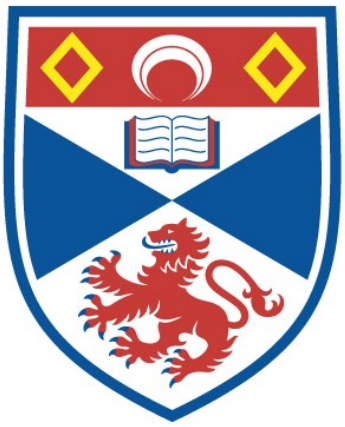
STRUCTURAL STUDIES ON TYROCIDINE A AND THE SPECIFIC CHEMICAL FISSION OF PEPTIDE LINKS

Joseph Court Robinson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1955

STRUCTURAL STUDIES ON TYROCIDINE A.

and

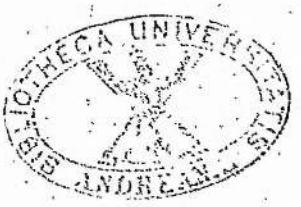
THE SPECIFIC CHEMICAL FISSION OF PEPTIDE LINKS.

being a Thesis

presented by

JOSEPH COURT ROBINSON, B.Sc.

to the



UNIVERSITY OF SAINT ANDREWS

in application for the

Degree of

DOCTOR OF PHILOSOPHY.

April, 1955.

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DECLARATION

I hereby declare that the following Thesis is a record of experiments carried out by me, that the Thesis is my own composition and has not previously been presented for a Higher Degree.

The investigations were carried out in the Chemical Research Laboratory of the University of St. Andrews, and in the Department of Organic Chemistry, The University of Bristol, under the direction of A.R.Battersby, B.Sc., M.Sc., Ph.D.

28th. April, 1955.

CERTIFICATE

I hereby certify that Mr. J.C.Robinson, B.Sc., has spent nine terms at Research work under my direction, that he has fulfilled the conditions of Ordinance No.16 (St.Andrews), and that he is qualified to submit the accompanying Thesis in application for the Degree of Ph.D.

28th April, 1955.

Director of Research.

UNIVERSITY CAREER AND RESEARCH EXPERIENCE

I entered the University of St. Andrews, under a Grant from the Further Education and Training Scheme, in October, 1948, pursuing the course of study leading to the Degree of B.Sc., and was awarded Second Class Honours in Chemistry in 1952.

In October, 1952, I was admitted as a Research Student of the University of St. Andrews, and was engaged on Research there, under the direction of Dr. A.R.Battersby, until January, 1954. From that date this work was continued in Bristol, under the same Supervisor. During the whole period of Research work I was maintained by an Assistantship from the Medical Research Council.

I wish to sincerely thank my Supervisor, Dr. Allan R.Battersby for the help and guidance he has so willingly given to me during the course of this Research work, and without whom the work could not have been begun in the first place. I also wish to thank Mr. B.S.Noyes of Bristol University for the micro-analyses, Mr. Z.M.Zochowski of the University of St. Andrews for his help with some of the synthetic work, and, finally, the Medical Research Council for an Assistantship.

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(I) Introduction

The structural investigation of a polypeptide involves isolation of the substance in a pure state, followed by the determination of the relative amounts and the order of the amino acids in the molecule. The problems of isolation and amino acid composition have been greatly simplified by the introduction of counter-current distribution and ion-exchange chromatography. With pure materials available, the sequence of the amino acids can be studied by partial hydrolysis with acid or enzymes, and by stepwise chemical degradation. A number of methods are available permitting the latter approach to be used, but generally the degradation can remove in turn only a few amino acid residues before complications occur. It seems desirable therefore that the peptide chain should first be specifically cleaved at known points along its length and the various isolated fragments then studied by the stepwise procedure. The object of some of the researches to be described was to discover methods for specifically cleaving the peptide chain at those points where glutamic acid and aspartic acid residues

occur.

It was planned that any methods found to be successful with simple model peptides should be tested on a naturally occurring polypeptide for an assessment of their true value. The polypeptide chosen for this purpose was tyrocidine A, which is readily available, and can be obtained pure. All the structural features of this molecule are known save one; it was not known, at the outset of this work, whether the glutamic acid and aspartic acid residues present were linked into the peptide chain by their side-chain or their α-carboxyl groups. The study of this problem forms the first part of the work described in this thesis.

(II) The Chemistry of Tyrocidine Hydrochloride

(a) Early Work

(i) Isolation

In 1939 Dubos (1) described the preparation from autolysed cultures of B.brevis, of a substance which exerted a bactericidal effect on certain micro-organisms. The active substance could be precipitated in close association with a protein by adjusting the filtered culture mediums to pH. 4.5 with hydrochloric acid. The precipitate was soluble in neutral buffer, but if heated or treated with proteolytic enzymes was rendered insoluble. This treatment did not in any way effect its lytic or bactericidal properties. It seemed probable therefore that the active substance was not a protein, and that the protein with which it was associated determined its solubility properties. This proved to be the case.

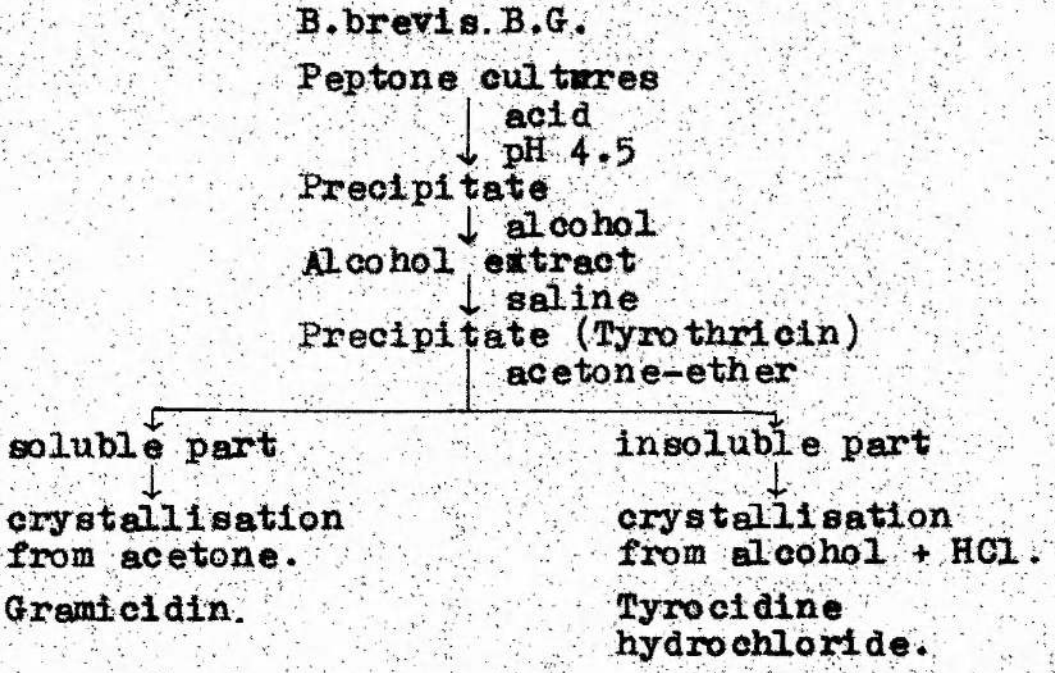
Of the two procedures which have been published (2, 3) for the further fractionation of this material, the simplest employs solvent extraction with 95% ethanol, which leaves the protein undissolved. The latter fraction

did not possess the bactericidal powers of the original mixture, whilst the alcohol solution on dilution with aqueous sodium chloride afforded a precipitate which was fully active. This crude product, which was insoluble in water, but soluble in acetone and alcohol was called tyrothricin.

Tyrothricin was further fractionated by Hotchkiss and Dubos (4-7), and these authors succeeded in isolating two crystalline compounds from it, designated gramicidin and tyrocidine. The latter is a basic substance and is present in the mixture as the hydrochloride, whilst the former is a neutral antibiotic. All processes for the fractionation of tyrothricin are based on this difference. Thus, after removing fats and waxes from tyrothricin by ether extraction, the gramicidin was extracted into 1:1 acetone-ether whilst the crude tyrocidine hydrochloride remained insoluble. This portion, about 85% of the tyrothricin, was dissolved in boiling ethanol, and precipitated as the crystalline hydrochloride by addition of ethanolic hydrogen chloride. After recrystallisation from methanol, the tyrocidine hydrochloride was obtained as fine colourless needles melting unsharply and

with decomposition at about 240°C (corr.), $[\alpha]_D^{25} = -101°$ (C=1% in 95% alcohol). The yield of tyrothricin varies with the different strains of B.brevis, and with the culture methods. More than 0.5 g./l has been produced by the B.G.strain. When fractionated by the above methods a content of approximately 10-20% gramicidin, and 40-60% tyrocidine hydrochloride was indicated in tyrothricin, of which perhaps two-thirds could ordinarily be obtained in crystalline form. The portion of tyrothricin unaccounted for appears to be largely made up of "gramicidin-like" and "tyrocidine-like" components, speaking in both a chemical and biological sense.

The above method of fractionation may be summarised in the following scheme (Hotchkiss, 8).



An attempt by Gordon, Martin and Synge to fractionate tyrothricin by partition chromatography on silica gel (9) resulted in imperfect separation.

(ii) The Chemistry of unfractionated tyrocidine hydrochloride

The investigations of R.D.Hotchkiss (10), and independent work by H.N.Christensen et al.(11), showed that tyrocidine hydrochloride was the salt of a polypeptide. In the course of these studies gramicidin was shown to be of like nature, but the results for this substance will not be presented here. It should be noted that the tyrocidine hydrochloride used by these and subsequent workers until 1947 was not a chemical individual, Synge and Tiselius (17) showing in that year that the material isolated as above was a family of tyrocidines. The results obtained on the unfractionated material are consequently only of qualitative value.

Electrometric titration of intact tyrocidine hydrochloride by Hotchkiss (10) showed the presence of basic amino groups, and also weak acidic groups, probably phenolic since acetylated tyrocidine was neutral. Tryptophan and tyrosine were estimated colorimetrically, and the

presence of amide groups was indicated by the ammonia produced on brief hydrolysis. Hotchkiss and Dubos (7) on the basis of the elementary composition of tyrocidine hydrochloride suggested an empirical formula $C_{63}H_{83}N_{13}O_{13}.HCl$ corresponding to a minimum molecular weight 1267. Determinations of the weak acidity and easily hydrolysable ammonia by Hotchkiss (10) indicated that the true molecular weight is 2534 or some multiple of this. The same worker studied by titration the hydrolysis products of the polypeptide and his results indicated that it contains about twenty amino acid residues, indicating tryptophan, tyrosine, and aspartic acid. These are apparently combined in such a way that two basic amino groups, three amide groups, and one carboxyl or phenolic group are free in the molecule of weight 2534. The minimum unit proposed by Christensen et al. (11) on the basis of independent work was in reasonable agreement with that suggested by Hotchkiss above. Christensen gave evidence for the presence in tyrocidine hydrochloride of phenylalanine, alanine, and basic amino acids in addition to those found by Hotchkiss.

The limited study of the amino acid composition of

the polypeptide carried out by the above workers was extended by Gordon, Martin and Synge (12), who studied the products of acidic hydrolysis of tyrocidine hydrochloride by partition chromatography. The following amino acids were isolated from the hydrolysate, and characterised as their acetyl derivatives: phenylalanine, leucine, proline, valine, tyrosine, ornithine and glutamic acid. Additional evidence was obtained for the occurrence of tryptophan and aspartic acid, but the presence of alanine could not be confirmed. The ornithine was isolated as its diacetyl derivative, and this appears to be the first isolation of ornithine by acidic hydrolysis of a polypeptide. The recovery of tryptophan from the hydrolysates was very variable, similar results being obtained when tryptophan, in admixture with other amino acids, was heated with acid under the conditions of hydrolysis used for tyrocidine hydrochloride. The errors in this work entirely prevented the stoichiometric calculation of a "minimum molecule" of tyrocidine hydrochloride. These authors concluded however that if the results for tryptophan and ammonia, as determined by Hotchkiss (10) were combined with those they had obtained for other constituents, 88-105% of the nitrogen of tyrocidine hydrochloride had been identified

in its hydrolysate.

Not only did this work succeed in identifying more amino acids in the polypeptide, but gave evidence of th their optical configuration. The phenylalanine isolated had mainly the D-configuration and the other amino acids were mainly of the L-form. Lipmann, Hotchkiss and Dubos (13) had previously shown by enzymatic assay with D-amino acid oxidase that tyrocidine appeared to contain D-amino acids amounting to 15% of its total nitrogen. The total amount of D-amino acid (13% of the total nitrogen) found by Gordon, Martin and Synge (12) was thus in agreement with this value.

The work which has been discussed so far was admirably reviewed by Hotchkiss (8) in 1944, who showed that all the available analyses were in fair agreement with the 26 nitrogen unit mentioned above. At that time, however, no reliable figure could be given for the molecular weight of tyrocidine hydrochloride. Ogston (14) had attempted the determination in dilute acetic acid with the ultracentrifuge. Sedimentation was slow and comparable in magnitude with diffusion, and the molecular weight could only be estimated as probably between

1,000 and 3,000. However on the basis of 26 nitrogen atoms in the molecule the analyses indicated a polypeptide composed of 3 phenylalanine and 3 ammonia residues, and 2 residues each of glutamic acid, aspartic acid, ornithine, tryptophan, tyrosine, proline, leucine and valine. Such a structure, less 22 molecules of water, would have the empirical formula $C_{127}H_{166}N_{26}O_{26}\cdot 2$ HCL and a molecular weight of 2546. This calculated formula was in good agreement with the elementary analyses, differing by a single carbon only from the double molecule previously deduced from the elementary composition alone. Hotchkiss visualized a likely arrangement of the residues as a peptide chain in which 19 α-amino and carboxyl groups were combined, presumably formed in a cycle. The two basic groups of the ornithine side chains were said to be free to account for the basic nature of the molecule, and three of the four acidic side chains of the dicarboxylic acids were presumably combined with ammonia as amide groups. The free weak acid group found when tyrocidine hydrochloride was titrated with alkali in alcoholic solution was thought to be either an α-or side chain

carboxyl from one of the dicarboxylic acids, a change of the original opinion that this grouping was phenolic.

The assumption and approximations which had been made at this stage were acknowledged by Hotchkiss, who pointed out that until their use was rendered unnecessary by the availability of the more precise analyses required for these larger molecules, they could be said to have helped in the drawing of the broad structural outlines.

More information about the amino acid composition of tyrocidine was published in the year following Hotchkiss's review by Christensen et al.(15). These workers removed all doubt about the presence of aspartic acid in tyrocidine by isolating this amino acid, as the benzoyl derivative, from the hydrolysed polypeptide. They also examined the hydrolysate by microbiological assay and found that the small amount of tryptophan which survived was of the L-form. An important outcome of these new experiments was the withdrawal by Christensen et al. of their earlier claim that alanine was present in tyrocidine, thus bringing their work into agreement with that of Gordon, Martin and Synge (12).

A paper which followed, by Christensen (16), on the free functional groups of tyrocidine showed quite clearly that the only amino group present is the δ-amino group of ornithine. The ornithine residue was converted practically completely to an L-arginine residue by the action of S-methylisothiourea, without producing detectable quantities of other alkyl guanidinium compounds. Further, treatment of tyrocidine with p-toluenesulphonyl chloride, followed by acid hydrolysis and acetylation enabled α-acetyl-δ-(p-toluene sulphonyl)-L- Ornithine to be isolated. An alkaline hydrolysate of the tosylated tyrocidine gave no evidence for the presence of the N-p-toluenesulphonyl derivatives of the other amino acids present in tyrocidine. The absence of any other amino group, except the δ-amino group of ornithine, gave strong support to the view, that the molecule is cyclic. This work also showed that the phenolic hydroxyl group of tyrosine is free in the polypeptide. When tyrocidine was treated with p-toluenesulphonyl chloride in pyridine, followed by acid hydrolysis of the product O-(p-toluenesulphonyl) -L-tyrosine was isolated from the resultant mixture.

II.(b).Isolation and characterisation of a single peptide.

The tyrocidine hydrochloride used in the foregoing studies had been repeatedly recrystallised, and the constancy of its physical and chemical properties indicated homogeneity. However, Synge and Tiselius (17) considered that the product might still consist of two or more different peptides crystallising together in proportions unchanged by crystallisation. They therefore subjected tyrocidine hydrochloride to adsorption analysis, using first the frontal analysis method of Tiselius.

Frontal analysis on charcoal immediately showed that a number of components were present, moreover the increasing fluorescence of the effluent solution in U.V. led them to suspect that the successive components had differing tryptophan contents. Three components were recognised, only the first two being sharply defined. Advantage was taken of the Erhlich colour test for tryptophan when the least strongly adsorbed component (I) gave no colour reaction, whereas components (II) and (III) gave colours, which however were not identical. Recycling of the components on a

similar column gave the same type of pattern, showing that it was very unlikely that the charcoal had had an effect on the tyrocidine, and that the material was in fact heterogeneous. Although frontal analysis could be used to isolate component(I), the overlapping of zones common to this procedure (18) made it necessary to use elution and displacement development to separate the more strongly adsorbed components. Elution with ethanol separated components (I) and (II) on a column which was not overloaded. Component (III) was finally displaced, using stearic acid in ethanol. The recoveries were low when simple elution was used (30-40%), but increased to 73% with stearic acid as displacing agent.

Before discussing the characteristics of the different components it is interesting to note that tyrothricin, the parent mixture from which tyrocidine hydrochloride and gramicidin were isolated, was also subjected to frontal analysis. Hydrolysis of the total products from the analysis, together with two dimensional paper chromatography, revealed only amino acids already known to be present in either gramicidin

or tyrocidine. Any peptides other than these two in tyrothricin must therefore have similar amino acid compositions.

Characterisation of the tyrocidine components (I), (II) and (III) was attempted, but apart from their colours with Erhlich's reagent, and differing fluorescence no striking difference could be detected in their properties. They were all obtained as gums, which, upon hydrolysis and paper chromatography, revealed the presence of all the eight amino acids common to tyrocidine, (ornithine, valine, leucine, phenylalanine, proline, tyrosine, glutamic acid and aspartic acid) in each hydrolysate. Tryptophan was destroyed during the period of hydrolysis. Furthermore the relative proportions of the amino acids did not seem very different in the different fractions. Pedersen and Synge (19) attempted to ascertain the molecular weights of the tyrocidine components, and also of unfractionated tyrocidine hydrochloride, by diffusion experiments in aqueous acetic acid and ethanolic solutions. Their data suggested for the latter[a] molecular weight of 1900-5100, a wide span

which included the figure calculated by Hotchkiss (8) for his postulated molecule of 19 amino acids, namely 2473 for the free unhydrated base. The different components of tyrocidine did not differ much from one another in their diffusion characteristics and their molecular weights were also thought to lie in the range given above.

Adsorption experiments thus definitely established the existence of several "tyrocidines" which differ in their tryptophan content. This undoubtedly accounts in part for the very variable figures found previously in quantitative determinations of tyroptophan on unfractionated tyrocidine, the partial destruction of this amino acid however under conditions of acidic hydrolysis, must also be taken into account.

The heterogeneity of tyrocidine hydrochloride was also demonstrated by Craig, Gregory and Barry (20), who fractionated the polypeptide by countercurrent distribution (C.C.D.21), using a small hand operated machine. After several hundred transfers the distribution pattern indicated a minimum of five

components, of which three could be isolated in crystalline form. The tryptophan contents of the latter differed, one contained little tryptophan, whereas the other two contained appreciable amounts of this amino acid. The other amino acids in the three fractions were the same, and corresponded with those for unfractionated tyrocidine.

Tyrothricin and gramicidin were also subjected to C.C.D. at this time. The latter was shown to contain four peptides, three of which were isolated, whereas the former contained at least three more peptides not present in either gramicidin or tyrocidine. Altogether then, at least eleven different polypeptides had been separated from tyrothricin. Countercurrent distribution was thus shown capable of separating mixtures of closely related polypeptides, indeed the method showed resolving power where other methods had failed. This was clearly demonstrated in the case of gramicidin which could not be resolved by frontal analysis(17).

One difficulty in the early work was the small number of transfers and hence the number of

extractions, which could be applied by hand operation. Thus, if two substances with very similar structures were present in a mixture being studied the pair might not be separated in the limited distribution. The number of tubes present in the apparatus is also important for efficient fractionation. In a machine with a small number of tubes the spreading of the bands which occurs with large numbers of transfers can often cause difficulty. With these points in mind Craig designed an improved form of the apparatus containing 220 tubes, which was automatically operated at each stage of the distribution (22).

Making use of this apparatus, Battersby and Craig (23) were able to isolate from tyrocidine hydrochloride three major components called tyrocidines A, B, and C. Comparison of the relative absorption of the three components at 290 mμ showed that part of the differences between them could be ascribed to differences in tryptophan or tyrosine content. After hydrolysis and paper chromatography B and C gave strong spots corresponding to tryptophan, whilst A gave only a faint spot in this position. All three gave

distinct spots corresponding to phenylalanine, leucine, tyrosine, valine, proline, ornithine, glutamic acid and aspartic acid.

The tyrocidine A obtained from the first distribution was extensively fractionated by further C.C.D. in two different solvent systems; in the first system 1600 transfers were applied and in the second, 2140 transfers. The final product contained no tryptophan, but all the other amino acids mentioned above were still present. The pure polypeptide was obtained crystalline as the hydrochloride and this salt had m.p. 240-242°, $[\alpha]_D^{25}$ -111°(C 1.37 in 50% ethanol). The analytical figures for C,H,N and Cl agreed well with the empirical formula $C_{66}H_{87}O_{13}N_{13}.HCl$ corresponding to a minimum molecular weight of 1307. Determination of the free amino nitrogen by Van Slyke's method gave a value which agreed with the presence of a single primary amino group in a mol. wt. of 1307. Amide nitrogen was determined by hydrolysing the salt in hydrochloric acid, and estimating the ammonia formed. The amount found, corresponded to two amide groups in a mol. wt. of 1307. In a separate experiment

the ammonia was isolated and characterised as ammonium chloride. Finally in these experiments on the intact tyrocidine A, the U.V. absorption was measured, and it was shown that one residue of tyrosine in a minimum molecular weight of 1307 could account completely for the absorption of the polypeptide.

The amino acid composition of tyrocidine A was determined by complete hydrolysis followed by fractionation of the hydrolysate by C.C.D. All the constituent amino acids were readily isolated crystalline from the various separated bands in the machine save aspartic acid which was characterised by the method of Van Slyke *et al.*(24). The amounts of the different amino acids present were calculated from the areas under the curves obtained from the distribution. The molar proportions found, on the basis of a molecular weight of 1307 for tyrocidine A hydrochloride were, phenylalanine 3 moles, and 1 mole each of tyrosine, valine, leucine, proline, ornithine, glutamic acid and aspartic acid. Agreement between the amount of

amino acid recovered, and the theoretical quantity on this basis was for the most part good, although in some cases the weight recovered was lower than the theoretical. However, no other amino acids could be detected by C.C.D. or paper chromatography. The optical form of the isolated amino acids showed that the L-form had been obtained in all cases except that of phenylalanine. The optical rotation in this case corresponded to two moles of the D-, with one of L-isomer.

