteriomethylated mixture gave the following:

High Resolution Data:

m/e206 = $C_9H_8NO_4D_6$ which is Ac(CD_3)–Glu(OCD_3)–

m/e452 = $C_{22}H_{26}N_3O_6D_{12}$ which is Ac(CD_3)–Glu(OCD_3)–Val(CD_3)–Leu(CD_3)

Metastable Transitions:

m/e651 ⟶ m/e452 ⟶ m/e322

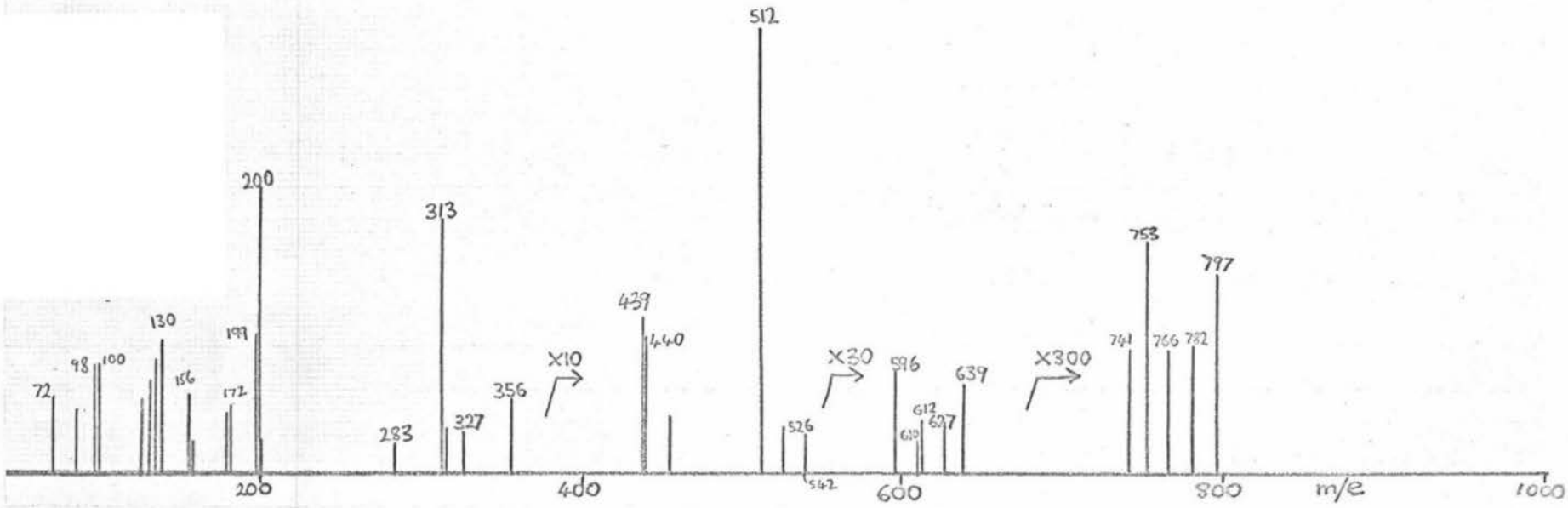


Fig. 48 Mixed Mass spectra obtained from the permethylated cheese peptides at a sample temperature of 200°C (Exp. (52)). Predominantly peptide (II) with molecular ion at m/e797; see Fig. 6(a) between pages 53 and 54.

The corresponding spectrum from the perdeuteriomethylated mixture gave the following:

High Resolution Data:

m/e208 = $C_9H_{15}N_2O_3D_9$ which is Ac—Asn(CD_3)$_2$— (CD_3)

m/e536 = $C_{23}H_{14}N_5O_8D_{24}$ which is Ac—Asn(CD_3)$_2$—Glu(CCD_3)—Asn(CD_3)$_2$— (CD_3)

m/e666 = $C_{30}H_{24}N_6O_9D_{27}$ which is Ac—Asn(CD_3)$_2$—Glu(CCD_3)—Asn(CD_3)$_2$—Leu— (CD_3)