In these experiments much more evidence was presented in support of the view that the polypeptide was of a cyclic nature. Mild acetylation of tyrocidine A with acetic anhydride in pyridine gave the crystalline O,N-diacetyltyrocidine A, m.p. 289-292°, and this product was shown to be neutral in reaction. Thus there can be no free carboxyl group in the molecule. Battersby and Craig (25) also treated the polypeptide with 1-fluoro-2,4-dinitrobenzene, and showed that this reagent attacks tyrocidine A only at two points, the terminal amino group of ornithine, and the phenolic hydroxyl group of tyrosine. From the

hydrolysed product a crystalline dinitrophenyl (DNP) derivative of ornithine was isolated, as the monohydrochloride, which was shown to be the δ - and not the α -DNP-derivative by the ninhydrin-CO_2 method (24). Furthermore the hydrolysate when examined by paper chromatography showed no spots corresponding to ornithine or tyrosine, however two new spots corresponding to δ-DNP-ornithine and O-DNP-tyrosine were found. All the other amino acids were present unchanged. Thus no α - amino group occurs free in tyrocidine A, the basic group being the δ - amino group of ornithine; also the phenolic hydroxyl group of tyrosine is free in the intact peptide. These results are in agreement with the insolubility of tyrocidine A in aqueous sodium carbonate and its ready solubility in aqueous sodium hydroxide.

On the basis of this evidence these workers concluded that the molecule must be cyclic and put forward the amino acid residue formula of tyrocidine A as (D-phenylalanine)$_2$(L-phenylalanine), (L-valine),(L-tyrosine),(L-leucine).(L-proline), (L-ornithine),(L-glutamine or L-isoglutamine) and

(L-asparagine or L-isoasparagine), which corresponds to the empirical formula $C_{66}H_{87}O_{13}N_{13}$.

As shown previously, attempts to determine the molecular weight of tyrocidine by different methods gave very variable values probably due to the known tendency of this type of substance to associate, and also to hold solvent of crystallisation. However, following their work on the isolation and characterisation of tyrocidine A, Battersby and Craig (25,26) put forward a method for the determination of the molecular weights of polypeptides. This is based on partial substitution of a polyfunctional molecule followed by fractionation of the resultant mixture by C.C.D. Regarding the partition ratio (K) as an overall balance of the hydrophobic and hydrophilic properties of the substance it can be seen that any change affecting a polar group in a solute molecule will have a striking effect on the partition ratio. If several polar groups are involved, each will produce its own contribution to the change in K; the various stages of substitution in a polyfunctional

molecule should thus be separable by C.C.D.

If a partial substitution of a polyfunctional molecule is carried out leaving some of the starting material unchanged, the mono- and di-substituted products would be expected to occur. Distribution of the product in a suitable system would then give a number of bands, depending on the number of functional groups able to react with the substituting reagent. With one group, two bands would be expected, namely unchanged starting material and the mono-substituted derivative. With two groups, four bands in the pattern are possible. One band containing the disubstituted product would be furthest removed from that containing the unchanged material. Two possible intermediate monosubstituted products would be expected to have very similar partition ratios and could form overlapping bands unless a very high number of transfers had been applied. The monosubstituted product would be expected to lie next to the unchanged material, the disubstituted product next to the monosubstituted. Analysis for the substituting group in a particular

band would then give the molecular weight, since the number of substituents is known from the position of the band in the distribution pattern. One difficulty of course is that the monosubstituted material might react much more rapidly than the starting material, and unless special precautions are taken the former might not be detected in the final mixture. The results are therefore normally confirmed by carrying out a second partial reaction using different functional groups in the molecule.

When applied to tyrocidine A, two partial reactions were used, namely methanolysis and treatment with Sanger's reagent, 1-fluoro-2,4-dinitrobenzene (FDNB). In the first case, pure tyrocidine A was treated with methanolic hydrogen chloride and distribution of the crystalline product gave two well separated bands. One proved to be unchanged tyrocidine A whilst the other contained a product formed by methanolysis of one or more amide residues. On the basis of the above argument the new product was interpreted as being the mono-methylester, since it lay next to the unchanged material. From the methoxyl content a molecular weight

of 1300 was indicated. The amide-nitrogen content corresponded approximately to half that found for the original peptide, thus methanolysis had converted one $-CONH_2$ group to a $-COOCH_3$ group. This value was in direct agreement with the minimum molecular weight of 1270, derived from amino acid analysis.

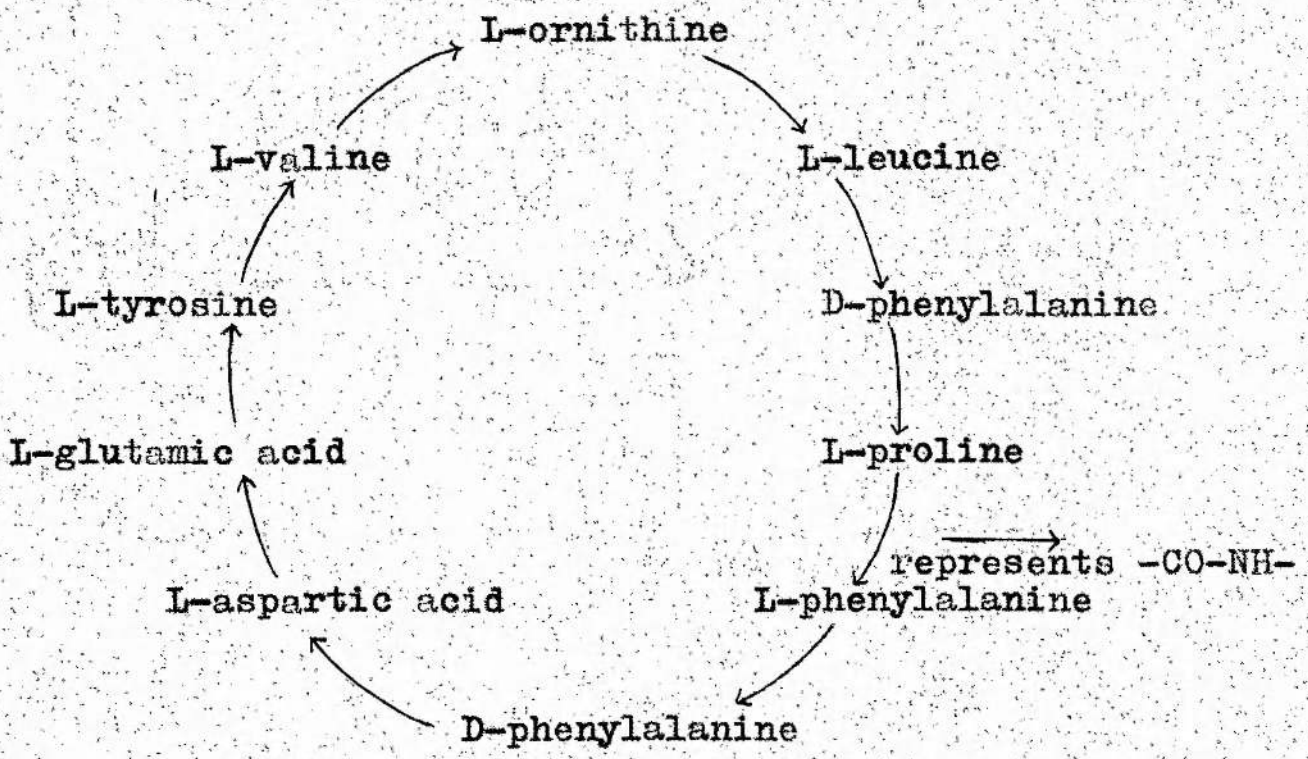
Confirmation of this result was obtained by partial substitution of tyrocidine A with FDNB which can attack the δ-amino group of the ornithine residue and the phenolic hydroxyl group of the tyrosine residue. Distribution of the product gave three well separated bands. Since the first band proved to be unchanged tyrocidine A, the next band, yellow in colour, was interpreted as being the mono-substituted N-DNP-tyrocidine A. This was confirmed by hydrolysis and paper chromatography. From the known molar extinction coefficient (ϵ) of N-DNP derivatives at 350mμ, and the absorption of N-DNP-tyrocidine A at this wavelength, it was possible to calculate the molecular weight which was found to be 1620. Though this value is somewhat higher than the theoretical figure (1436) for the mono DNP-derivative of a peptide of molecular

weight 1270 (i.e., that corresponding to the free base) it confirms that the minimum molecular weight of 1270, derived from amino acid and elementary analyses, is the true molecular weight of tyrocidine A.

The next band in the pattern contained the fully substituted DNP-tyrocidine A. Again using absorption measurements, a value of 1840 was obtained for the molecular weight of the N,O-disubstituted tyrocidine A. This value is somewhat higher than the theoretical figure (1603) for the N,O-disubstituted DNP-derivative of a peptide of molecular weight 1270.

The determination of the sequence of the amino acids in tyrocidine A was accomplished by Paladini and Craig (27a), using partial hydrolysis. When the polypeptide was partially hydrolysed for three hours at 80°C in 10 N-hydrochloric acid, a complex mixture of peptides was obtained, which were clearly separated by the use of C.C.D. and ion exchange chromatography. The many small peptides which were isolated and thoroughly characterised by analysis and molecular weight determinations (25), were then studied by the methods employed by Sanger and his colleagues in their work

on the structure of insulin (27b). In this way all the linkages shown in the structure (I) below were established. Moreover, by isolation of the phenylalanine from a number of the small peptides which contained it, it was possible to show that the two D-residues and the one L-residue were distributed as shown.



(I) The sequence of amino acids in tyrocidine A.

The only unknown feature in this structure is the mode of linkage of the glutamic acid and aspartic acid

residues in the molecule. Each possesses two carboxyl groups and linkage could occur in each case through either of the two groups. The most probable linkage is by way of the α-carboxyl groups, although linkage through the side-chain carboxyls is not unknown in peptides from bacteria, for example the poly D-glutamic acid from B.subtilis. The study of this remaining point will form part of the work described in this Thesis.

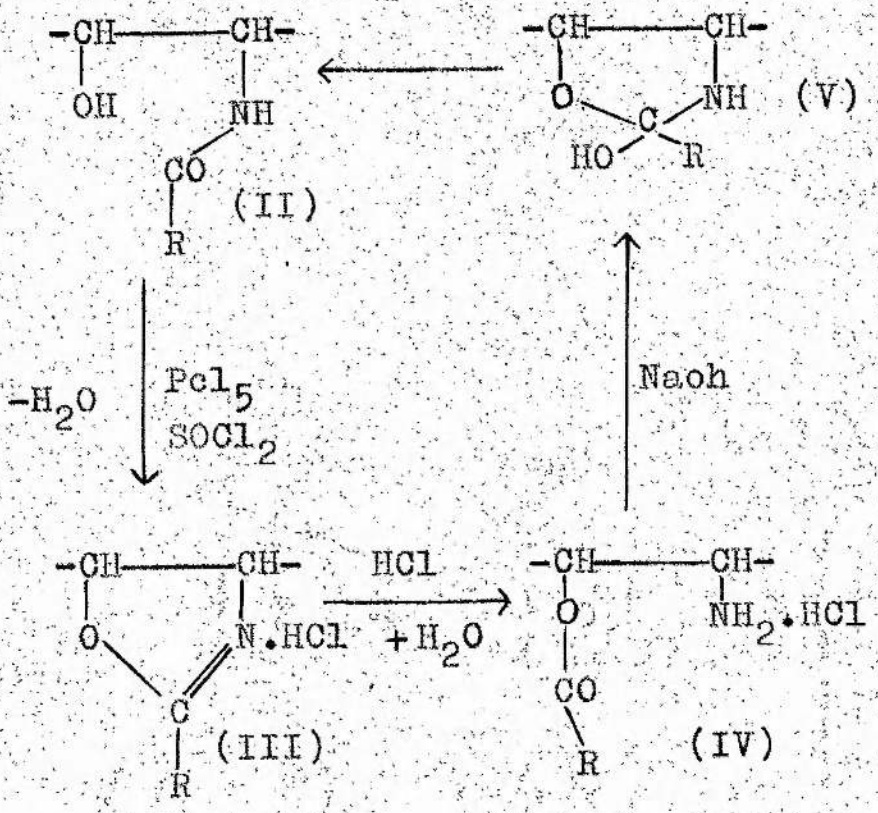
(III). The Specific Fission of Peptide Links by Chemical Means.

(a). Specific fission at points in the peptide chain remote from either end.

Peptides may be degraded for structural study by partial hydrolysis with acids and enzymes, and by stepwise degradation from the ends of the chain. A number of methods are available which permit the latter approach to be used. The structure of a small peptide however is more readily determined by these methods than that of a large one and for this reason it is desirable that the peptide chain should be cleaved specifically at known points along its length.

Elliott (28) appears to have been the first to study this problem, and succeeded in cleaving the peptide chain in silk fibroin at the serine residues. This protein was chosen for study because of its availability, its relatively simple amino acid composition, and its high serine content. Bergmann *et al.* (29a) had observed the migration of acyl groups in acyl derivatives of substances having an amino group and an hydroxyl group on adjacent carbon atoms. The migration of the acyl

group from nitrogen to oxygen, and the reverse transformation, involves a number of steps shown by these workers to be as follows (29b).



Elliott aimed to convert the N peptidyl form (II) of the serine residues in silk fibroin to the O peptidyl form (IV), and to hydrolyse the resulting product at the ester linkages. Desnuelle and Casal (30) had shown that in proteins the peptide bonds involving the nitrogen atoms of serine and threonine were more labile than other peptide bonds to acid hydrolysis, and interpreted their results on similar lines.

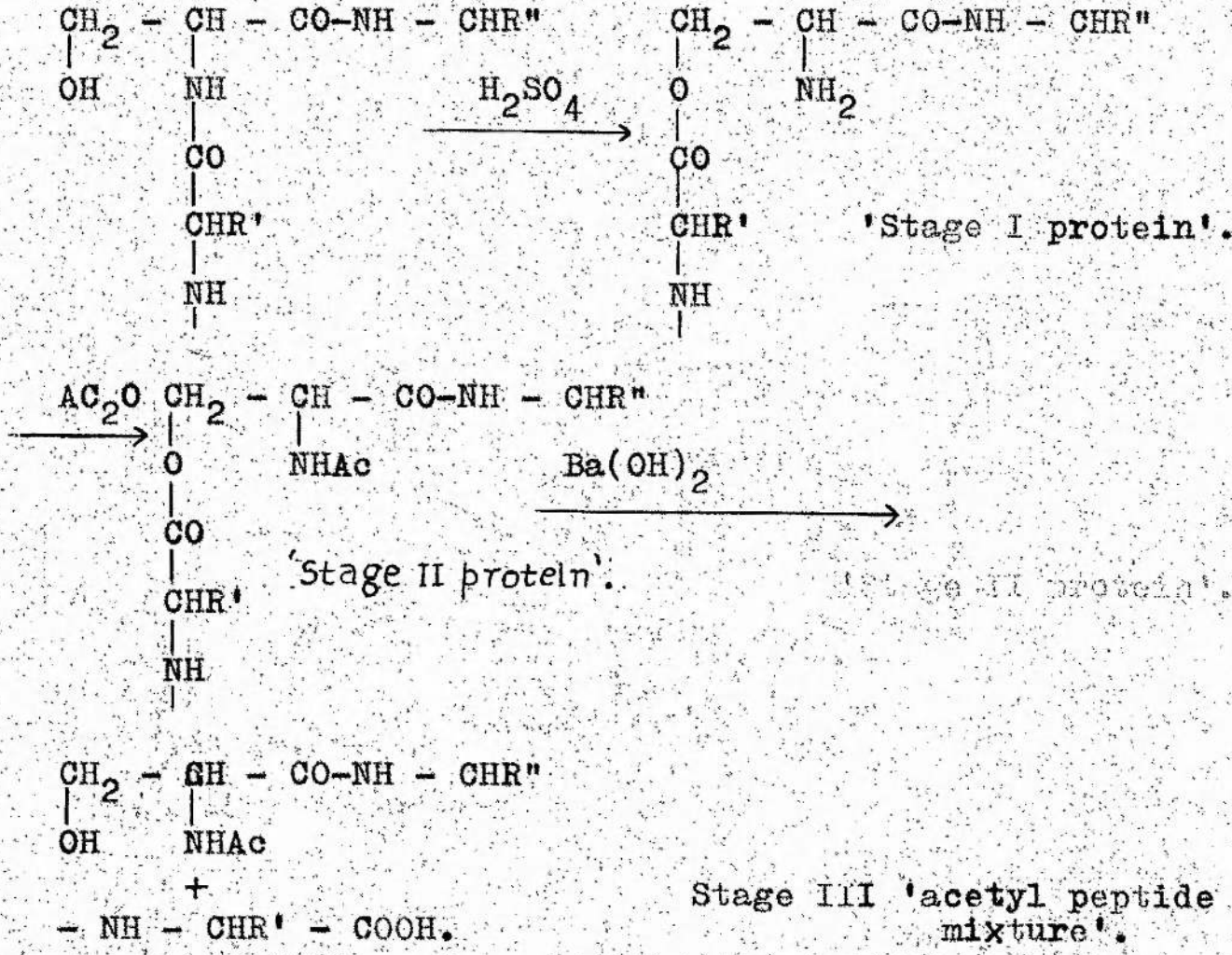
The reagents used previously to bring about the N→O acyl migration had been of a rather drastic nature. (PCl_5, $SOCl_2$, ethanolic HCl), and it was essential for work in the protein field that a reagent should be found which would effect the rearrangement without causing fission of peptide bonds. Reitz and coworkers (31) have shown that if proteins are treated for a short period with anhydrous sulphuric acid no peptide bond fission occurs, Elliott's scheme utilized these conditions in the first step, subsequent stages are shown below.

(i). Treatment of silk fibroin with concentrated sulphuric acid, which gave 'Stage I protein'.

(ii). Acetylation of the product at pH5 to give 'Stage II protein'.

(iii). Hydrolysis of the latter with dilute alkali forming Stage III 'acetyl peptide mixture'.

The reactions used throughout may be illustrated as follows:



Treatment of silk fibroin with 97.5% sulphuric acid at 21° for 3 days gave a product which had the expected properties for the O-peptidyl form. For example the amino nitrogen value (9.2%) was much higher than that of silk fibroin itself (0.55%). This value was much reduced on keeping the product at pH9 for several hours as would be expected due to rearrangement to the N-peptidyl form. Elliott estimated that the number of serine residues involved in the transformation was 62% of the total number present.

The 'Stage I protein' was then treated with acetic anhydride in acetate buffer at pH5 which brought about acetylation of most of the amino groups, only a small proportion resisting this treatment. Having blocked the nitrogen atoms in this way, hydrolysis of the O-peptidyl bonds was possible with dilute alkali, without reversal of the original rearrangement. The resulting 'Stage II protein' was hydrolysed by treatment with excess alkali for 1.5 hours when the expected number of carboxyl groups were released. The last step also converted an indiffusible substance

into a product of which 86% of the nitrogen was diffusible. Elliott's attempts to substitute the amino groups in 'Stage I protein' with 1-fluoro-2, 4-dinitrobenzene were not wholly successful as the number of such groups introduced was much lower than expected. However partial hydrolysis of the DNP-'Stage I protein' with acid gave a mixture of DNP-peptides, which after separation and complete hydrolysis yielded DNP-serine in each case.

Elliott's method has not yet been applied to other substances by workers in the protein and polypeptide field and this may be because the rearrangement and subsequent hydrolysis affect only about 60% of the serine residues.

A second interesting example of the specific fission of a peptide bond was discovered by du Vigneaud *et al.* (34-36), during their studies of the structures of Oxytocin and Vasopressin. They showed that treatment of the hormones or their performic acid oxidation products with bromine water caused a cleavage of the peptide bond between tyrosine and the amino acid joined to its carboxyl group. If this reaction is found to be general for tyrosyl peptides it will clearly be a most valuable one.

III.(b) Selective Chemical Degradation of peptide chains from the amino and carboxyl ends.

The presence of free functional groups at the ends of a peptide chain renders these sites susceptible to attack by chemical reagents, and a number of methods have been described in which a chemical reagent is attached to the terminal amino acid and in subsequent steps the latter is removed from the chain. A method is satisfactory only if it involves few stages, if the reactions used at each stage proceed specifically and in high yield, and if the conditions throughout are mild enough to leave the remainder of the peptide chain unaffected. The older methods used have been reviewed by Fox (35), and the more recent developments by Khorana (36), since this latter publication (1952) several useful procedures have appeared in the literature.

All the methods used in the stepwise degradation of peptide chains which have been described so far may be divided into two groups.

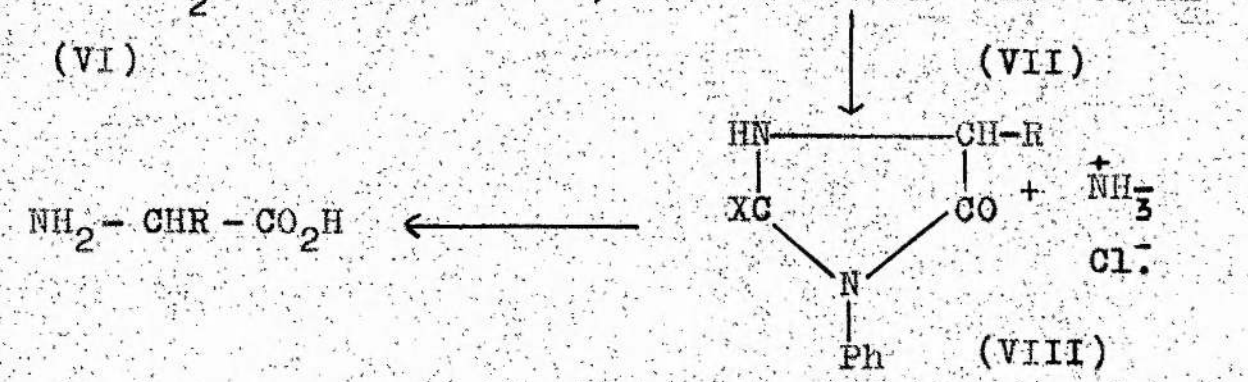
(a) The terminal peptide bond is rendered labile by a fundamental structural change in the terminal amino acid.

(b) A chemical reagent is attached to the free functional group of the terminal amino acid, and cleavage of the terminal peptide bond occurs due to reaction between the attached residue and the terminal amino acid.

Degradation from the amino end.

(i) The use of Aryl Isocyanates or Isothiocyanates.

Bergmann, Miekeley and Kann (37), showed that phenyl isocyanate ((VI), X=O) reacted with the amino group of an amino acid at room temperature or below to yield products which gave hydantoins by hydrolysis.