Metastable Transitions:

m/e830 ⟶ m/e666 ⟶ m/e536

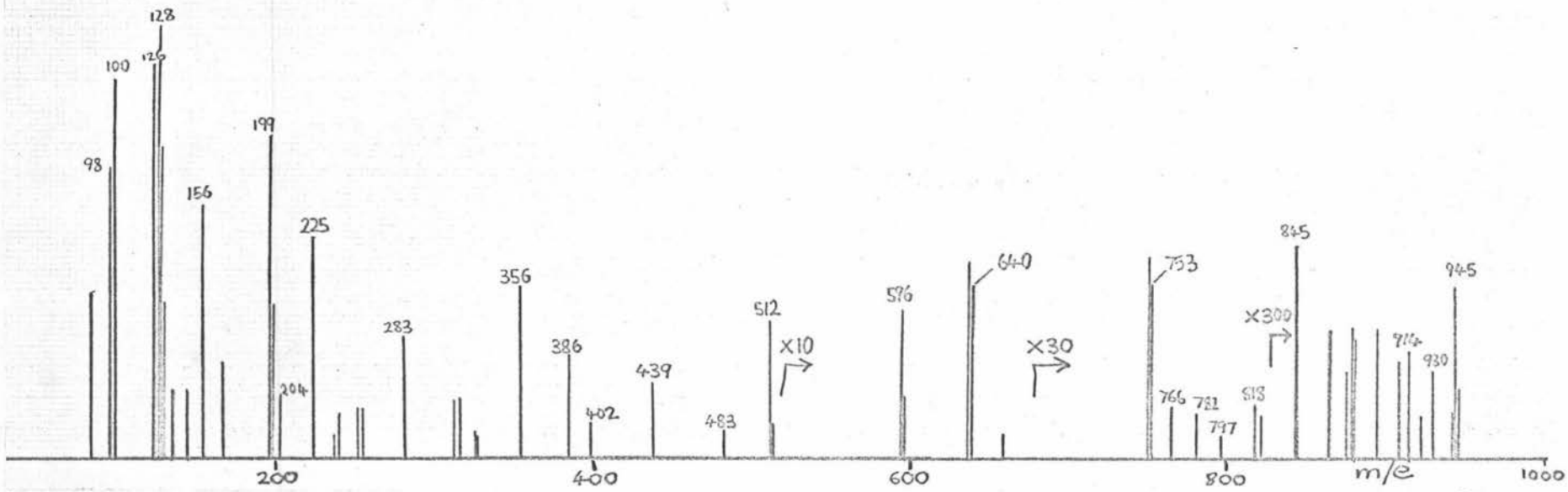


Fig. 49 Mixed mass spectra obtained from the permethylated cheese peptides at a sample temperature of 260°C (Exp. (52)). Predominantly peptide (III) with molecular ion at m/e945; see Fig. 7(a) between pages 53 and 54.

Metastable Transitions:

m/e640 ⟶ m/e483 ⟶ m/e386 ⟶ m/e225

The corresponding spectrum from the perdeuteriomethylated mixture gave the following:

High Resolution Data:

m/e131 = $C_6H_7NO_2D_3$ which is Ac—Ala(—CD_3)—

m/e228 = $C_{11}H_{14}N_2O_3D_3$ which is Ac—Ala(—CD_3)—Pro—

m/e392 = $C_{21}H_{22}N_3O_4D_6$ which is Ac—Ala(—CD_3)—Pro—Phe—

Metastable Transitions:

m/e768 ⟶ m/e652 ⟶ m/e489 ⟶ m/e392

Permethylation of His-Ser-Gln-Gly-Thr-Phe:

Exp. (53) To His-Ser-Gln-Gly-Thr-Phe (M.R.)(1.5 mg) was added acetic acid (1.25ml) - the peptide dissolved at once - water (0.5 ml) and acetic anhydride (1.25 ml). The solution was kept at room temperature for 2 hours, evaporated at room temperature, redissolved in water and re-evaporated. The acetyl-peptide product was dried under vacuum ($\leq$ 0.3mm Hg, liquid-air trap, 20 minutes), "Suba" sealed under nitrogen and dissolved in DMSO (0.4 ml).

NaH/oil dispersion (21 mg, 50% NaH) was used to generate dimsyl sodium in DMSO (0.20 ml) as previously described, stirring at 67°C for 1 hour. After cooling to room temperature, the dried acetyl-peptide in DMSO was added by syringe. After 10 minutes, chilled I_2-free CH_3I (0.30 ml) was rapidly injected into the vigorously stirred solution. After a further 10 minutes stirring at room temperature, water (1 ml) was injected to stop the reaction, the product extracted into chloroform, washed, evaporated and applied to the direct insertion probe for mass spectrometry.

There was no evidence of any spectrum at source temperatures up to 300°C.

It was found that some compounds eluted from TLC absorbents would not give mass spectra, e.g. Exp. (3) : neither leucine nor Leu-Gly-Leu eluted from Silica gel G with chloroform would give a mass spectrum. Exp. (6) : the compound in "fraction 1" eluted from silica gel H with methanol would not give a mass spectrum. After permethylation, the eluted material gave the mass spectrum of permethylated Ac-Ala-Gly-Gly.