The possibility of using this reaction as a stepwise procedure was investigated by Abderhalden and Brockmann (38). Results on simple synthetic peptides, using methanolic hydrogen chloride in the hydrolysis step, showed that although the terminal amino acid was split off as the 3-phenylhydantoin, peptide bonds further along the chain were also hydrolysed to a small extent.

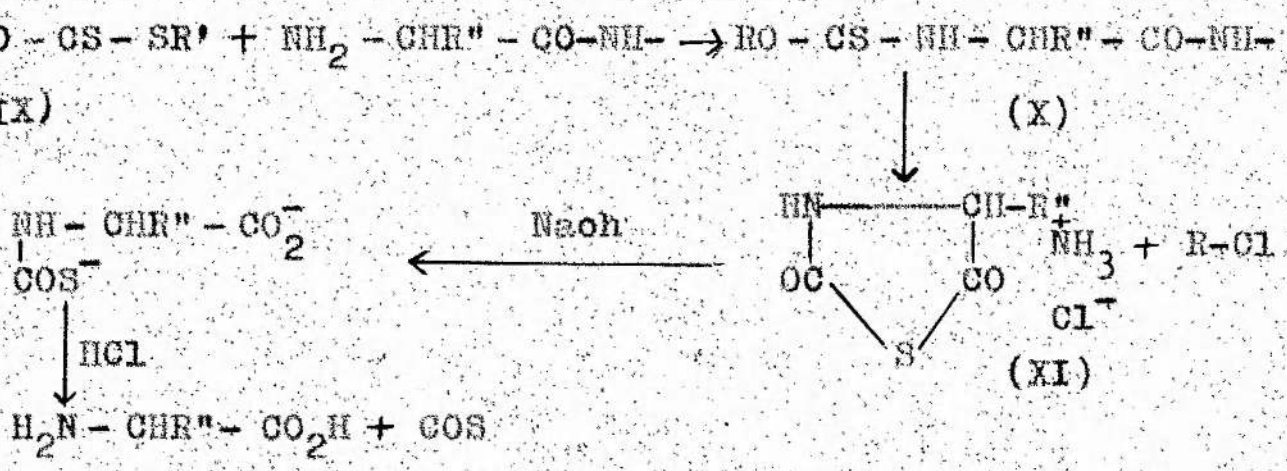
The method has since been improved by Edman (39) using phenyl isothiocyanate (VI, X=S). This substance reacts with the amino group of a peptide at about pH9 to form the phenylthiocarbamyl derivative (VII, X=S). Cleavage of the latter in anhydrous nitromethane saturated with hydrogen chloride affords the 3-phenylthiohydantoin (VIII, X=S), and the hydrochloride of the amino acid or peptide lacking the terminal amino acid.

Edman's method has been studied more fully than any other, and although applied successfully to a number of simple synthetic peptides, certain difficulties are encountered when used on larger molecules. Landmann, Drake and Dillaha (40) used the method to determine the N-terminal pentapeptide sequences of lysozyme and insulin, but in neither case was it possible to continue the degradation. It was only partly successful in the degradation of the hexapeptide alanylglycylvalylaspartylalanylalanine liberated during the transformation of ovalbumin into plakalbumin (41a) and of oxidised vasopressin (34). These difficulties are due to the impossibility at present of forming the thiohydantoin ring without causing a small degree of peptide bond fission.

The very low solubility of the phenylthiocarbamyl derivatives of peptides was an initial disadvantage of the method since the cleavage step had to be carried out using a suspension in anhydrous acid. Fraenkel-Conrat and Harris (41b) have shown however, that a solution of the derivatives in aqueous acid may be employed in this step. Despite these difficulties however the method is a valuable one, made more so since paper chromatographic methods are now available for the identification of the 3-phenylthiohydantoin derivatives of amino acids.

(ii). Dialkyl xanthates.

The possible use of these substances in the stepwise degradation of peptide chains was investigated by Khorana, and Kenner and Khorana (42), who reacted the sodium salts of peptides at room temperature with dialkyl xanthates. (IX, $R' = CH_3$).

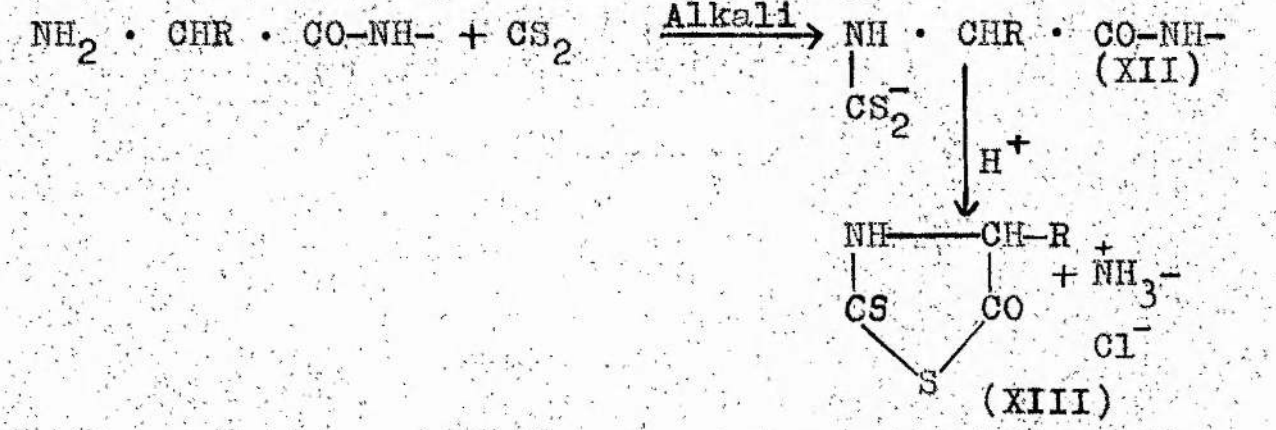


The N-thioncarbalkoxy derivatives (X) which are formed are fairly soluble in organic solvents, and can thus be separated from any unreacted peptide by extraction of (X) into organic solvents, generally ethyl acetate containing acetic acid. The derivatives (X) are fairly soluble also in anhydrous inert solvents, and when their solution in anhydrous nitromethane is treated with hydrogen chloride, as in Edman's method, easier cleavage occurs than with the ~~less~~ soluble phenylthiocarbamyl derivatives (VII, X=S). Since the hydrochloride of the degraded peptide or amino acid is insoluble, the soluble cyclic intermediate (presumably XI), can be isolated, and the terminal amino acid regenerated after a period of brief hydrolysis. This method seems to be one of the best available and has been applied successfully

to a number of simple synthetic peptides.

(iii). Formation of N-dithiocarboxypeptides.

The reaction of carbon disulphide under alkaline conditions with the amino group of a peptide to give the N-dithiocarboxy-peptide (XII), and subsequent removal of the terminal amino acid as a 4-alkyl-2-thiothiazolid-5-one (XIII), was utilized by Levy (43) in a scheme of degradation, shown below

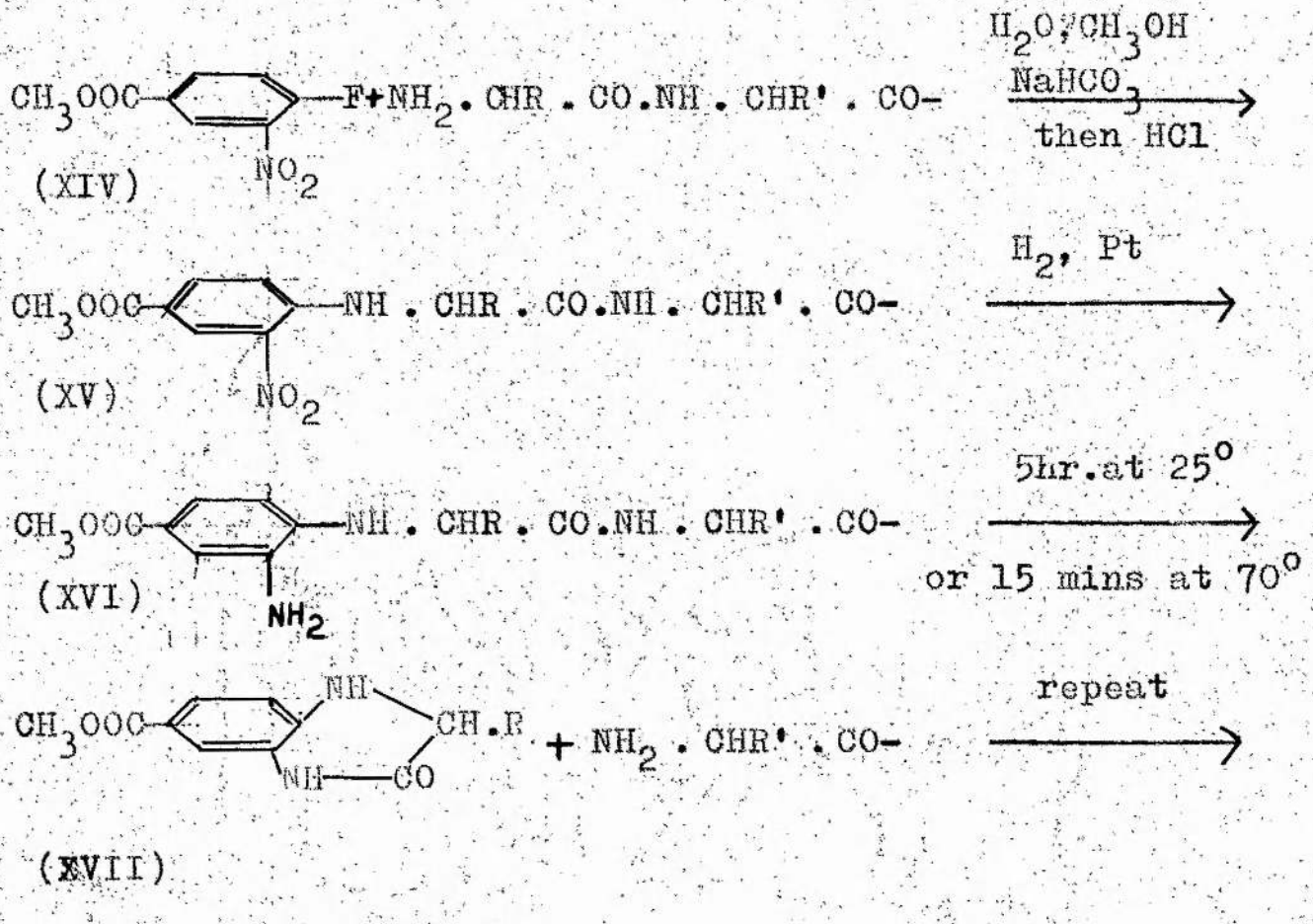


The reaction was shown to occur with simple synthetic peptides, but as the formation of (XII) and the cleavage step are carried out in the same aqueous solution, contamination of the degraded peptide with the original peptide is possible.

(IV). The use of 4-carbomethoxy-2-nitrofluorobenzene.

The introduction of this reagent by Holley and Holley (44) for the stepwise degradation of peptide chains differs

from the approaches discussed so far but still involves the use of cyclic intermediates. The steps in the degradation may be outlined as follows:



4-Carbomethoxy-2-nitrofluorobenzene (XIV) gives practically quantitative yields of the N-(4-carbomethoxy -2-nitrophenyl) peptide (XV) after reaction with the peptide for 24 hours at 35°. After acidification, the derivative is removed

by filtration or extraction, and in this way unreacted peptide is not carried forward, nor do inorganic salts accumulate. The sodium salts of (XV) is smoothly reduced by catalytic hydrogenation over platinum oxide, and affords after neutralisation the N-(2-amino-4-carbomethoxyphenyl) peptide (XVI). Cyclisation to 7-carbomethoxy-3,4-dihydro-2(1H)-quinoxalone (XVII) takes place rapidly in aqueous solution, and is complete in 5 hours at room temperature or 15 minutes at 70°. The sparingly soluble dihydroquinoxalone is filtered off, and the process can be repeated on the residue obtained from the filtrate by evaporation. Dihydroquinoxalones are nicely crystalline compounds, and are identified by comparison with authentic samples prepared directly from amino acids. The derivatives retain their optical activity when prepared under these conditions.

The method has been successfully applied to the degradation of simple synthetic peptides on a small scale, and in these the average yield for each amino acid residue removed was claimed to be 84%. This would permit repetition for 10 amino acid residues with an over-all yield of 17%, and the repetition for 20 amino

acids with an over-all yield of 3%. So far the degradation has only been applied to peptides containing the simplest amino acids, and the yield may fall when more complex residues are present. The sulphur containing amino acids, cysteine, cystine, or methionine might well cause difficulty in the reduction step, but this is not insurmountable. The presence of amino or hydroxyl groups on the side chains of certain amino acids will lead to other problems arising from the 2-amino-4-carbomethoxyphenyl groups substituted in these positions. The application of this method to larger naturally occuring peptides is awaited with much interest.

Degradation from the Carboxyl end.

This has proved to be relatively difficult, although several methods have been proposed. The older methods which have been reviewed by Fox (35) probably suffice only for the identification of the carboxyl end group; the following procedures however do offer hope that at least several stages of a given degradation may be possible from the carboxyl end.

(V). The Bergmann-Zervas Degradation (45).

The benzoylated peptide ester (XVIII) is converted through the hydrazide and azide to the benzyl-urethane (XIX). Hydrogenation of the latter gives the aldehyde with one less carbon than the terminal amino acid. The benzoyl peptide amide (XX) is left as a residue, and the process can then be repeated.

$$\underset{\text{(XVIII)}}{Ph.CO.NH.CHR.CO_2.R'} \xrightarrow{N_2H_4} Ph.CO.NH.CHR.CO.NH.NH_2 \xrightarrow{HNO_2}$$

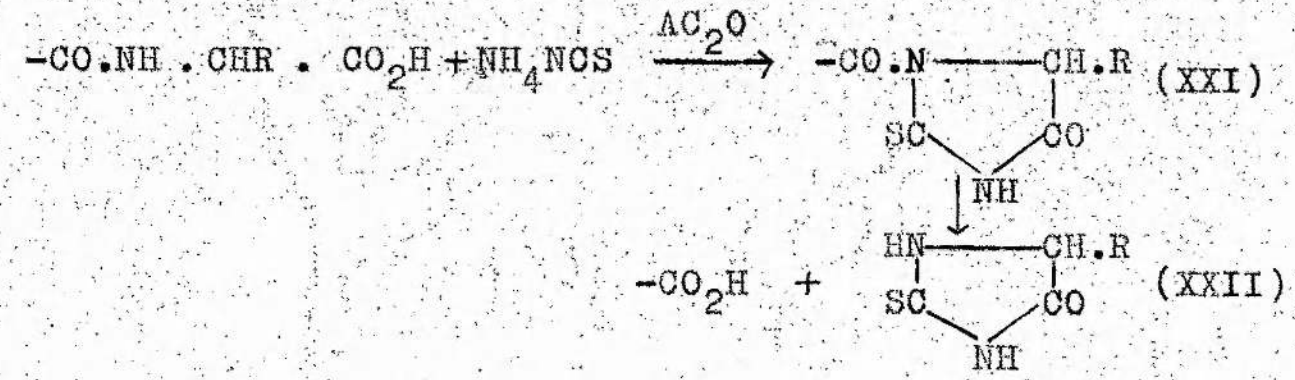
$$Ph.CO.NH.CHR.CON_3 \xrightarrow{PhCH_2OH} \underset{\text{(XIX)}}{Ph.CO.NH.CHR.NH.CO.O.CH_2Ph}$$

$$\xrightarrow{H_2/Pd} \underset{\text{(XX)}}{Ph.CO.NH_2} + R.CHO + NH_3 + CO_2 + Ph.CH_3$$

In this way Bergmann and Zervas successfully degraded the tetrapeptide glycyl-L-alanyl-L-leucyl-L-glutamic acid. The isolation of β-amino-propionic aldehyde in the first step of the degradation shows that the side chain carboxyl group of glutamic acid does not cause difficulties in the degradation when the latter is the terminal amino acid, an important advantage of this method.

(VI). Thiohydantoin formation.

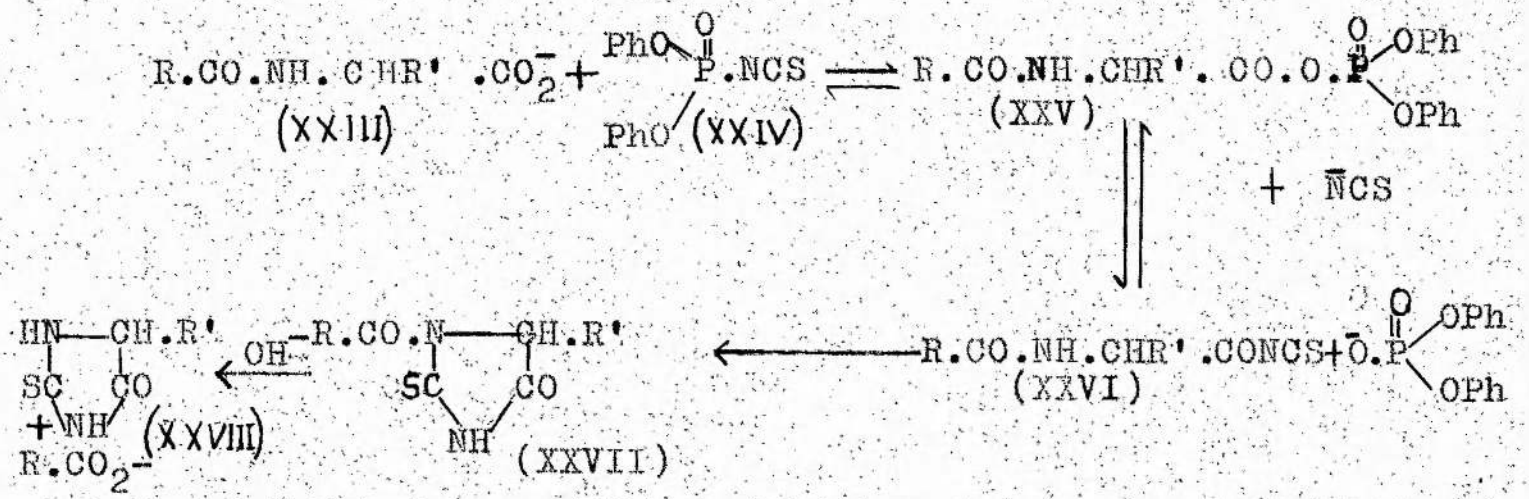
Schlack and Kumpf (46) were the first workers to attempt the degradation of peptide chains from the carboxyl end by way of thiohydantoins. In their method, an N-acyl peptide is heated with acetic anhydride and ammonium thiocyanate to form (XXI) which decomposes readily in alkali to the thiohydantoin (XXII) derived from the terminal amino acid, and the degraded peptide.



Although used successfully on model di-and tripeptides by the original workers, one questions the usefulness of the method when applied to larger naturally occuring molecules, since the conditions and reagents used throughout are vigorous ones. Moreover the yield of thio-hydantoin by this method is far from quantitative. The method has been re-examined by Whaley and Watson (47) and Tibbs (48). The former workers showed that

the hydrolytic fission of (xxi) by N-alkali used by Schlack and Kumpf was unnecessarily drastic, and that much more dilute alkali would suffice. Acid hydrolysis however appears to be preferable in this step to alkaline hydrolysis which leads to opening of the thiohydantoin ring. (49, 51). The findings of Baptist and Bull (49a) that the method appears to fail when aspartic acid, glutamic acid, lysine or arginine form the end groups, and that crystalline thiohydantoins from histidine and arginine have not been prepared, are also important (49b). As a qualitative method for end group determination it appears useful, and has given unequivocal results on lysozyme, bovine plasma albumin, and ovomucoid (50). The identification of the thiohydantoins is now greatly simplified by paper chromatographic methods.

Attempts to form acylthiohydantoins in high yield under mild conditions were made by Kenner, Khorana and Steadman (51), using diphenylphosphoroisothiocyanatidate (XXIV). Their method of degradation may be shown as follows:



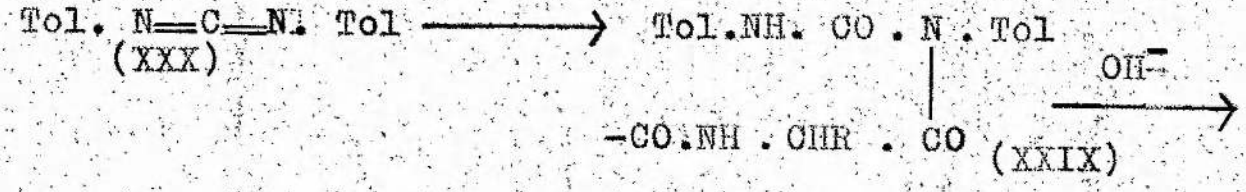
A solution of the triethylammonium salt of an N-acyl-peptide is treated with the reagent (XXIV), a mixed anhydride. The anion (XXIII) of the relatively weak oxyacid displaces the isothiocyanate ion forming an acyl-phosphate (XXV), which in turn is attacked by the isothiocyanate ion giving the desired acyl-isothiocyanate (XXVI). The subsequent isomerisation of (XXVI) into the acylthiohydantoin (XXVII) is effectively irreversible, and so the whole process goes to completion. A number of simple acylthiohydantoins were prepared in high yield (87-100%).

Following the results of Whaley and Watson (47), the acylthiohydantoins were hydrolysed by two equivalents of 0.01N sodium hydroxide for 30 minutes at room temperature, which gave satisfactory but by no means quantitative yields of the desired products. It was found that the thiohydantoin ring in both (XXVII) and

(XXVIII) is attacked by alkali. The method had only been applied to peptides containing the simplest amino acids, and difficulties may arise in those cases where aspartic acid or glutamic acid form the end group. Despite these drawbacks however the method seems one of the best yet proposed for stepwise degradation from the carboxyl end of the peptide chain.

(VII). <u>The use of Carbodi-imides</u>.

Khorana (52) discovered that N-acyl-peptides react with di-p-tolylcarbodi- imide (XXX) at room temperature to give acylureas (XXIX) in good yield.



$-CO_2H$ + Tol. NH . CO .NH . CHR . CO.NH.Tol
(XXXI)

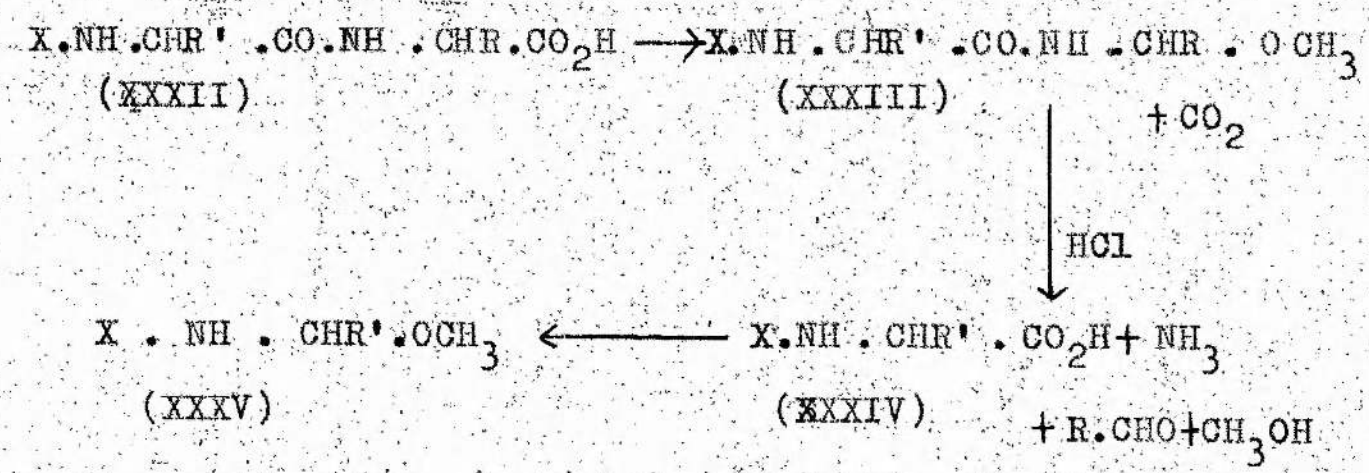
Tol = p-C_6H_4Me.

The acylureas are immediately split by cold dilute alkali to form (XXXI), and the degraded peptide or amino acid; alkaline hydrolysis of (XXXI) liberates the terminal amino acid. The conditions used are

mild, and the method has been applied to a number of small synthetic peptides. A distinct disadvantage is that the degraded peptide is sometimes seriously contaminated with the starting material (36, 51).

(VIII). Degradation by Anodic Oxidation.

A novel method for the degradation of peptide chains from the carboxyl end was introduced by Boissonnas (53). His scheme which is based upon the work of Linstead et al.(54). may be depicted as follows:

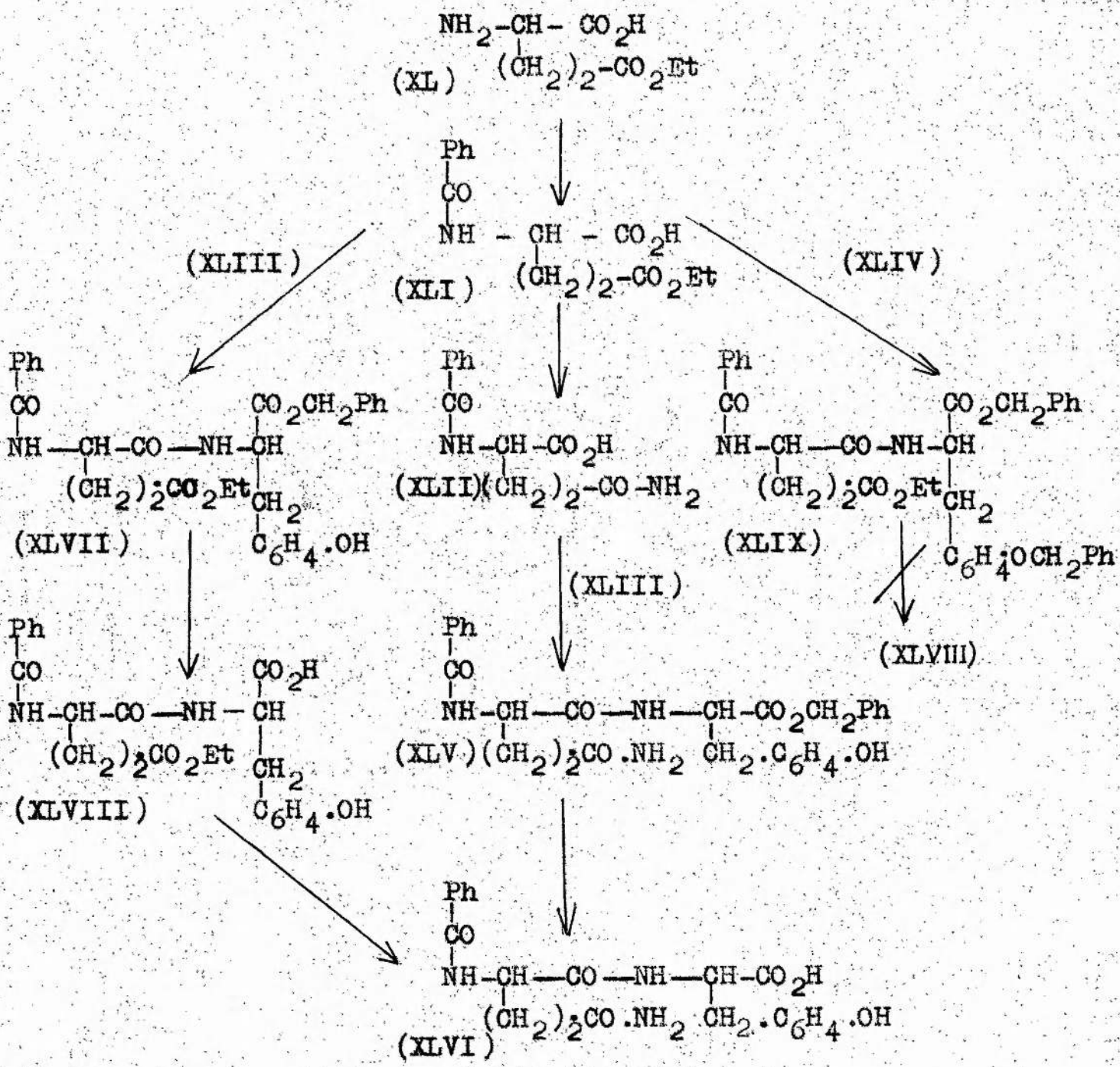


X= dinitrophenyl or $Ph.CH_2.O.CO-$

The protected peptide (XXXII) is electrolysed in anhydrous methanol at 0° for 1-3 hours, to form the methoxy-compound,(XXXIII). Brief acid hydrolysis (20% HCl, 15 mins, 100°) of the latter liberates the

aldehyde, and the amide function is converted to a carboxyl group; and the degraded peptide (XXXIV) may be isolated. When a totally hydrolysed portion of (XXXIV) is compared on a paper chromatogram with the hydrolysis products of the original peptide (XXXII), the missing spot corresponds to the C-terminal amino acid in (XXXII). The degraded peptide may be further anodically oxidised to give (XXXV), and the procedure repeated. The method has the advantage that it can be used on very small quantities (a few mgm), and has been successfully used to degrade a tripeptide. So far only peptides containing simple amino acids have been used as models; when hydroxy-amino acids are present, non-specific hydrolysis may occur in the treatment with hydrochloric acid.

It will be seen from the foregoing outline that the more promising methods of stepwise degradation of peptides are those starting from the amino-end of the chain. Although many of the methods have been applied successfully to simple synthetic models, the assessment of their true value in structural studies can only be judged when they have been tried on larger naturally occuring molecules.



$NH_2-CH(CH_2-C_6H_4-OR)-CO_2CH_2Ph$

(XLIII), R=H.
(XLIV), $R=CH_2Ph$

Fig.1.

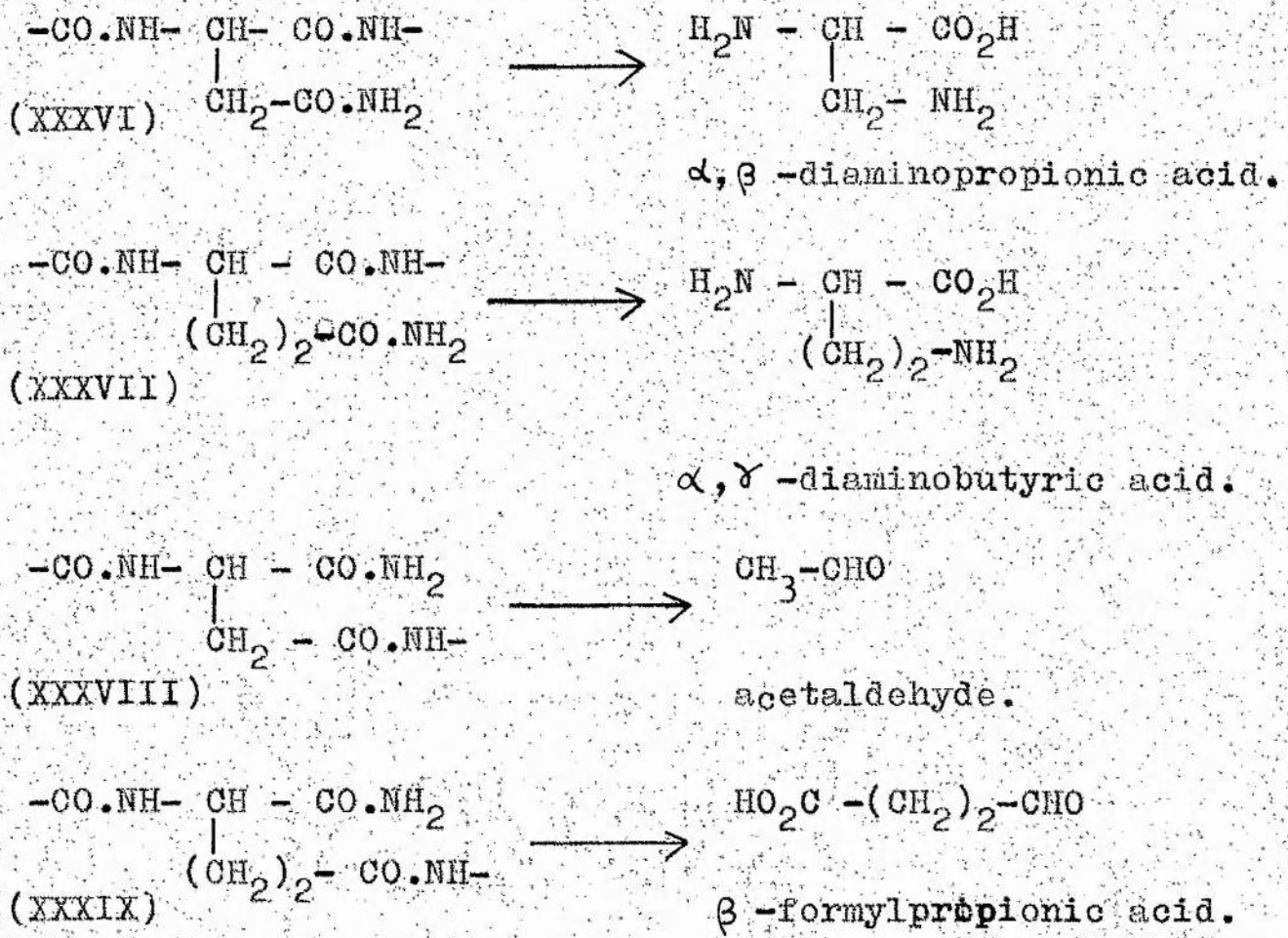
The preparation of N-Benzoylglutaminyl-L-tyrosine (XLVI)

(IV). Theoretical Section.

The work which is to be described falls into two parts. The first deals with the linkage of the aspartic acid and glutamic acid residues in tyrocidine A. The second part covers the chemical methods which have been devised with the object of specifically cleaving the peptide chain at known points along its length.

(a). The elucidation of the mode of linkage of the aspartic acid and glutamic acid residues in tyrocidine A.

It will be apparent from the description of the chemical features of tyrocidine A which has already been given that asparagine (XXXVI), glutamine (XXXVII), isoasparagine (XXXVIII) and isoglutamine (XXXIX) residues may occur in the polypeptide. It seemed therefore that a solution to this problem might well be obtained if tyrocidine A was submitted to the Hofmann degradation, and the product subsequently hydrolysed. Under these conditions structures (XXXVI) and (XXXVII) would be expected to give α,β-diaminopropionic acid and α,γ-diaminobutyric acid respectively, whilst the alternative structures (XXXVIII) and (XXXIX) should yield acetaldehyde and β-formylpropionic acid respectively.



In view of the difficulty in preparing tyrocidine A in quantity, it was decided to carry out the preliminary experiments on a simple synthetic peptide of glutamine. N-Benzoyl-glutaminyl-L-tyrosine (XLVI) was chosen because tyrosine seemed to be the only amino acid in tyrocidine A likely to interfere in the Hofmann reaction (55).

γ -Ethyl L-glutamate (XL) was prepared by the method of Miller and Waelsch (56). Attempts to benzoylate (XL) using benzoyl chloride and potassium carbonate gave very unsatisfactory results, γ -ethyl N-benzoyl-L-glutamate (XLI) being produced only in about 10% yield. These difficulties were to be expected however as the result of partial racemisation under the alkaline conditions used (57). A suitable method of preparing (XLI) was found however by using the conditions of Heghedus (58) for the preparation of the corresponding γ -ethyl N-benzyloxycarbonyl-L-glutamate. A solution of γ -ethyl-L-glutamate (XL) in water containing sodium bicarbonate and magnesium oxide when treated with a slight excess of benzoyl chloride at 0° gave γ -ethyl N-benzoyl-L-glutamate (XLI) in satisfactory yield.

The first method of preparing the glutaminyl dipeptide (XLVI) from (XLI) and the benzyl ester of L-tyrosine (XLIII) is shown in the left hand limb of fig. 1. The latter component (XLIII) was prepared in admixture with the benzyl ester-benzyl ether of L-tyrosine (XLIV) by a modification of a recent method

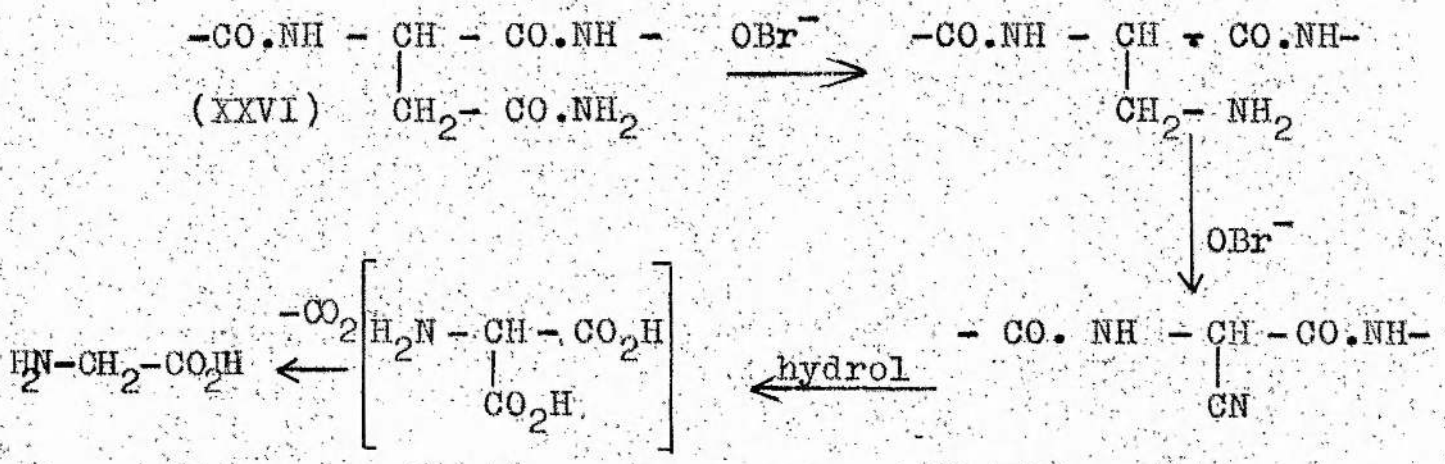
of direct esterification using p-toluenesulphonic acid as catalyst (59). The two products were readily separated by virtue of the differing solubilities of their hydrochlorides in ethanol. Both esters were hydrolysed to tyrosine by mild treatment with dilute hydrochloric acid, which is in accord with the known sensitivity of benzyl ethers to acidic hydrolysis (60). Also the benzyl groups in both tyrosine derivatives (XLIII) and (XLIV) were cleaved quantitatively by hydrogenation over palladium-charcoal catalyst at room temperature without the phenolic nucleus suffering reduction.

Coupling of (XLI) and (XLIII) was accomplished by the mixed anhydride method (61). The components were dissolved in dioxan-toluene to avoid racemisation (Vaughan, (62)), and a fair yield of the dipeptide γ-ethyl N-benzoylglutamyl-L-tyrosine benzyl ester (XLVII) was obtained. Catalytic hydrogenation of (XLVII) in ethanol-glacial acetic acid using palladium-charcoal catalyst afforded a resin, which on crystallisation from aqueous-ethanol gave γ-ethyl N-benzoylglutamyl-L-tyrosine (XLVIII) in low yield. Treatment of

(XLVIII) with saturated alcoholic ammonia yielded a product, a portion of which after hydrolysis gave a positive test for ammonia (63), thus indicating that at least some of the glutaminyl dipeptide (XLVI) had been formed. The other route to this latter compound, represented by the right hand limb of fig.1. was less successful, for although the dipeptide γ-ethyl N-benzoyl-glutamyl-L-tyrosine benzyl ester-benzyl ether (XLIX) could be prepared in the same way as used in the preparationnof (XLVII), difficulty was experienced when attempts were made to hydrogenate the former compound. Complete removal of the benzyl groups by catalytic hydrogenation did not occur, despite using platinum oxide and palladium charcoal in succession as catalysts. The small amount of acidic product isolated was different from (XLVIII).

The amide derived from (XLVIII) by the first method was submitted to the Hofmann degradation, using sodium hypochlorite to avoid halogenation of the phenolic nucleus. Using a slight excess of the reagent solution, (1.2 equivalents of sodium hypochlorite/amide group) (55), the reaction was carried out at 70-80^{0}. The

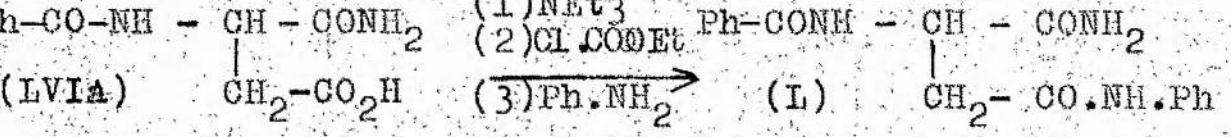
product was isolated and hydrolysed, and a portion of the hydrolysate chromatographed. In addition to the spots corresponding to glutamic acid and tyrosine, a new spot in the right position for α,γ-diaminobutyric acid was present. Tyrocidine A, which had been obtained pure by fractionating tyrocidine hydrochloride by C.C.D. (23), was treated with sodium hypochlorite using the conditions which had been found sucessful on the simple model. The Hofmann degradation product when hydrolysed and examined by two-dimensional paper chromatography, gave no evidence that either α,β-diaminopropionic acid or α,γ-diaminobutyric acid were present in the hydrolysate. Methanol had however been used as solvent, and as this was found to react with the sodium hypochlorite, the experiment was repeated using purified dioxan (64) as solvent. The hydrolysate when chromatographed again showed no spots corresponding to the desired basic amino acids. However, a new spot, in the right position on the chromatogram for glycine was noted. This amino acid is not a constituent of tyrocidine A, but a possible source is from the asparagine residue (XXXVI) by the following reactions:



Higher aliphatic amines are known to be largely oxidised to nitriles by an excess of hypobromite (65). An attempt to hydrogenate the intermediate nitrile which could arise by this series of reactions, was made in a separate experiment. The Hofmann degradation product of tyrocidine A was hydrogenated prior to hydrolysis, using Raney-nickel as catalyst; the activity of which had been previously demonstrated in the successful reduction of cyanoacetic ester. The product from the hydrogenation was then hydrolysed and chromatographed; the spot corresponding to glycine was still present, furthermore no new spot in the right position for α , β -diaminopropionic acid had been produced.

Attention was next turned to the possibility that isoasparagine (XXXVIII) and isoglutamine (XXXIX) residues

may occur in tyrocidine A. N-Benzoyl-DL-isoasparagine (LVIa, fig.2.) was submitted to Hofmann degradation and the product hydrolysed, using Barker's conditions (66) for the formation and isolation of acetaldehyde from isoasparagine derivatives. Also N-benzoyl-DL-isoasparagine β -anilide (L) was used in extended investigations, and was prepared from N-benzoyl-DL-isoasparagine (LVIa), and aniline by the mixed anhydride method.



Acetaldehyde was produced from both model substances (LVIa) and (L)under the above conditions, and was easily isolated on a very small scale, and characterised as its p-nitrophenylhydrazone. The degradation was then applied to tyrocidine hydrochloride, being more readily available than tyrocidine A, but no trace of acetaldehyde could be detected. On account of the ready production of acetaldehyde by Hofmann degradation followed by hydrolysis, of (LVIa) and (L), it was thought that this result from tyrocidine hydrochloride

was more reliably negative than the experiments described above in which the basic amino acids α,β-diaminopropionic acid and α,γ-diamino-butyric acid had been sought. Thus it was decided to study the effect of varying the concentration of OBr^- on N-benzoyl-glutaminyl-L-tyrosine (XLVI) to find the best concentration for the production of α,γ-diaminobutyric acid.

A much improved preparation of (XLVI) was found when the middle route of fig.1. was used to prepare more of (XLVI) for the trial experiments. N-Benzoyl-L-glutamine (XLII) was prepared in very good yield by treating γ-ethyl N-benzoyl-L-glutamate (XLI) with aqueous ammonia at room temperature. This product was then coupled by the mixed anhydride method with the benzyl ester of L-tyrosine (XLIII), using dioxan\toluene as before to avoid racemisation. N-benzoylglutaminyl-L-tyrosine benzyl ester (XLV) was ohtained in three crops which differed in their solubility properties and m.p.'s. That all three were in fact (XLV) was shown by hydrolysing portions of them, and examining the hydrolysates by paper chromatography. Spots corresponding to glutamic acid and tyrosine were obtained in each case. One crop

was catalytically hydrogenated in ethanolic solution using palladium-charcoal catalyst, and gave N-benzoyl-glutaminyl-L-tyrosine (XLVI) in excellent yield. A portion of the product was hydrolysed under acidic conditions, and the glutamic acid and tyrosine were separated by paper chromatography in the system formic acid/sec-butanol/water. The portion of the paper containing the glutamic acid was cut out, eluted with water, and the glutamic acid recovered from the aqueous eluate by evaporation. The optical rotation was determined on a solution of the glutamic acid obtained, $[\alpha]_D^{17} = -20.3^\circ$(C,1.479 in 8-N HCl). This value assumes that the glutamic acid was eluted as the hydrochloride. L-glutamic acid has $[\alpha]_D^{22.4} = +31.2^\circ$(C,1.0 in 6N-HCl) (67). This result suggests that the glutamic acid residue in (XLII) had been partially racemised during the formation of (XLV) by the mixed anhydride method, although the published precautions were taken to avoid such racemisation.