A peptide was applied under various conditions to the direct insertion probe for mass spectrometry.

Exp. (54) Mass Spectrometry of Leu-Gly-Leu:

A. A small sample of Leu-Gly-Leu (M.R.) was applied to the direct insertion probe for mass spectrometry.

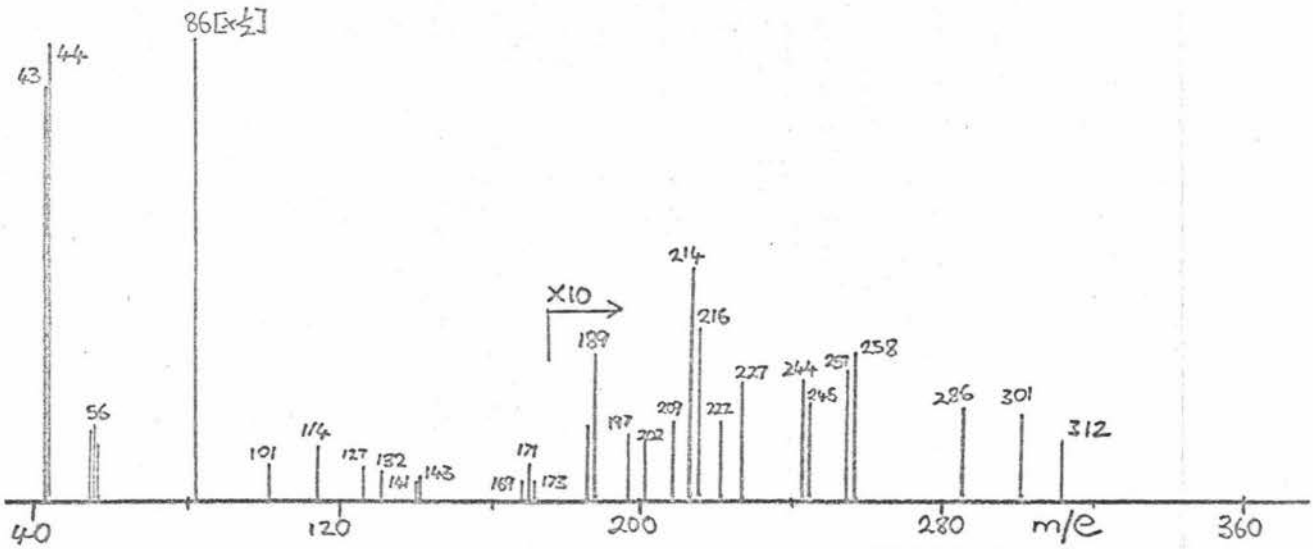


Fig. 50 Mass spectrum of Leu-Gly-Leu (M.R.) (Exp. (54)).

Expected molecular ion at m/e301.

High Resolution Data:

m/e312 = $C_{15}H_{26}N_3O_4$

m/e301 = $C_{14}H_{27}N_3O_4$ the expected molecular ion.

Spectra obtained on strong heating of Leu-Gly-Leu applied under various conditions showed m/e265 as the apparent molecular ion:

High Resolution Data:

m/e265 = $C_{14}H_{23}N_3O_2$ m/e222 = $C_{11}H_{16}N_3O_2$

Metastable Transitions:

m/e265 ⟶ m/e222; m/e265 ⟶ m/e209

The molecular ion m/e265 apparently is a bicyclic pyrolysis product from the tripeptide formed by the loss of two molecules of water.

The spectrum obtained had molecular ion at m/e 301, with some M + 11 at m/e 312. On heating to above 220°C, an ion at m/e 265 was observed (Fig. 50).

B. A small sample of Leu-Gly-Leu (M.R.) in 10% isopropanol was applied to a layer of silica gel H. The sample was eluted from the layer with methanol. evaporated and applied to the direct insertion probe in 10% isopropanol.

Initially ions at m/e 86, 114 were obtained. On stronger heating, an ion at m/e 265 was observed. There was no sign of the normal spectrum with molecular ion at m/e 301.

C. A small sample of Leu-Gly-Leu (M.R.) was dissolved in 0.1N aqueous HCl and applied to the direct insertion probe for mass spectrometry, evaporating the solution with a hot air stream.

The expected spectrum was not obtained, though on heating above 200°C, an ion at m/e 265 was observed.

D. A small sample of Leu-Gly-Leu (M.R.) was dissolved in 0.15N aqueous ammonia and applied to the direct insertion probe, evaporating the solution in a hot air stream.

The expected spectrum, with molecular ions at m/e 301, 312 was obtained.

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