When the Hofmann degradation of (XLVI) was repeated using increasing concentrations of OBr^-, it was found that, quite contrary to the literature (55),

an excess of sodium hypobromite gives the best results with this type of compound. The best yield of α,γ-diaminobutyric acid obtained was of the order of 20%, as judged by the intensity of the spots on the paper chromatogram. The conditions which had given this yield were then used on pure tyrocidine A. The hydrolysate when examined by two dimensional paper chromatography showed that two new spots had been produced by the Hofmann reaction, the positions of which coincided exactly with those of α,β-diaminopropionic acid and α,γ-diaminobutyric acid on a replicate paper chromatogram. The spot corresponding to glycine and already noted on a previous chromatogram (p.58) was also present on this paper. In addition the intensity of the ornithine spot had been much diminished, whereas the glutamic acid spot was much stronger than usual. This result supports both the chromatographic evidence that the above spot is glycine and also its method of formation from the asparagine residue, given on p59. If the mechanism suggested there is correct the δ-amino group of ornithine would be expected to behave similarly,

and glutamic acid would result from the ornithine residue. The paper chromatogram also showed that the tyrosine spot had disappeared, the other amino acids which occur in tyrocidine A were all present.

In order to establish that the spots corresponding to α,β-diaminopropionic acid, and α,γ-diaminobutyric acid were in fact these basic amino acids it was necessary to isolate and characterise them. The picrate was chosen as a suitable derivative, and that from L-α,γ-diaminobutyric acid is known (68). The picrate of L-α,β-diaminopropionic acid was prepared, and microanalysis of the derivative gave figures in close agreement with those for the dipicrate monohydrate; although the monopicrate hemihydrate could not be excluded by analysis alone. The m.p.(141.5 -144°) of the specimen rose on standing to 184°, and a repeated microanalysis on this specimen gave figures which agreed closely with those found on the lower melting form. The molecular weight of the derivative melting at 184° was determined by a spectrophotometric method due to Springet al. (69); the result obtained showed that the dipicrate monohydrate had been formed. Thus L-α,β-diaminopropionic acid forms a

dipicrate monohydrate, which is dimorphous, the lower melting being converted to the higher melting form on standing.

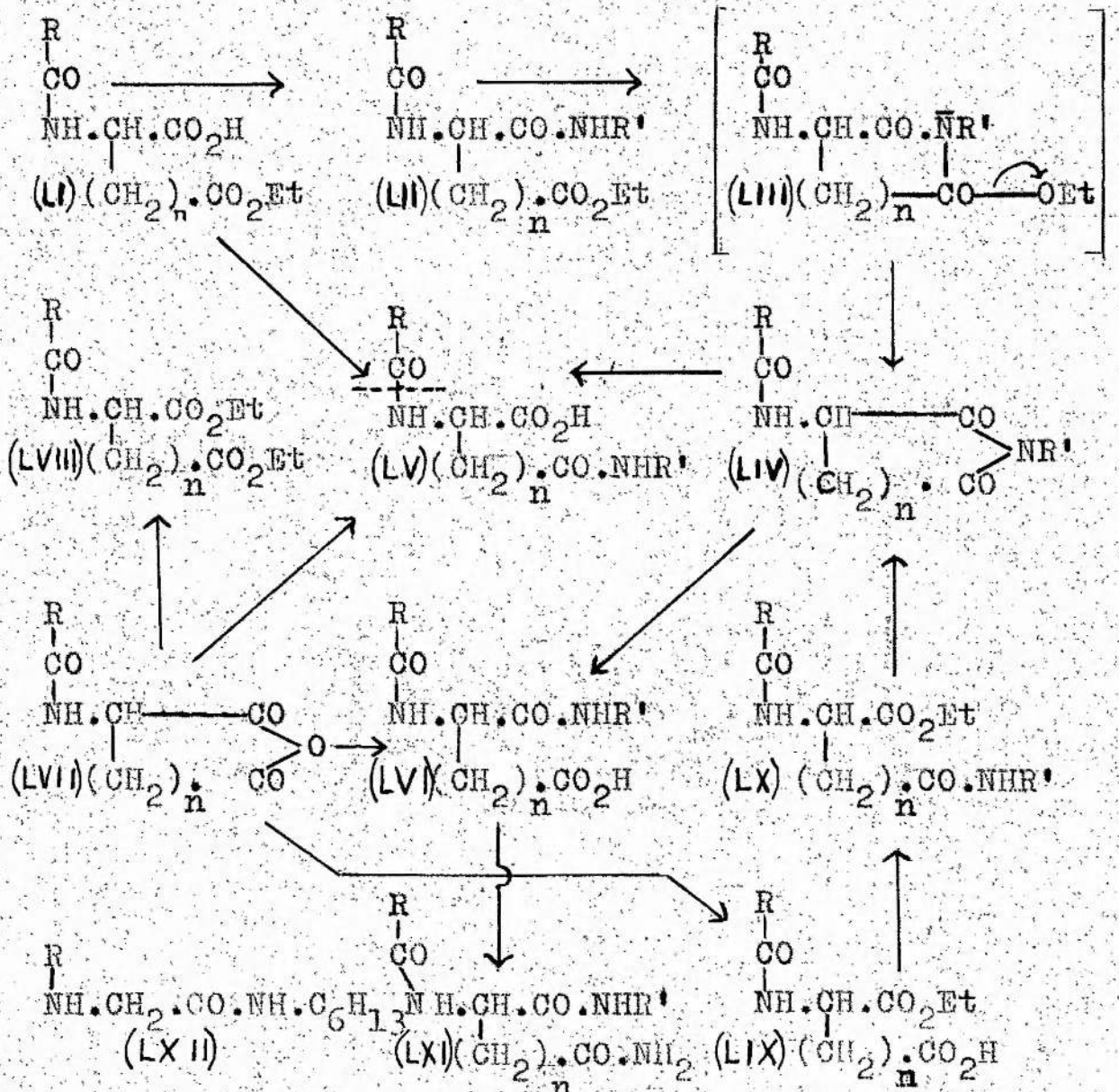
Tyrocidine A was submitted to Hofmann degradation using the conditions of the preceeding experiment. The hydrolysate when examined by two dimensional paper chromatography, showed that the supposed α,β-diaminopropionic acid and α,γ-diaminobutyric acid were present to the extent of some 20-25 mgm. each (5% yield). One half of the hydrolysate was chromatographed in the system formic acid/sec-butanol/water, and the basic amino acids were isolated from the paper by elution with water. They were then separated on a further paper-chromatogram using the system phenol/water/ammonia, the portions containing the basic amino acids sought being cut out and separately eluted. Evaporation of the aqueous eluates gave residues which were treated with picric acid. The residue from the supposed α,β-diaminopropionic acid yielded a picrate the m.p. of which indicated that it was L-α,β-diaminopropionic acid dipierate.monohydrate (low melting form). The m.p. was raised to that of the higher melting form in admixture

with an authentic sample of this form. The supposed α,γ-diaminobutyric acid gave a picrate whose m.p. and mixed m.p. showed it to be L-α,γ-diaminobutyric acid dipicrate.

Better evidence that α,β-diaminopropionic acid was present in the hydrolysate was obtained by ion-exchange chromatography of the remaining half (p.65) using IRC-50 (70). Ornithine, α,γ-diaminobutyric acid, and α,β-diaminopropionic acid were shown to be retained on a buffer free column of this resin at pH 4.7, when it was eluted with water. These amino acids were quantitatively removed by elution with hydrochloric acid. When the hydrolysate above was treated in the same way, and the acidic eluate examined by two dimensional paper chromatography it was apparent that losses of the basic amino acids had occured during the elution of the column with water some 75% (based on the original estimate, p.65) being lost in this way. Nevertheless it was possible to isolate them from the acidic eluate by paper-chromatography and elution with water as before. The picrate of the supposed α,β-diaminopropionic acid which was obtained was shown by m.p.

and mixed m.p. to be L-α,β-diaminopropionic acid dipicrate monohydrate, (low melting form).

The fact that α,β-diaminopropionic acid and α,γ-diaminobutyric acid arise by Hofmann degradation of tyrocidine A, followed by hydrolysis, shows clearly that asparagine (XXXVI) and glutamine (XXXVII) residues occur in the polypeptide, and it follows that the linkage of these amino acids in the peptide chain is in both cases by way of the α-carboxyl group.



Series (a): n=1, R=Ph, R'=H.

Series (b): n=1, R=Ph, R'=$CH_2.CO.NH.C_6H_{13}$.

Series (c): n=2, R=Ph, R'=H.

Series (d): n=2, R=Ph, R'=$CH_2.CO.NH.C_6H_{13}$.

Series (e): n=2, R=$CH_2Ph.O.CO.NH.CH_2.CO.NH.CH_2$, R'=$CH_2.CO.NH.C_6H_{13}$.

Series (f): n=2, R=$NHAc.CH_2CO.NH.CH_2$, R'=$CH_2.CO.NH.C_6H_{13}$

Fig. 2. The rearrangement of aspartyl and glutamylpeptides.

(IV).(b). The specific fission of peptide links by chemical means.

A possible method for specifically cleaving the peptide chain at the nitrogen atoms of aspartic acid and glutamic acid involves first the rearrangement of the normal α-aspartyl and α-glutamyl peptides (LVI; n = 1 and 2) to give the β- and γ-isomers (LV; n = 1 and 2) respectively. The latter possess the group $-CO.NH.CHR.CO_2H$ and thus differ from the carboxyl end of a peptide chain only in the nature of the group R. The methods which are available for the selective removal of the C-terminal residue, and of which an outline has already been given (see pp. 44-51), should therefore be applicable to structures of this type, the peptide chain being cleaved as indicated in (LV).

(i). The rearrangement of aspartyl and glutamyl peptides.

The obvious intermediate for the required rearrangement is the imide of type (LIV), the ready hydrolysis of which under mild alkaline conditions is already well known (Titherley and Stubbs (71)). It remained to establish that the desired hydrolysis product of type (LV) was obtained in satisfactory yield. Attack by hydroxyl ion on the above imide should be governed in

part by the relative electrophilic character of the two carbonyl groups in the ring, and should occur to a greater extent on the α -carbonyl group (corresponding to the stronger carboxyl group). The amount of the acidic peptide of type (LV) formed by the hydrolysis of the imide would thus be expected to be greater than that of the isomeric acid of type (LVI). However this direction of ring opening, the desired one in this case, may be adversely affected by steric hindrance (cf. Emery and Gold (72)).

Cherbuliez and Chambers (73) hydrolysed DL-benzamidosuccinimide (LIVa) with hot saturated barium hydroxide, and obtained N-benzoyl-DL-asparagine (LVa) but the yield was not recorded. Battersby (74) showed that these conditions are unnecessarily drastic, and hydrolysed the imide (LIVa) with cold dilute sodium hydroxide or equally effectively with warm 0.28 N-sodium carbonate. Almost a quantitative yield was obtained of a product which was mainly the desired benzoylasparagine (LVa), though a small amount of N-benzoyl-DL-isoasparagine (LVIa) was also obtained. Cherbuliez and Chambers (loc.cit.) prepared the imide (LIVa) by heating benzoyl-

asparagine (LVa) at 200°, but it was necessary for the present purpose to find a suitable method for its preparation using much milder conditions. Attempts were therefore made to cyclise N-benzoyl-DL-isoasparagine ethyl ester (LIIa) to the related imide (LIVa) under acidic conditions. A solution of the isoasparagine derivative in anhydrous nitromethane when treated at 0° with dry hydrogen bromide failed to give the imide; the same result was obtained when a solution of the isoasparagine derivative in absolute ethanol was treated at 0° with dry hydrogen chloride.

A suitably mild method of preparation was found however following the observation of Battersby (74) that N-benzoyl-DL-isoasparagine ethyl ester (LIIa) when treated with 0.5 equivalent of dilute sodium hydroxide, or with sodium ethoxide in ethanol, gave the desired imide (LIVa). These reactions are probably initiated by removal of a proton to give (LIIIa), followed by cyclisation. Accordingly N-benzoyl-DL-isoasparagine ethyl ester (LIIa) was warmed at 50° with an excess of 0.28 N-sodium carbonate in order that the formation of the imide (LIVa) and subsequent hydrolysis could be

achieved in one operation. The acidic products, a mixture of N-benzoyl-DL-asparagine (LVa) and N-benzoyl-DL-isoasparagine (LVIa) were isolated and an assessment of the composition of the mixture was made. For this purpose pure specimens of (LVa) and (LVIa) were required, and were prepared by the action of ammonia on N-benzoyl-DL-aspartic anhydride (LVIIa). The two amides (LVa) and (LVIa), obtained in this way differ appreciably in their acidic strengths and were therefore separable by fractional precipitation from sodium carbonate; a larger amount of the isoasparagine (LVIa) was obtained than the asparagine derivative (LVa). This result is in accord with the relative strengths of the two carbonyl groups concerned (cf.p.70). Trial experiments with mixtures of the pure amides (LVa) and LVIa) showed that complete separation could be achieved by addition of a solution of the mixture to a column of IR-4B resin (in the chloride phase) (cf.Drake (80)), and eluting the resin with N-acetic acid followed by N-hydrochloric acid. The isoasparagine derivative (LVIa) being the weaker acid was eluted with the N-acetic acid. The mixture of (LVa) and (LVIa) obtained by the alkaline

hydrolysis of (LIIa) was shown by this procedure to contain some 65% of N-benzoyl-DL-asparagine (LVa).

In order to be sure that all the benzoylasparagine had been produced by rearrangement during the alkaline hydrolysis of (LIIa) it was necessary to show that the β -ethyl N-benzoyl-DL-aspartate (LIa) used in the preparation of (LIIa) was in fact free from the α -ethyl ester (LIXa). It was also necessary to show that no rearrangement had occured in the formation of (LIIa) from (LIa) by the mixed anhydride method (61). β -ethyl N-benzoyl-DL-aspartate (LIa) was fractionated by counter-current distribution alone and in admixture with the α -ethyl ester (LIXa) (graphs I and II respectively). A single peak was obtained in the first case and two peaks in the second, thus showing that the β -ethyl N-benzoyl-DL-aspartate (LIa) was pure. The α -ethyl N-benzoyl-DL-aspartate (LIXa) was prepared by the action of absolute ethanol on N-benzoyl-DL-aspartic anhydride (LVIIa), and was obtained almost pure by fractional extraction (Le Quesne and Young (81)). It was completely purified by countercurrent distribution (graph III). Both pure esters (LIa) and (LIXa) were

converted to the corresponding amido esters by the mixed anhydride method, which were shown to be homogeneous and different, and thus must be (LIIa) and LXa) respectively. Thus rearrangement has not occured by way of the mixed anhydrides, but only in the treatment of (LIIa) with alkali.

In order to study the rearrangement further the ethyl esters (LIIb) and (LXb) of N-benzoyl-α-and-β-DL-aspartylglycine n-hexylamide were prepared from (LIa) and LIXa) respectively by the mixed anhydride method using ethyl chloroformate and glycine n-hexylamide (LXII, R=H). The glycine n-hexylamide (LXII; R=H) was prepared from the known compound phthaloylglycylchloride (82) and n-hexylamine. The phthaloyl group was removed from the resulting phthaloylglycine n-hexylamide by treating this compound with N-ethanolic hydrazine hydrate. Both esters (LIIb) and (LXb) were treated with warm 0.28 N-sodium carbonate and the acidic products were fractionated by countercurrent distribution (graphs V and VI respectively). The component (K = 1.05) which was present to the extent of 75% in each case was assigned the structure (LVb) since it had the

lower partition ratio in the buffered solvent system amd must be the stronger acid. This was confirmed by acidic hydrolysis (83) of the ester (LIIb) of unequivocal structure to give the acid (LVIb), which was identical with the weaker acid (K= 5.15.) from the rearrangement. The structures of both acids (LVb) and (LVIb) were confirmed by synthesis from N-benzoyl-DL-aspartic anhydride (LVIIa) and glycine n-hexylamide (LXII, R=H); the weaker acid (LVIb) predominated in the product.

The fact that both esters (LIIb) and (LXb) are rearranged by alkali to give mixtures of (LVb) and (LVIb) of much the same composition suggests that simple hydrolysis of the ester group is not a serious side reaction in the aspartic acid series. When the alkaline hydrolysis of both esters (LIIb) and (LXb) was interrupted it was possible to isolate the imide (LIVb) from the reaction mixture; thus hydrolysis of the imide is the rate controlling step in the aspartic acid series.

In order to see if rearrangement occurs in the asparaginyl series, N-benzoyl-DL-asparaginyl-glycine n-hexylamide (LXIb) was prepared from N-benzoyl-α-DL-

aspartylglycine n-hexylamide (LVIb) and ammonia by the mixed anhydride method. A sample of (LXIb) was warmed with 0.28 N-sodium carbonate as in the previous experiments, and a further portion shaken with 0.1N-sodium hydroxide over several hours. In both cases about 80% of the material remained unchanged by this treatment. That rearrangement had occured to a small extent however was shown by fractionation of the combined acidic material from the two experiments by counter-current distribution (graph VII). The same two peaks were obtained as in the rearrangement of the esters (LIIb) and (LXb); the component present to the extent of 75% was the rearranged acid (LVb).

In the glutamic acid series the alkaline hydrolysis of an optically active peptide, N-acetylglycyl-glycyl-α-L-glutamylglycine n-hexylamide ethyl ester (LIIf) was studied, and the preparation of this compound will be briefly discussed. Firstly the tripeptide γ-ethyl benzyloxycarbonylglycylglycyl-L-glutamate (LIe) was prepared by the mixed anhydride method from benzyloxy-carbonylglycylglycine (84) and γ-ethyl-L-glutamate (XL, fig 1.) (56). The product was fractionated by

countercurrent distribution and gave a single peak, (graph VIII). The tripeptide (LIe) was then coupled with glycine n-hexylamide (LXII; R=H) by the mixed anhydride method. The coupled product, N-benzyloxy-carbonylglycylglycyl-α-L-glutamylglycine n-hexylamide ethyl ester (LIIe) could not be used as such in the study of the alkaline rearrangement of the glutamic acid residue because Wesselęy et al. published[a] series of papers after this work was well advanced showing that benzyloxycarbonyl groups may be involved in other rearrangements (85). The protective group was therefore removed by hydrogenation, and the product acetylated with acetic anhydride in aqueous solution to give the desired acetyltetrapeptide (LIIf). This compound was shaken with 0.1N-sodium hydroxide for several hours; aqueous sodium carbonate was not used since the glutamylpeptide esters appear to be more resistant than the aspartyl analogues. From the acidic products N-acetylglycylglycyl-γ-DL- glutamylglycine n-hexylamide (LVf) was isolated in 56% yield. That it was the rearranged peptide was shown by its acidic strength, $pK_a = 3.45$;, this structure being confirmed by anodic

oxidation, (see Section IV,(b),(ii)). A product isomeric with (LVf), and probably the weaker acid (LVIf), was also isolated; the small quantity available however and, its difficult solubility properties precluded satisfactory identification. The product (LVf) isolated was optically inactive, racemisation was to be expected however under the alkaline conditions used.

The yield of rearranged peptide (LVf) is in good agreement with that obtained by treating N-benzoyl-α-DL-glutamylglycine n-hexylamide ethyl ester (LIId) with 0.1N sodium hydroxide and fractionating the acidic products by countercurrent distribution (Battersby (74)). This worker isolated a small amount of neutral matter during the alkaline hydrolysis of (LIId), and showed it to be unchanged ester; ring closure to the imide (LIVd) is therefore the rate controlling step in the glutamic acid series, in contrast to the aspartic acid series.

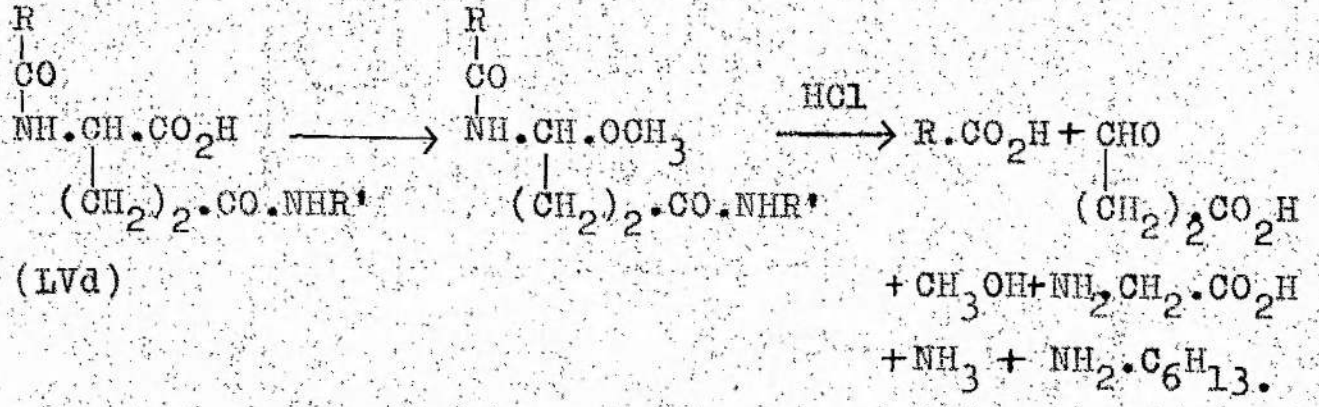
The yield of rearranged peptide (LVd) obtained by the alkaline hydrolysis of (LIId), is appreciably lower than that of the aspartyl analogue (LVb) obtained in the same way from the ester (LIIb). One or both of the following factors may be responsible for this difference:

(a) the desired direction of ring opening at the α-carbonyl group in (LIVd) is only slightly favoured over the alternative direction; (b) simple hydrolysis of the ester group in (LIId) competes significantly with cyclisation to the imide (LIVd). However, esterification of the acid (LVId), followed by alkaline hydrolysis would give a better over-all yield of the desired peptide (LVd).

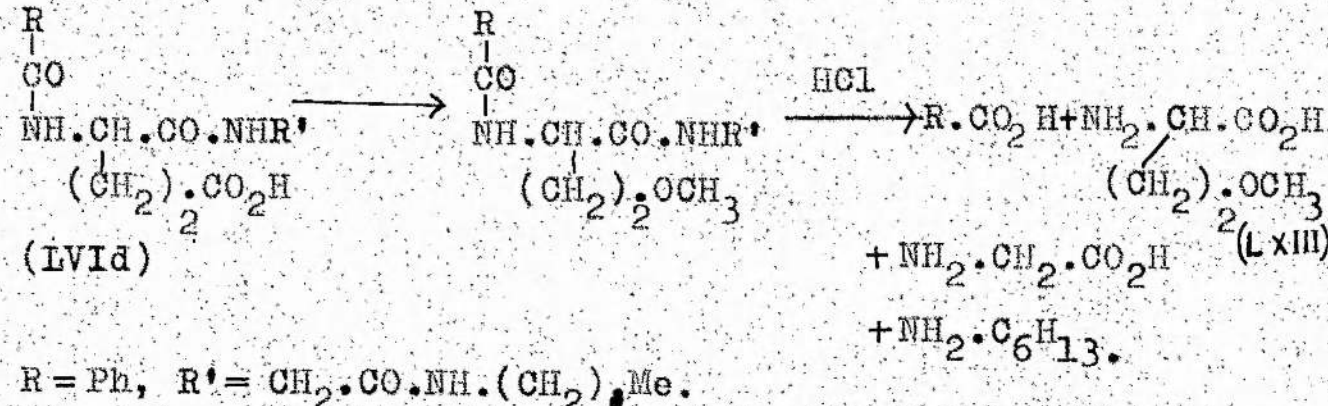
(IV).(b).(ii). <u>The specific fission of γ-glutamyl peptides.</u>

In order to show that the group -CO.NH . CHR. CO_2H which occurs in γ-glutamyl peptides is susceptible to ~~the~~ degradation by the methods available for the selective removal of the C-terminal residue (cf.p 44), the anodic oxidation (Boissonnas (53)) of several peptides prepared above was studied. N-acetyl-glycylglycyl-γ-DL-glutamylglycine n-hexylamide (LVf) was anodically oxidised in anhydrous methanol and the product was hydrolysed and chromatographed. The chromatogram showed that the glutamic acid residue has been almost completely destroyed, and that no new amino acid had been produced. N-benzoyl-γ-DL-

glutamylglycine n-hexylamide (LVd) when similarly treated showed the same result; in this case the glutamic acid residue had been completely destroyed. However N-benzoyl-α-DL-glutamylglycine n-hexylamide (LVId) differed from (LVf) and (LVd) on anodic oxidation. After hydrolysis and chromatography of the products, it was found that glutamic acid had been almost completely destroyed, with the production of a new amino acid (probably α -amino-γ -methoxybutyric acid (LXIII)). These reactions may be illustrated by the following equations:



(LVd)

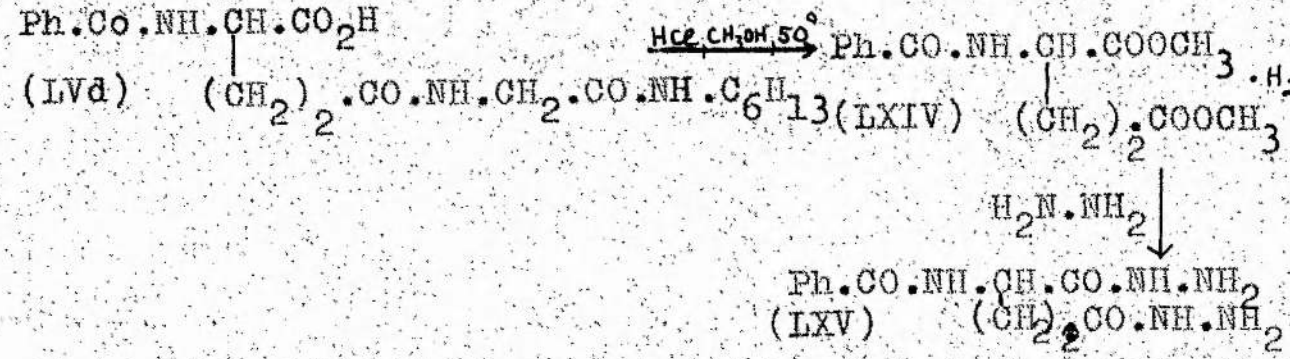


(LVId)

R = Ph, R' = $CH_2.CO.NH.(CH_2)_5Me$.

The behaviour of the group $-CO.NH\ CHR.CO_2H$ in (LVd) on anodic oxidation is thus seen to be the same as that of the C-terminal residue in a peptide chain. This result is a promising one in that it gives hope that specific cleavage of the peptide chain in structures such as (LVd) may be achieved by application of anodic oxidation on a macro-scale under the right conditions.

In order to study alternative methods an attempt was made to degrade the peptide N-benzoyl-γ-DL-glutamyl-glycine n-hexylamide (LVd) by the Bergmann-Zervas method. (45). (cf.p.45). A portion of the peptide (LVd) was esterified with methanolic hydrogen chloride by heating at 50° for 5 hours. The neutral product obtained was shown to be dimethyl-N-benzoyl-DL-glutamate monohydrate (LXIV).



Treatment of (LXIV) with hydrazine hydrate gave N-benzoyl-DL-glutamyldihydrazide (LXV) in the normal way.

Undoubtedly the conditions of esterification are too drastic, and milder methods will have to be used to prepare the mono-methyl ester of (LVd) for the first step in the Bergmann-Zervas degradation. A satisfactory method of esterification might be by the use of diazomethane, or possibly by the use of methanolic hydrogen chloride at room temperature. In this latter connection the aspartyl analogue (LVb fig.2.) gave a satisfactory yield of the mono-ethyl ester (LXb) by treatment with ethanolic hydrogen chloride at room temperature.

(IV). (b).(iii). <u>The structure and attempted rearrangement of tyrocidine A methyl ester</u>.

Battersby and Craig (25) (p.25) showed that if a solution of tyrocidine A hydrochloride in methanol was treated at 0^{o} with dry hydrogen chloride for a short time, methanolysis of one of the two amide groups present occured. It was not known at that time however which residue had been affected by this treatment. The product obtained by partial methanolysis of tyrocidine A in this way is called tyrocidine A methyl ester, and

it was of interest to compare the product obtained by Hofmann degradation followed by hydrolysis, of this compound with those obtained in the same way from pure tyrocidine A; those from the latter have been shown to be α,β-diaminopropionic acid and α,γ-diaminobutyric acid (p.67). Consequently tyrocidine A methyl ester was subjected to Hofmann degradation using the conditions found to be successful for the degradation of tyrocidine A (p.120). The product was hydrolysed with constant boiling hydrochloric acid at 110°, and the hydrolysate examined by two-dimensional paper chromatography in the two systems described on p.114 . The chromatogram showed the spot corresponding to α,β-diaminopropionic acid, and this coincided in position with that from an authentic sample of this basic amino acid on a replicate chromatogram. The spot corresponding to glycine, and already noted when tyrocidine A was degraded in the same way, was also present. No spot corresponding to α,γ-diaminobutyric acid was present on the chromatogram. The fact that α,β-diaminopropionic acid had been produced by Hofmann degradation, followed by hydrolysis, of tyrocidine A

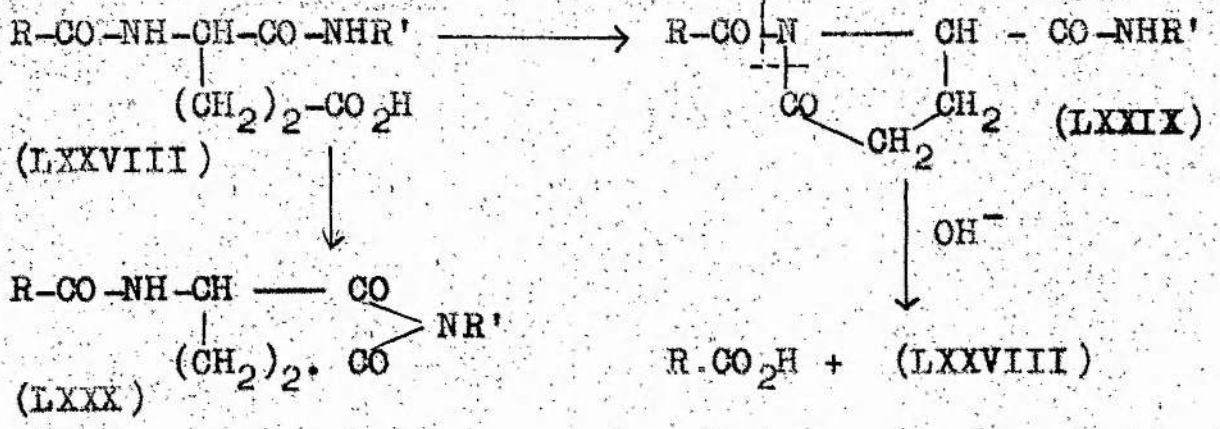
methyl ester, shows clearly that the asparagine residue (XXXVI, p.54) is present in this compound; the probable presence in the hydrolysate of glycine supports this conclusion. The absence of α,γ-diaminobutyric acid in the same hydrolysate shows that the glutamine residue (XXXVII, p.54) is not present in tyrocidine A methyl ester, and it follows that methanolysis of the glutamine residue had occured in the initial treatment of a methanolic solution of tyrocidine A hydrochloride with hydrogen chloride.

The glutamic acid residue in tyrocidine A methyl ester should thus be rearranged by alkali in the way which has been described above for the simple glutamyl peptide esters; the asparagine residue should be largely unaffected by this treatment (cf.p. 76). Exploratory experiments have been begun with this object in view. A larger amount of tyrocidine A methyl ester was prepared and separated from unchanged tyrocidine A by countercurrent distribution (25). A portion of the product was shaken with 0.1N-sodium hydroxide, and the acidic products were fractionated by countercurrent distribution in a buffered solvent system. The distribution pattern did not show

discrete peaks, but rather a broad band; this indicated that at least two components are present, which is to be expected if rearrangement has occured. Further work is necessary on the fractionation of these acidic products by countercurrent distribution, for example other solvent systems will have to be studied.

(IV) (b) (iv). <u>The preparation and alkaline cleavage of 1-acylpyrrolid-2-one derivatives from α-glutamyl peptides</u>.

The formation of the acylpyrrolidone derivative (LXXIX) from the corresponding α-glutamyl peptide (LXXVIII), followed by alkaline hydrolysis of the former, provides an alternative method to the one already given (p.69) for specifically cleaving the peptide chain. Two directions of hydrolysis are possible, as illustrated in(LXXIX), giving rise to fission products on the one hand and to recovered starting material on the other.

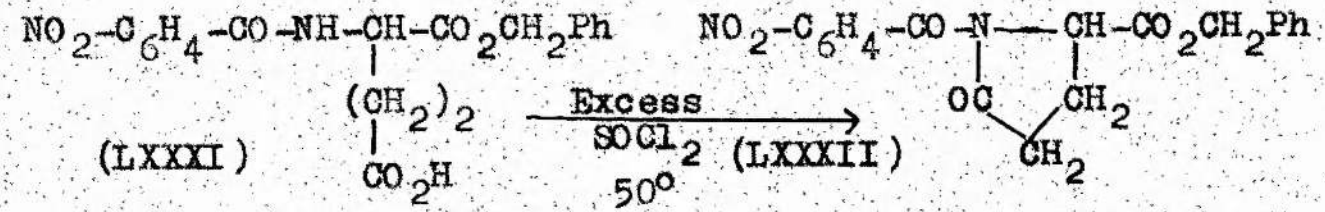


This second approach to the problem of specifically cleaving the peptide chain at the nitrogen atom of glutamic acid is simpler than the first (p.69) in that only two stages are involved. A study of the formation and

hydrolysis of acylpyrrolidones was, therefore, undertaken. In the formation of acylpyrrolidones from compounds of type LXXVIII, the possibility of cyclisation to the imide (LXXX), must not be overlooked.

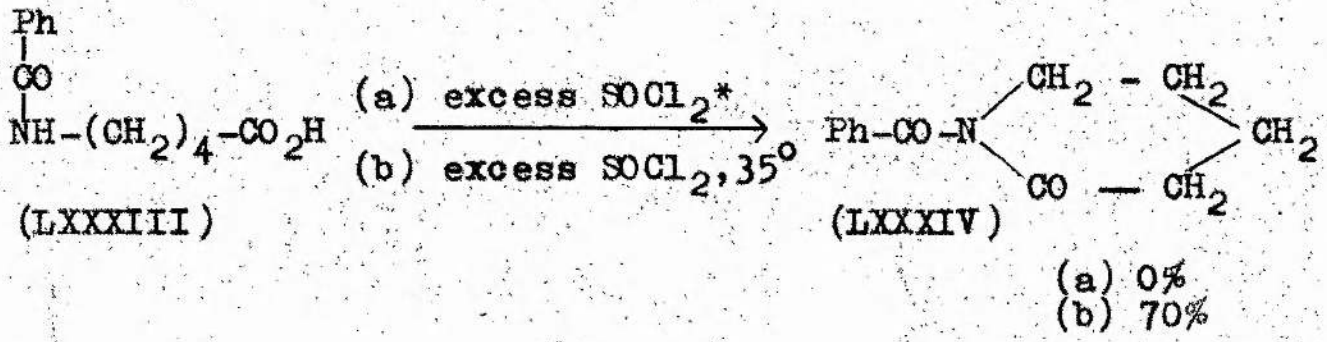
The preferential formation of the acid $R.CO_2H$ is to be expected during the alkaline hydrolysis of the acylpyrrolidone (LXXIX), since the carboxyl group of this acid, being generally the α-carboxyl group of an amino acid, is stronger than the γ-carboxyl group of the glutamic acid residue. Again, however, attack by hydroxyl ions on the acylpyrrolidone (LXXIX) to give the expected acid $R.CO_2H$ in this way could be seriously affected by steric hindrance (72).

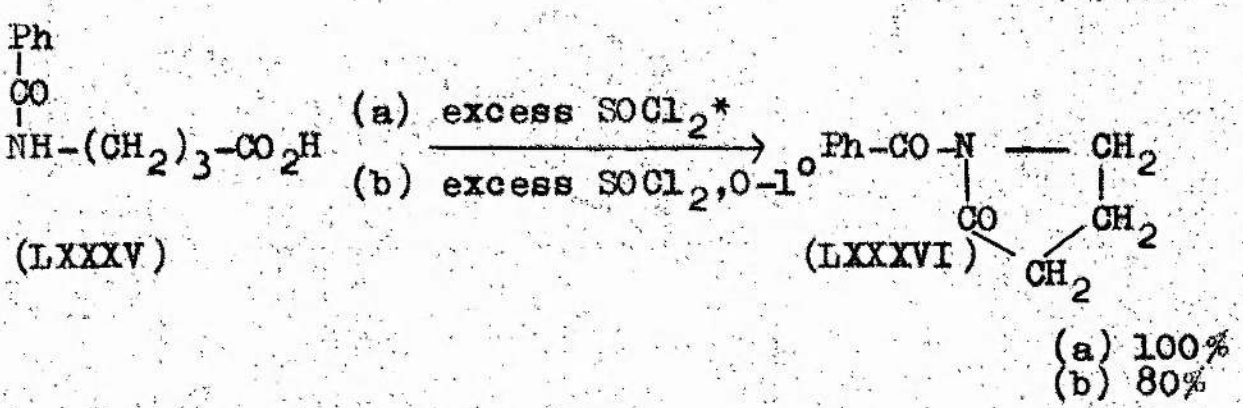
King and Spensley (88) prepared the acylpyrrolidone (LXXXII) by warming the acid (LXXXI) with an excess of thionyl chloride to 50°. In this case, only acylpyrrolidone formation is possible.



The results obtained by Kanewskaja (89) however

suggested that if the α-glutamyl peptide (LXXVIII) were treated under these conditions the 6-membered ring (LXXX) could well be formed in addition to the desired acylpyrrolidone (LXXIX).





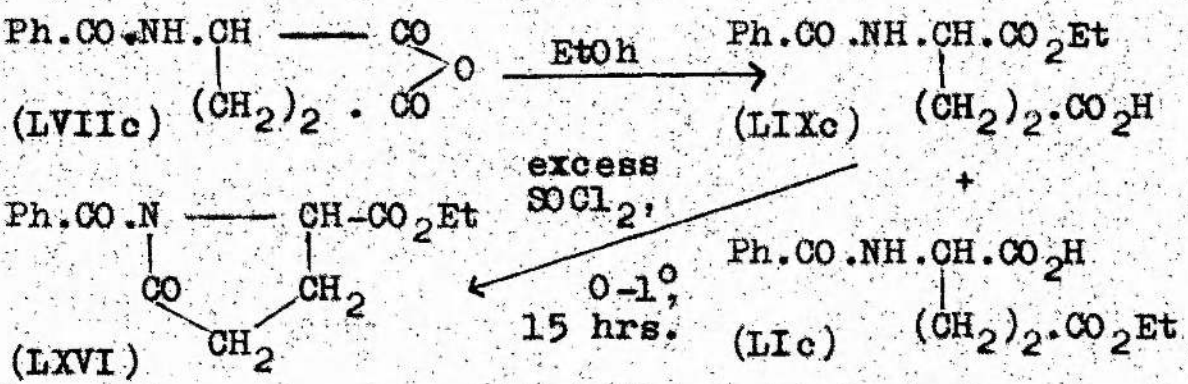
* at room temperature.

This worker treated the acid (LXXXIII) with an excess of thionyl chloride at room temperature and obtained the corresponding acid chloride. When the latter was heated at 35° cyclisation occurred and a 70% yield of N-benzoyl-piperidone (LXXXIV) was obtained. Ring closure was much easier however in the case of the acid (LXXXV) which gave

an 80% yield of N-benzoylpyrrolidone (LXXXVI) when treated with an excess of thionyl chloride at 0-1°, and a quantitative yield of the acylpyrrolidone resulted when the reaction was carried out at room temperature. Kenner and Clayton (90) have shown more recently that when a solution of the α-glutamyl peptide (LXXVIII. $R=CH_3.C_6H_4.SO_2.NH.CH_2.CO.NH.\underset{|}{C}H.CH_2.C_6H_5$, $R'=-CH_2.CO.NH.C_6H_{11}$) in dimethylformamide is treated with thionyl chloride (1.2 equiv.) and pyridine (2.4 equiv.) at 20° for 2 hours the corresponding imide (LXXX) is formed in 74% yield. It seemed, therefore, that a suitable method might well be found for preparing the acylpyrrolidone (LXXIX) from the α-glutamyl peptide (LXXVIII), without the formation of the imide (LXXX), by using an excess of thionyl chloride at 0°.

In order to examine this reaction and also to determine the yield of the acid $R.CO_2H$ formed by alkaline hydrolysis of the expected acylpyrrolidone (LXXIX), it was necessary to prepare suitable glutamyl peptides to serve as models. In the simplest case, 5-carbethoxy-1-benzoyl-DL-pyrrolid-2-one (LXVI) was formed from α-ethyl N-benzoyl-DL-glutamate (LIXC). The latter compound

was prepared in admixture with the γ-ethyl ester (LIC), from N-benzoyl-DL-glutamic anhydride (LVIIc) and ethanol.

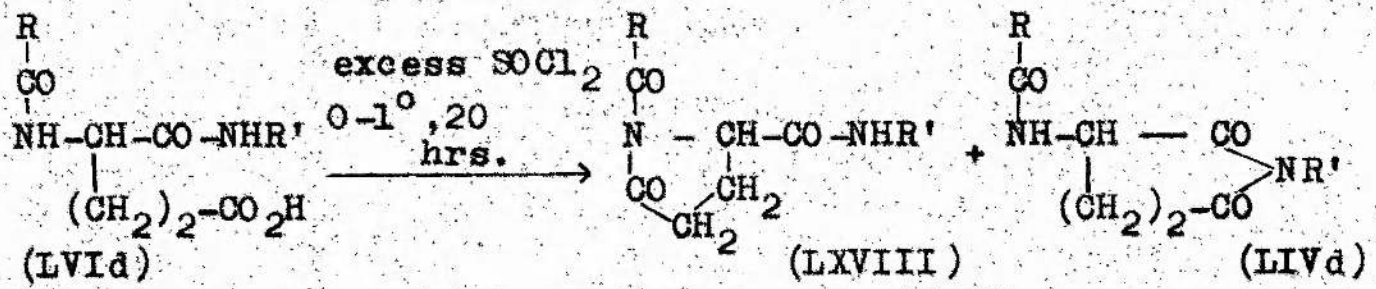


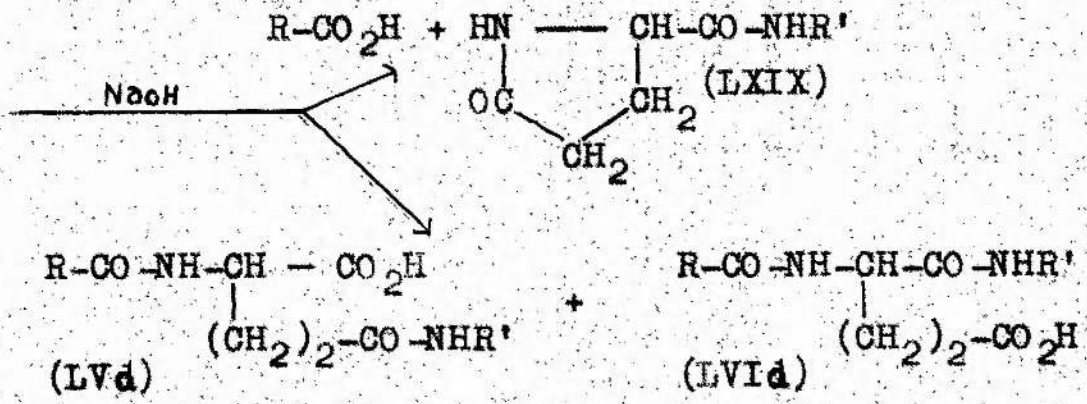
The two isomeric esters (LIXc) and (LIc) were separated by countercurrent distribution, (graph IX). The α-ethyl ester (LIXc) was treated with 14 equivalents of thionyl chloride at 0-1° for 15 hours, and gave an 84% yield of 5-carbethoxy-1-benzoyl-DL-pyrrolid-2-one (LXVI). When a solution of (LXVI) in ethanol was titrated with 1 equivalent of 0.1 N sodium hydroxide it was found that about 0.4 equivalent of alkali was consumed quickly, but thereafter the rate at which alkali was consumed decreased. The neutral product isolated after hydrolysis was shown to be diethyl N-benzoyl-DL-glutamate (LVIIIc, Fig.2), probably formed by the base-catalysed ring opening of (LXVI) with ethanol. In a further experiment an ethanolic solution of (LXVI) was titrated with 0.64

equivalents of 0.1 N-sodium hydroxide, and the products were separated into an acidic and a neutral fraction. From the former, benzoic acid was obtained in 34% yield. The neutral product in ethanolic solution was treated with 0.36 equivalents of 0.1 N-sodium hydroxide, when alkali was consumed very slowly; from the hydrolysis products diethyl N-benzoyl-DL-glutamate (LVIIIc, fig.2) was isolated as before. The fact that the diethyl ester is formed indicates clearly that ethanol is unsuitable as a solvent for the alkaline hydrolysis of (LXVI). When a solution of (LXVI) in purified dioxan and water was treated with 1 equivalent of 0.1 N-sodium hydroxide, a 46% yield of benzoic acid was obtained.

The effect of thionyl chloride on N-benzoyl-α-DL-glutamylglycine n-hexylamide (LVId) at 0° was next studied. (LVId), prepared as described on p.148, was treated at 0-1° with 20 equivalents of thionyl chloride for 20 hours. The neutral product from the reaction was obtained in 96% yield, and on analysis gave figures which agreed closely with those for the acylpyrrolidone (LXVIII). In further experiments, samples of (LVId) were treated for varying lengths of time with 20 equivalents of thionyl chloride. After 2 hours, only a small yield

(34%) of the neutral product was obtained, the yield after 10 and 15 hours was little different from that obtained after 20 hours. Finally a portion of (LVId) was treated at -20° with 20 equivalents of thionyl chloride for 19 hours, a 90% yield of neutral product being obtained in this case.





R=Ph; R'=$-CH_2.CO.NH.C_6H_{13}$

The neutral product, obtained by treating (LVId) at 0-1° with 20 equivalents of thionyl chloride for 20 hours, was dissolved in aqueous dioxan, and the solution titrated with 1.02 equivalents of 0.1 N-sodium hydroxide

over 20 minutes. After hydrolysis in this way, the neutral and the acidic fractions were separated, and the former was shown to be the pyrrolid-2-one derivative (LXIX). From the acidic products benzoic acid was isolated in 36% yield. It was thought at this time that the acidic material remaining after removal of the benzoic acid was (LVId), arising by attack by hydroxyl ion on the alternative carbonyl group in (LXVIII). However, when this material was fractionated by counter-current distribution (graph X) two components were seen to be present. One component, (K 0.47, 49%), was N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd). The other component (K 2.7, 51%) was the weaker isomeric acid (LVId). The acid (LVd) can only arise in this series of reactions by the alkaline hydrolysis of the intermediate imide (LIVd). This fact, together with the isolation of benzoic acid and the pyrrolid-2-one derivative (LXIX) above, shows clearly that treatment of the α-glutamyl peptide (LVId) with an excess of thionyl chloride at 0-1° for 20 hours results in a mixture of the acylpyrrolidone (LXVIII) and the imide (LIVd).

The relative amounts of (LVd) and (LVId) formed

by the alkaline hydrolysis of the imide (LIVd) are not known at present, thus, despite knowing the amount of N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd) formed by hydrolysis of the total neutral fraction above, it is not possible to calculate the amount of the imide (LIVd) present. A.R.Battersby (74), however, showed that alkaline hydrolysis of the ester (LIId, fig.2) gave a mixture of (LVd) and (LVId) which contained 57% of the former. The acid (LVId) may arise from the ester (LIId) in one or both of two ways, namely, simple hydrolysis of the ester group in (LIId), or hydrolysis of the imide (LIVd). If simple hydrolysis of the ester group in (LIId) is not a serious side reaction, a maximum value for the amount of the imide (LIVd) in admixture with the acylpyrrolidone (LXVIII) above can be calculated, since it is then assumed that alkaline hydrolysis of the imide (LIVd) alone gives a mixture of which 57% is (LVd). The amount of N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd) present was calculated from the area under the curve (K 0.47) in graph X and from this the amount of the imide (LIVd) present originally in the mixture was calculated to be 38%. If simple hydrolysis of the ester

group in (LIId) occurs, hydrolysis of the imide (LIVd) alone would give a mixture of (LVd) and (LVId) which contained more than 57% of (LVd), and if this higher value was used to calculate the amount of the imide (LIVd) in admixture with the acylpyrrolidone (LXVIII) clearly a smaller amount of the imide would be indicated. Thus the amount of imide (LIVd) (38%) which has been calculated to be present in the mixture is a maximum value. The minimum amount of the acylpyrrolidone (LXVIII) must then be 62%. Taking this value of 62% for the amount of acylpyrrolidone in the mixture and knowing the amount of benzoic acid released by hydrolysis, it is possible to calculate that the maximum amount of acylpyrrolidone (LXVIII) which undergoes fission is 58%, and at least 42% is ring opened.

It is hoped to be able to fractionate the mixture of the acylpyrrolidone (LXVIII) and the imide (LIVd) by countercurrent distribution; if a separation can be achieved, the hydrolysis of (LXVIII) can be repeated and the yield of benzoic acid determined. Isolation of the imide (LIVd) and subsequent alkaline hydrolysis would give the relative amounts of the isomeric acids

(LVd) and (LVId) formed; it would then be possible to ascertain whether simple hydrolysis of the ester group is a serious side reaction in the rearrangement of glutamyl peptide esters with alkali.

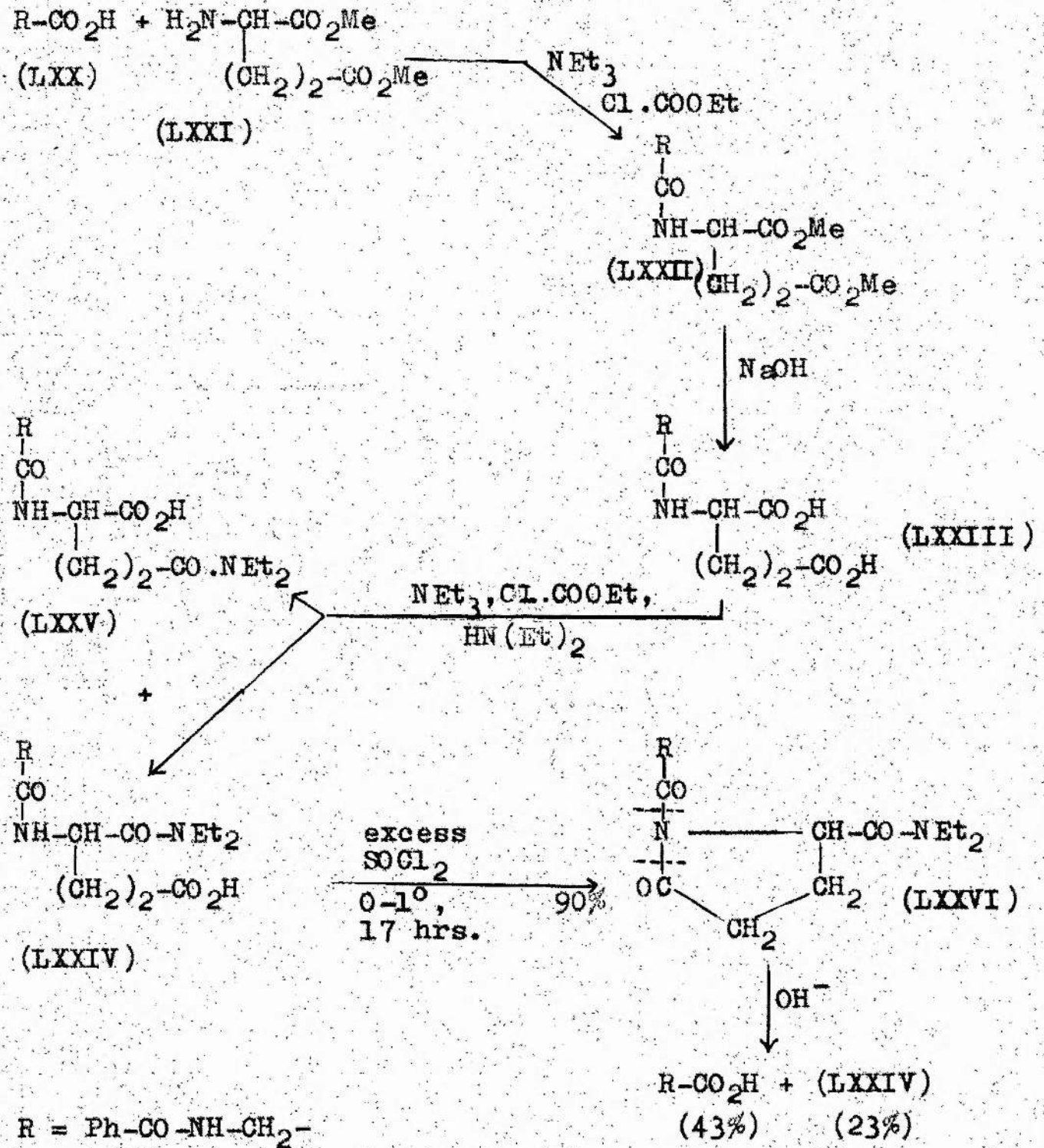
Treatment of the α-glutamyl peptide (LVId) with an excess of thionyl chloride at 0° for 10 and 15 hrs. and also at -20° for 19 hours gave a high yield of the mixture of the acylpyrrolidone (LXVIII) and the imide (LIVd) in all three cases. The neutral products were separately hydrolysed with 0.1 N-sodium hydroxide, and the yield of benzoic acid was determined. The results obtained, which are tabulated below, show that the acylpyrrolidone (LXVIII) content of all four neutral products is much the same.

Conditions	Yield of neutral matter, (LXVIII)+(LIVd)	Yield of benzoic acid
20 equiv. $SOCl_2$, $0-1^{\circ}$, 2 hrs.	34 %	-
" ,10 hrs.	94 %	33.6 %
" ,15 hrs.	94 %	35.0 %
" ,20 hrs.	96 %	36.0 %
20 equiv. $SOCl_2$ -20°, 19 hrs.	90 %	33.0 %

Fig.3. The action of thionyl chloride under different conditions on the α-glutamyl peptide (LVId).

The two acylpyrrolidones (LXVI) and (LXVIII) which have been described in the foregoing paragraphs are similar in that the acyl group is benzoyl in both cases. It was interesting, therefore, to prepare another acylpyrrolidone in which the benzoyl group was replaced by an N-acyl amino acid residue. The α-carboxyl group of the latter is stronger than the γ-carboxyl group of the glutamic acid residue and, in the absence of overpowering steric hindrance, cleavage of the peptide chain should occur in preference to ring opening of the pyrrolidone ring on treatment with alkali.

The acylpyrrolidone, 5-diethylcarbamoyl-1-benzoylglycyl-DL-pyrrolid-2-one (LXXVI), was prepared from N-benzoylglycyl-α-DL-glutamyldiethylamide (LXXIV) and thionyl chloride. The preparation of (LXXIV) and (LXXVI) may be illustrated by the following formulae.


R-CO2H + H2N-CH-CO2Me
(CH2)2-CO2Me
(LXX)
(LXXI)
NEt3
Cl.COOEt
R
CO
NH-CH-CO2Me
(CH2)2-CO2Me
(LXXII)
NaOH
R
CO
NH-CH-CO2H
(CH2)2-CO2H
(LXXIII)
NEt3, Cl.COOEt,
HN(Et)2
R
CO
NH-CH-CO2H
(CH2)2-CO.NEt2
(LXXV)
+
R
CO
NH-CH-CO-NEt2
(CH2)2-CO2H
(LXXIV)
excess
SOCl2
0-1°,
17 hrs.
90%
R
CO
N
CH-CO-NEt2
OC
CH2
CH2
(LXXVI)
OH⁻
R-CO2H + (LXXIV)
(43%) (23%)
R = Ph-CO-NH-CH2-

Benzoylglycine (LXX) was coupled with dimethyl DL-glutamate (LXXI), using the mixed anhydride method, and the resulting dimethyl N-benzoylglycyl-DL-glutamate (LXXII) was hydrolysed by treatment with N-sodium hydroxide at room temperature to give N-benzoylglycyl-DL-glutamic acid (LXXIII). N-Benzoylglycyl-α- and -γ-DL-glutamyldiethylamide, (LXXIV) and (LXXV) respectively, were formed from (LXXIII) and diethylamine by the mixed anhydride method. The α-diethylamide (LXXIV) separated almost pure when the mixture of (LXXIV) and (LXXV) was treated with ethyl acetate. This same acid precipitated first when a solution of the mixture of the acids in sodium hydroxide was partially acidified, and is thus shown to be the weaker acid. Treatment of (LXXIV) with an excess of thionyl chloride at 0-1° for 17 hours gave 5-diethylcarbamoyl-1-benzoylglycyl-DL-pyrrolid-2-one (LXXVI). The alkaline hydrolysis of this latter compound with 0.1 N-sodium hydroxide was carried out as described for (LXVIII). After hydrolysis the neutral fraction was removed and the sodium salts of the acids $R.CO_2H$ and (LXXIV) were dissolved in water; the solution was then partially acidified to precipitate

the weaker acid (LXXIV). More acid was then added to the solution to give two crops of benzoylglycine (LXX). The yield of (LXXIV), formed by ring opening of the acylpyrrolidone (LXXVI) with alkali, was found to be only 23%, whereas that of benzoylglycine, formed by direct fission of the peptide chain, was 43%. Although the acids (LXXIV) and (LXX) recovered in this way account only for some two-thirds of the acylpyrrolidone (LXXVI), it is quite clear that a valuable amount of specific fission does occur. Indeed, since (LXXIV) is readily isolated and only a small amount was obtained, it suggests that the main attack by hydroxyl ion on this particular acylpyrrolidone (LXXVI) occurs at the benzoylglycine residue.

It will be apparent from the results which have been given that the acylpyrrolidone route is one of considerable promise for specifically cleaving the peptide chain at the nitrogen atom of glutamic acid. More work is required, however, on this approach to the problem, and the formation and alkaline hydrolysis of a larger number and variety of acylpyrrolidones will have to be studied before the true value of this method is known.

Notes on the Experimental Section.

Analyses are mainly by Mr. B.S.Noyes of Bristol University. Analytical samples were dried at 100° in vacuo over phosphoric oxide, unless otherwise stated. Evaporations were carried out at 40° under reduced pressure. Congo-red was the indicator used for acidifications, unless otherwise stated.

* Denotes that the compound was prepared by Dr. A.R.Battersby.

V. Experimental Section.

(a) The Hofmann degradation of tyrocidine A, including models.

γ-Ethyl L-glutamate. (XL). H.K.Miller and H.Waelsch(56).

A stream of dry hydrogen chloride (32 g.) was passed into absolute ethanol (480 ml.), the solution cooled to room temperature, and L-glutamic acid (48.3 g.) added. The suspension was shaken until complete solution occurred (about 20 minutes). The solution was diluted to 1380 ml. with absolute ethanol, and triethylamine added dropwise until a test portion was alkaline. After standing at 0-5° for 16 hours the solution was filtered, and the solid washed with cold absolute ethanol, then ether, and dried in vacuo. (48.5 g.) m.p. 177°. This was recrystallised from water-ethanol (1:6) at 0° to give (XL) (37.1 g., 65%) m.p. 194-5°. Miller and Waelsch record m.p. 194°.

γ-Ethyl N-Benzoyl-L-glutamate (XLI)

(cf. Method for the benzyloxycarbonyl derivative. B.Heghedus (58)).

γ-Ethyl-L-glutamate (XL) (18.4 g.) was dissolved in water (140 ml.), and sodium bicarbonate (1.04 g.) added, followed by light magnesium oxide (8 g.). The mixture was stirred vigorously, cooled in ice-water, and benzoyl chloride (23.6 g., 1.6 equiv.) added over 25 minutes. The mixture was stirred for a further 10 minutes only, the solid filtered off, and the filtrate shaken with ether (150 ml.). The aqueous layer was acidified, and the precipitated oil taken up in 3 portions of ether (2 x 150 ml., 50 ml.). The ethereal solution was washed with water, dried, and evaporated in vacuo, the residue after standing overnight crystallised as needles, (18 g.). This was recrystallised by dissolving in hot ethyl acetate (75 ml.), and adding petrol-ether (60/80°) (80 ml.). When crystallisation was well underway the mixture was cooled to 0°, and further portions (total, 350 ml.) of petrol-ether were added over several hours. (15.04 g.), m.p. 81-84°, after previous sintering. A further recrystallisation in the same way gave γ-ethyl N-benzoyl-L-glutamate (XLI) (14 g., 48%), m.p. 87-87.5° (Found, in sample dried at 56°: C, 60.5; H, 6.2; N, 4.8. $C_{14}H_{17}O_5N$ requires C, 60.2; H, 6.1; N, 5.1%).

When recrystallised from 10% aqueous ethanol the

m.p. fell to 72-75°, which rose to 85-86° on a further recrystallisation from ethyl acetate/petrol-ether. Attempted preparations of this compound using potassium carbonate and benzoyl chloride (1 equiv.) in dioxan, added over 2 hours, gave very unsatisfactory results. Oily products, difficult to crystallise resulted, from which (XLI) was obtained in about 10% yield.

N-Benzoyl-L-glutamine (XLII)

A solution of γ-ethyl N-benzoyl-L-glutamate (XLI) (8 g.) in aqueous ammonia (250 ml.; d, 0.880) was kept in a well-stoppered flask at 45° for four days. The solution was evaporated to dryness in vacuo, bath temperature 40-50°, and the residue dissolved in water (30 ml.). The aqueous solution was acidified, and the precipitated oil crystallised after standing at 0° for several days. The product was collected, washed with a little water, and dried in vacuo. (6.49 g.), m.p. 151-152°, after sintering at 128°. A further recrystallisation from water gave N-benzoyl-L-glutamine (XLII) (5.73 g., 80%), m.p. 156.5°, unaltered by further recrystallisation from the same solvent. (Found: C,58.0; H, 5.6; N, 11.3. $C_{12}H_{14}O_4N_2$ requires C, 57.6; H, 5.6; N, 11.2%).

Benzyl Ester of L-Tyrosine (XLIII) and the Benzyl Ester-Benzyl Ether of L-Tyrosine (XLIV).

(c.f. H.K.Miller and H.Waelsch (59)).

To a solution of dried p-toluenesulphonic acid (22.5 g.) in warm redistilled benzyl alcohol (228 ml.) was added L-tyrosine (21.8 g.), and the mixture heated at 100° for 30 minutes with occasional shaking. Esterification was continued by distilling in vacuo with a bath temperature not greater than 130°. To the residue was added fresh portions (100 ml.) of benzyl alcohol, and p-toluenesulphonic acid (0.8 g.), and the operation repeated. The resulting hot mass was transferred to a mortar, and rubbed successively with ether (300 ml.), and water. The crude solid was then thoroughly shaken with potassium carbonate solution (50 ml., 30%) for 30 minutes, and the suspension extracted with chloroform (500 ml.). After filtration the residue was again extracted with a further portion (500 ml.) of chloroform, then dried in vacuo, (8.4 g.).

The chloroform solution was dried, and evaporated in vacuo with the exclusion of moisture, bath temperature 50°. The residue was dissolved in chloroform (25 ml.),

and ether (100 ml.) added. The solution was saturated with dry hydrogen chloride, a further portion (100 ml.) of ether added, and allowed to stand at 0° overnight. The solid (20.7 g.) crystallised from boiling ethanol (90 ml.), gave the benzyl ester-benzyl ether of L-tyrosine hydrochloride (XLIV) (2.16 g., 4.9%), m.p. 187-199° after sintering at 187°; raised to 211° by a further recrystallisation from ethanol. (The m.p. varies with the rate of heating). (Found, in sample dried at 110°: C, 69.2; H, 6.1; N, 3.5. $C_{23}H_{23}O_3N.HCl$ requires C, 69.4; H, 6.1; N, 3.5%). This compound (XLIV) absorbed 2 moles of hydrogen on microhydrogenation, using palladium-charcoal as catalyst.

To the mother-liquor was added a further portion (40 ml.) of ethanol, followed by ethyl acetate (250 ml.). After standing at 0° overnight the benzyl ester of L-tyrosine hydrochloride (XLIII) was collected, (7.69 g.) m.p. 196-197°, unchanged by recrystallisation. (The m.p. varies with the rate of heating). The residue obtained by evaporation of the mother-liquor was crystallised from ethanol-ethyl acetate to give two further crops of (XLIII), (3.3 g., m.p. 193°, and 1.29 g., m.p.

189-190°) respectively. (Total yield of (XLIII): 12.28 g., 38%). (Found in sample dried at 110°: C, 63.0; H, 5.9; N, 4.4. $C_{16}H_{17}O_3N.HCl$ requires C, 62.4; H, 5.9; N, 4.6%). (XLIII) absorbed 1 mole of hydrogen on microhydrogenation, using the same catalyst as above. Portions of both esters(XLIV) and (XLIII) were heated with 2N-hydrochloric acid for 3 hours. The hydrolysates, when examined by paper chromatography, showed that tyrosine was present in each.

N-Benzoylglutaminyl-L-tyrosine Benzyl Ester.(XLV)

To an ice-cold solution of N-benzoyl-L-glutamine (XLII) (3.5 g.) and triethylamine (1.9 ml.) in dioxan (650 ml.) and toluene (200 ml.) was added ethyl chloroformate (1.52 g.), and the solution was stirred for 20 minutes at 0°. A solution of the benzyl ester of L-tyrosine hydrochloride (XLIII) (4.3 g.) and triethylamine (1.9 ml.) in dioxan (450 ml.) and toluene (20 ml.) was added and stirring continued for a further 2 hrs. at 0°. The triethylamine hydrochloride was filtered off, and the filtrate concentrated in vacuo, bath temperature 45°, small portions of water being added to

assist in the removal of the organic solvent. The suspension was extracted with two portions of ethyl acetate (100 ml., 200 ml.), filtered, and the solid collected and dried in vacuo. 1.815 g., m.p. 166-168°, raised to 191-191.5° by recrystallisation thrice from ethanol. (Found: C, 66.1; H, 5.8; N, 8.0. $C_{28}H_{29}O_6N_3$ requires C, 66.8; H, 5.8; N, 8.3%).

The ethyl acetate solution was extracted successively with aqueous sodium carbonate, hydrochloric acid, and water, dried and evaporated in vacuo. The residue (3.57 g.), m.p. 122-130° was recrystallised from aqueous-ethanol (50%. 50 ml.), and the solid collected (2.42 g.) m.p. 126-139°, which flowed at 163°. Fractional crystallisation of this product from the same solvent mixture gave two crops, melting at 152-162° and 131-135° respectively.

10 mgm. specimens of the two crops of neutral material, together with the water-ethyl acetate insoluble product, were hydrolysed in 20% hydrochloric acid in sealed tubes at 110° for 15 hours. Paper-chromatography of the hydrolysates in the system formic acid/sec-butanol/water showed them all to contain tyrosine and glutamic acid. (Total yield of (XLV), 5.39 g.,76%).

N-Benzoylglutaminyl-L-tyrosine (XLVI)

A solution of N-benzoylglutaminyl-L-tyrosine benzyl ester, (XLV) (0.92 g., part of the foregoing product m.p. 191-191.5°) in ethanol-glacial acetic acid (3:1) (150 ml.), and water (5 ml.) was shaken with palladium-charcoal (10%, 0.7 g.), and hydrogen. Hydrogenolysis of the benzyl group occurred smoothly, the theoretical volume of the hydrogen being taken up in 12 minutes. The catalyst was filtered off, washed well with ethanol, and the filtrate evaporated to dryness in vacuo, bath temperature 50-60°. The residue, a colourless gum, was dissolved in aqueous sodium carbonate, from which, after acidification, the product crystallised (0.718 g., 96%) m.p. 162-167°. Recrystallisation thrice from water raised the m.p. to 167-168.5°. (Found: C, 57.7; H, 5.9; N, 9.2. Loss on drying at 120°, 5.4. $C_{21}H_{23}O_6N_3.1\frac{1}{2}H_2O$ requires C, 57.2; H, 6.0; N, 9.5; H_2O, 6.13%. A portion of the product (80.6 mgm.) was hydrolysed by refluxing with constant boiling hydrochloric acid (110 ml.) under nitrogen for 48 hours. The residue after evaporation was dissolved in water (1 ml.), the solution spread over the starting line on

two Whatman No.542 filter papers (24" x 24"), and the chromatograms were developed in the system formic acid/sec-butanol/water for 33 hours. The portions containing glutamic acid were eluted with water, and the aqueous eluate on evaporation yielded 25.9 mgm. of a white crystalline residue upon which the optical rotation was determined. $[\alpha]_D^{17} = -20.3^\circ$ (C, 1.479 in 8N-HCl), after correction for a blank. The blank solution was obtained by running two clean sheets of the same filter paper in the same solvent system and eluting a strip of appropriate width from each. Evaporation of the combined aqueous eluates gave a residue (3.4 mgm.), the solution of which in 8N-hydrochloric acid served for blank readings.

Attempted Syntheses of N-Benzoylglutaminyl-L-Tyrosine (XLVI) by Alternative Routes.-

Coupling of γ-ethyl N-benzoyl-L-glutamate (XLI) with (XLIII) and (XLIV) in the same way as described above for the preparation of (XLV) gave γ-ethyl N-benzoylglutamyl-L-tyrosine benzyl ester (XLVII), and γ-ethyl N-benzoylglutamyl-L-tyrosine benzyl ester-

benzyl ether (XLIX), respectively. The former crystallised from aqueous-ethanol as rods (57%), m.p. 141-143.5°, raised to 147-148.5° by further recrystallisation from the same solvent. (Found: C, 68.0; H, 6.0; N5.1; $C_{30}H_{32}O_7N_2$ requires C, 67.7; H, 6.1; N, 5.3%).

Hydrogenation of (XLVII) in ethyl alcohol, glacial acetic acid over 10% palladium-charcoal gave an almost quantitative yield of γ-ethyl N-benzoylglutamyl-L-tyrosine (XLVIII), as a resin. Crystallisation from aqueous-ethanol gave needles (36%), m.p. 133-135°, raised to 140-141° by further recrystallisation. (Found: C, 59.2; H, 6.0; H_2O, by drying at 120°, 3.8; equiv. 469. $C_{23}H_{26}O_7N_2.H_2O$ requires C, 60.0; H, 6.1; H_2O, 3.9%; equiv. 460. Found: N, in sample dried at 120°, 5.4. $C_{23}H_{26}O_7N_2$ requires N, 6.3%). Treatment of (XLVIII) with saturated alcoholic ammonia for 2 days at 30° gave an oily product which later solidified (70%), m.p. 123-126°, flowing at 130-131°. Hydrolysis of a portion of the product, followed by treatment with alkali gave a positive test for ammonia (63) indicating that at least some of the glutaminyl peptide (XLVI) had been formed.

The γ-ethyl N-benzoylglutamyl-L-tyrosine benzyl ester-benzyl ether (XLIX) crystallised from aqueous-ethanol as rosettes of needles (55%), m.p. 95-96°, raised to 132.5-133° by recrystallisation twice from the same solvent. (Found in sample dried at 78°: C, 71.4; H, 6.2; N, 4.1. $C_{37}H_{38}O_7N_2$ requires C, 71.4; H, 6.2; N, 4.5%). Hydrogenation of (XLIX) in ethyl alcohol, glacial acetic acid, over platinum oxide gave only about 30% of an acidic product, which, after recrystallisation twice from aqueous-ethanol, had m.p. 154°, depressed in admixture with (XLVIII). The neutral product crystallised from aqueous-ethanol as needles, m.p. 87.5-95.5°. Hydrogenation over 10% palladium-charcoal resulted in one mole of hydrogen being absorbed, indicating that the initial hydrogenation over platinum oxide had removed the O-benzyl group. The acidic product (50%) crystallised in small yield from aqueous-ethanol as needles, m.p. 130-140 depressed in admixture with (XLVIII).

N-Benzoyl-DL-isoasparagine β-Anilide (L).

To an ice-cold solution of N-benzoyl-DL-isoasparagine

(LVIa, fig.2) (1.34 g.) and triethylamine (0.8 ml.) in dimethylformamide (30 ml.) was added ethyl chloroformate (0.54 ml.) and the solution stirred for 20 minutes at 0°. Redistilled aniline (0.57 ml.) was added, and stirring continued for a further 3 hours at 0°. The solution was evaporated to dryness in vacuo, bath temperature 70°, and the residual oil treated with aqueous potassium carbonate. The insoluble matter solidified, and was filtered off (0.33 g.), m.p. 224-225°. Crystallisation from ethanol, and then aqueous dimethylformamide afforded N-benzoyl-DL-isoasparagine β-anilide (L) as needles (0.19 g., 10%), m.p. 248-249°. (Found in sample dried at 110°: C, 65.4; H, 5.4; N, 13.8. $C_{17}H_{17}N_3O_3$ requires C, 65.6; H, 5.5; N, 13.5%).

Hofmann Degradation of the Amide derived from (XLVIII).

To the amide (62 mgm.) was added an ice-cold hypochlorite solution (4 ml., Cl_2, 1.2 mole; OH^-, 4 equiv.) prepared by passing chlorine (0.3 g.) into water (80 ml.) containing $Ba(OH)_2.8H_2O$ (3.79 g.). After shaking for several minutes almost the whole of the solid dissolved. The solution was warmed to 70-80° for 20 minutes, the

requisite amount of 0.1N-sulphuric acid then added to precipitate all the Ba^{++} ions. The barium sulphate was centrifuged down, washed well with ethanol and hot water, and the combined supernatant liquid evaporated in vacuo to an oil, which was hydrolysed in constant boiling hydrochloric acid (3 ml.) in a sealed tube at 110° for 24 hours. The hydrolysate was evaporated to dryness, dissolved in water, and the solution extracted thrice with ether. Attempts to precipitate any basic amino acid from the solution as the picrate were unsuccessful. Paper-chromatography of the hydrolysate in the system phenol/water/ammonia gave a spot (R_f 0.68) brown in colour, turning purple, corresponding to α,γ-diaminobutyric acid.

Attempted Degradation of Tyrocidine A using Equivalent Amounts of Hypochlorite or Hypobromite.

(i) Essentially the same procedure as above using pure tyrocidine A (0.13 g.) and barium hypochlorite solution (5.0 ml., Cl_2, 1.3 mole; OH^-, 15 equiv.); methanol being used to keep the polypeptide in solution. The solution was heated at 70-80° for 20 minutes, then

at 85-97° for one hour. Paper-chromatography of the hydrolysate in the two systems formic acid/sec-butanol/water, and phenol/water/ammonia gave no spots corresponding to α,γ-diaminobutyric acid (α,γ-D.A.B.) or α,β-diaminopropionic acid (α,β-D.A.P.). Two unidentified spots were however present on the base line, possibly unhydrolysed peptide, since on further hydrolysis only one remained. Ornithine was detected, as were all the other amino acids, which occur in tyrocidine A. (Methanol was later shown to be unsatisfactory as solvent.)

(ii) The experiment was repeated using pure tyrocidine A (0.26 g.), and barium hypobromite solution 1.1 ml., Br_2, 1.3 mole, OH^-, 16 equiv.); purified dioxan (64) being used as solvent. The solution was heated at 50-55° for 20 minutes, then at 80-90° for 1 hour. Paper-chromatography of the hydrolysate in the two systems used in (i) again gave no evidence for the presence of α,γ-D.A.B. or α,β-D.A.P. An unidentified spot (R_f 0.22, 0.43) in the systems respectively was noted which was probably glycine since this amino acid could be added to the hydrolysate and still only one spot occurred in this position after two dimensional chromatography. In a

further experiment using the same concentrations (except that sodium hypochlorite was used), the spot corresponding to glycine was again observed but no spots corresponding to α,γ-D.A.B. or α,β-D.A.P. were present, when a portion of the product was hydrolysed, and chromatographed in the two systems. The remaining portion was hydrogenated over Raney-nickel, then hydrolysed. Paper-chromatography of this hydrolysate showed that the spot corresponding to glycine was still present; furthermore, no new spot corresponding to α,β-D.A.P. had been produced.

Attempted Isolation of Acetaldehyde from the Hofmann Degradation Product of Tyrocidine A.

(a) Experiments with models (cf. (66))

(i) Hofmann degradation of N-benzoyl-DL-isoasparagine (LVIa fig.2) - N-benzoyl-DL-isoasparagine (0.33 g.) was dissolved in 2N-sodium hydroxide (4.1 ml.) and to the solution was added ice-cold sodium hypobromite (4.1 ml., Br_2, 1.1 mole; OH^-, 12 equiv.), prepared by adding bromine (1.1 ml.) to 2N-sodium hydroxide (52.5ml.). The solution was heated at 50° for 5 minutes; tests at

the end of this period for OBr^- with starch/potassium iodide were negative. Sulphuric acid (20%, 48 equiv.) (16 ml.) were then added, and the solution slowly distilled into p-nitrophenylhydrazine solution (3 ml.), prepared by dissolving p-nitrophenylhydrazine (0.17 g.) in water (6 ml.), with a few drops of conc.hydrochloric acid. The precipitate, a red solid, was collected. (21.2 mgm.), m.p. 159-170°. Attempts to crystallise this substance from aqueous-ethanol were unsuccessful. The filtrate was evaporated to dryness in vacuo, leaving a yellow solid, which was crystallised from aqueous-ethanol. (8.5 mgm.), m.p. 113-114° raised to 124-125° in admixture with an authentic sample of acetaldehyde-p-nitrophenylhydrazone (m.p. 128-129°). Trial experiments showed that if acetaldehyde was heated with sodium hydroxide under the above conditions, the solution acidified and distilled, only low yields of very impure acetaldehyde-p-nitrophenylhydrazone were obtained. Recovery as the p-nitrophenylhydrazone was good however (75%) if the sodium hydroxide was treated with an excess of sulphuric acid before the addition of acetaldehyde, the solution then being distilled.

(ii) Hofmann degradation of N-benzoyl-DL-isoaspargine β-anilide (L). - To an ice-cold solution of the anilide (L) (0.09 g.) in purified dioxan (30 ml.) and water (7 ml.) was added an ice-cold hypobromite solution (1.75 ml., Br_2, 1 mole; OH^-, 10 equiv.). The solution was heated at 50° for 10 minutes, negative tests being obtained at the end of this period for OBr^-. Enough sulphuric acid (28%, 20 ml.) to make the solution about 2.5 N with respect to sulphuric acid was added, and the solution was slowly distilled into p-nitrophenylhydrazine solution prepared as above. A faint yellow precipitate was noted at the beginning of the distillation. The reagent solution was concentrated in vacuo to remove dioxan, and the final volume brought to 5 ml. by the addition of water. The crystals formed were collected (26.5 mgm.) m.p. 119-120°. A solution in aqueous-ethanol (65%, 0.75 ml.) deposited a small amount of a red solution on cooling; which was removed by filtration. Addition of aqueous-ethanol (25%, 0.8 ml.) to the filtrate yielded acetaldehyde-p-nitrophenylhydrazone (14 mgm., 28%) m.p. 124°, raised to 126.5° in admixture with an authentic sample.

The purified dioxan when tested alone under the above conditions yielded no acetaldehyde.

(b) The Hofmann degradation of tyrocidine hydrochloride

An ice-cold solution of tyrocidine hydrochloride (2.2 g., containing about 0.7 g., tyrocidine A) in purified dioxan (6.6 ml.) and water (4.4 ml.) was treated with 2N-sodium hydroxide (10.75 ml.). An ice-cold hypobromite solution (2 ml., Br_2, 1.2 mole; OH^-, 15 equiv.) was then added, and the solution allowed to stand at 0° for 10 minutes. It was then heated at 50° for 5 minutes, excess sodium hypobromite being removed at the end of this period by the addition of sodium metabisulphite. The solution, made acid with sulphuric acid (28%, 26 ml.), was distilled into p-nitro-phenylhydrazine solution over five minutes, but no precipitate was obtained. The solution remaining after distillation was refluxed for 7 hours, and again distilled into p-nitrophenylhydrazine solution, but no precipitate formed.

The Hofmann Degradation of N-Benzoylglutaminyl-L-tyrosine

(XLVI)

To a solution of (XLVI) (0.1 g.) in purified dioxan (1 ml.) was added an ice-cold solution of sodium hypobromite (1 ml; Br_2, 1.2 mole; OH^-, 8 equiv.). The solution was cooled to 0° over 30 minutes, then heated at 70-80° for 20 minutes. The solvent was removed in vacuo, and the residue hydrolysed in constant boiling hydrochloric acid (7 ml.) in a sealed tube at 110° for 15 hours. The residue obtained by evaporation was dried thoroughly in high vacuum, and extracted with dimethylformamide (3 ml.) to remove sodium chloride. The dimethylformamide solution was evaporated to dryness in vacuo, and the residue dissolved in water (0.6 ml.). Two further samples of (XLVI) (0.1 g.) were treated exactly as above, except that the volumes of hypobromite were increased to 3 ml. and 6 ml. respectively.

Examination of all three hydrolysates by paper-chromatography in the formic acid/sec-butanol/water system showed that α,γ-D.A.B. (R_f, 0.05) was present in each, moreover the amount increased with increasing concentration of sodium hypobromite. The intensity of the spots indicated yields of α,γ-D.A.B. of 5, 10, and 20% respectively.

Hofmann Degradation of Tyrocidine A.

To a solution of pure tyrocidine A (0.3 g.) in purified dioxan (20 ml.) was added an ice-cold solution of sodium hypobromite (23 ml; Br_2, 15 mole; OH^-, 100 equiv./amide group). The solution was then treated exactly as in the degradation of (XLVI) above. The final residue was dissolved in water (1.5 ml.), and the solution examined by two-dimensional paper-chromatography in the systems described on p.114. A standard solution of ornithine, α,γ-D.A.B. and α,β-D.A.P. was run on a second paper in the same way. The chromatogram from tyrocidine A showed clearly the presence of two new basic amino acids, (R_f, 0.08, 0.69 and 0.06, 0.56) in the systems respectively, coinciding in position with α,γ-D.A.B. (R_f, 0.08, 0.67) and α,β-D.A.P. (R_f, 0.06, 0.55) on the replicate chromatogram. A new spot (R_f, 0.22, 0.46), already noticed on a previous chromatogram (see p.114) and probably glycine (R_f, 0.25, 0.49) was also present. No spot corresponding to tyrosine could be detected but all the other amino acids common to tyrocidine A were present. The concentration of ornithine was reduced, and that of glutamic acid increased. The

yield of either α,γ-D.A.B. or α,β-D.A.P. did not appear to exceed 10%.

L-α,γ-diaminobutyric acid dipicrate - A mixture of L-α,γ-diaminobutyric acid monohydrochloride (0.05 g.) and picric acid (0.17 g.) was dissolved in hot water (5 ml.). The dipicrate, as yellow needles, was collected (0.17 g., 91%), m.p. 181-182° dec., (sintered 128°). The m.p. was unaltered on recrystallisation from water. (D.W.Adamson (68), records m.p. 180-1°).

L-α,β-diaminopropionic acid dipicrate monohydrate - L-α,β-diaminopropionic acid monohydrochloride (0.05 g.) was dissolved in water (2 ml.) and a solution of picric acid (0.18 g.) in ethanol (1.5 ml.) added. The yellow crystals (rhombs) were collected and washed with picric acid solution (0.177 g., 85%), m.p. 141.5 - 144° dec. (sintered at 137°), unaltered on further recrystallisation from picric acid solution. (Found: C, 31.5; H, 2.9; N, 19.6. Loss on drying at 110°, 2.8%. $C_{15}H_{14}O_{16}N_8 \cdot H_2O$ requires C, 31.1; H, 2.8; N, 19.3; H_2O, 3.2%. $C_9H_{11}O_9N_5 \cdot \frac{1}{2}H_2O$ requires C, 31.6; H, 3.5; N, 20.5; H_2O, 2.6.

On standing in air for several days the m.p. of the crystals rose to 184° dec. (rhombs). (Found: C, 31.3; H, 3.2; N, 19.8%).

U.V. analysis on the latter specimen, m.p. 184° (cf. (69)). - A solution of the picrate in alcohol (17.15 mgm./litre) showed maximum absorption at 3575 A° where D = 0.961. Therefore the molecular weight for the monopicrate = 284.8 (Theor. for monopicrate. $\frac{1}{2}H_2O$ = 342); and the molecular weight for the dipicrate = 569.6 (Theor. for dipicrate.H_2O = 580). The molar extinction coefficient (max.) for picric acid at 3575 A° was found to be 16,140. The values for the molecular weight were confirmed exactly by determination of the absorption of the picrate and of picric acid at 3800 A°. $\epsilon_{3,800}$ for picric acid was found to be 12,950.

Isolation of basic amino acids after the Hofmann Degradation of tyrocidine A. - Tyrocidine A (4.5 g.) was used, and the degradation carried out exactly as above. A preliminary examination of the final hydrolysate by two-dimensional paper chromatography indicated that α,γ-D.A.B., and α,β-D.A.P. were present to the

extent of 20-25 mgm. each (5% yield).

One half of the solution was spread evenly across the starting lines on 12 Whatman No.1 filter papers (24" x 24") and run in formic acid/sec-butanol/water for 16 hours. The portions containing the basic amino acids were eluted with water (300 ml./paper), and evaporation of the total eluate in vacuo gave a clear gum (41.8 mgm.). This was dissolved in water (1 ml.) and chromatographed on 2 large Whatman No.1 filter papers, in the system phenol/water/ammonia for 13 hours. A portion of the paper when sprayed with ninhydrin showed a slight overlap of the ornithine and α,γ-D.A.B; the α,β-D.A.P. being well-separated from them. The portions of the paper containing the two latter basic amino acids were cut out, separately eluted with water, and the aqueous eluates evaporated in vacuo. The eluate containing α,γ-D.A.B. yielded a residue (9.8 mgm.) which was dissolved in water (0.3 ml.) and saturated aqueous picric acid solution (4.2 ml.) added. After standing at 0° for four days, the crystals were collected, (8 mgm.), m.p. 180-182°, undepressed in admixture with an authentic sample of L-α,γ-D.A.B. dipicrate (m.p.

181-183°) prepared in the same way.

Similarly, the α,β-D.A.P. eluate yielded a residue (8.3 mgm.) which was dissolved in water (0.3 ml.) and saturated aqueous picric acid solution (4.0 ml.) added. No crystallisation occurred at 0°, but on reducing the volume to 1.5 ml. in vacuo a crop of crystals was obtained (23 mgm.), m.p. 118-125°, with a small portion melting at 145°. After being thoroughly washed with ether, the crystals were dried (3.1 mgm.), m.p. 131-145°, after sintering at 123°; raised to 179-181° dec. in admixture with an authentic sample of L- α,β-D.A.P. dipicrate.H_2O, (higher melting form). The m.p. of the specimen rose to 170-175° on standing in air for 24 hours. When a mixture of the two forms of L-α,β-D.A.P. dipicrate.H_2O was gradually heated, fusion took place at 179-181°, that is, at the melting point of the higher melting form.

The remaining half of the solution (p.123) was adjusted to pH 5.3 with 2N-sodium hydroxide (5 ml.), evaporated, and the dried residue freed from sodium chloride by treatment with dimethylformamide, and dissolved in water (20 ml.). The solution was run on to a column (18.5 cm. x 1.8 cm.), of IRC-50 resin

buffered at pH 4.7 (70), and which had been previously well washed with water to remove buffer. After elution with water (300 ml.), the column was successively eluted with 0.5 N-hydrochloric acid (65 ml.), and 0.1 N-hydrochloric acid (150 ml.). The residue obtained after evaporation of the acid eluate was freed from sodium chloride, dissolved in water, and a portion run two-dimensionally on a paper-chromatogram in the two systems used previously (p.114). The chromatogram showed the three well-separated spots of ornithine, α, γ-D.A.B. and α, β-D.A.P., the colour intensities of the latter two corresponding to 2 and 1.5 mgm. respectively, (10 - 12.5 mgm. of each were expected, see p.123).

The basic amino acids were then isolated from the remaining part of the solution by paper-chromatography in exactly the same way as described on p.123. The α, γ-D.A.B. residue was dissolved in water (0.5 ml.), and picric acid (10 mgm.) added. After standing at 0° overnight, the crystals were collected (4.3 mgm.) m.p. 179-180.5°. Recrystallisation from water (0.1 ml.) yielded 2.2 mgm., m.p. 182.5 - 184° dec. (sintered at 134°). The mixed m.p. with authentic L-α, γ-D.A.B.

dipicrate (m.p. 180-182° dec.) was 180-182° dec. (sintered 178°). The α, β-D.A.P. residue was dissolved in water (0.4 ml.) and picric acid (10 mgm.) in ethanol (0.2 ml.) added. The volume was reduced in vacuo to about 0.4 ml., and after standing at 0° the crystals were collected (2.4 mgm.), m.p. 131° (sintered 118°), raised to 132-140° (sintered 130°) in admixture with an authentic sample of L-α, β-D.A.P. dipicrate.H_2O (low melting form, m.p. 141-142°). All specimens decomposed at 144-147°.

The recovery of the basic amino acids as the dipicrates was checked as follows. A mixture of ornithine (5 mgm.), L-α, γ-D.A.B. (5 mgm.) and L-α, β-D.A.P. (3 mgm.) was separated on a paper chromatogram in the phenol/water/ammonia system, and the two latter basic amino acids recovered as the dipicrates, by the above method. The yield of L-α, γ-D.A.B. dipicrate was 9.9 mgm. (53%), m.p. 173-177.5° (sintered 116°); that of L-α, β-D.A.P. dipicrate.H_2O was 6.7 mgm. (54%), m.p. 140-143° dec., with some melting at 118°.

V. (b)(i). The rearrangement of aspartyl and glutamyl peptides

β-Ethyl N-Benzoyl-DL-aspartate (LIa)*

A solution of β-ethyl-DL-aspartate hydrochloride (9.9 g., 0.05 mole.) (Puitti, (76); cf. Coleman (77)) and anhydrous potassium carbonate (10.4 g., 3 equiv.) in water (40 ml.) was stirred vigorously at 0° whilst benzoyl chloride (7 g.) in dioxan (20 ml.) was added during 1.5 hr. After being stirred for a further 2 hrs. at 0°, the solution was made alkaline with potassium carbonate (0.3 g.) and extracted with ether (3 x 100 ml.). The aqueous layer was acidified, and extracted with ethyl acetate (3 x 100 ml.). The latter extract after being washed with water, dried, and evaporated yielded a pale yellow gum (13.1 g.). When crystallised from 10% aqueous ethanol (300 ml.) this product gave β-ethyl N-benzoyl-DL-aspartate (9.99 g., 75%) m.p. 104-105°. A sample recrystallised twice from the same solvent gave diamond-shaped plates, m.p. 105-107° (Found: C,58.9; H,5.6; N,5.0. $C_{13}H_{15}O_5N$ requires C,58.9; H,5.7; N,5.3%).

A portion of the final product was fractionated by countercurrent distribution (30 transfers) between ehtylacetate and aqueous phosphate buffer made from 0.5M-KH_2PO_4(20 Vol) and 0.5M-K_2HPO_4(1 Vol). One slightly skewed peak (partition ratio K0.77) (graph I) was obtained, indicating the presence of a single component for which K increases with increasing concentration. The presence of two components in a mixture of the β-ethyl ester (LIa) and the α-ethyl ester (LIXa) (K2.0) was clearly visible after 30 transfers in the same solvent system (graph II).

Action of Ammonia on N-Benzoyl-DL-aspartic Anhydride.

A stream of dry ammonia was passed into a solution of the anhydride (6.7g) (Lawson, (78)) in warm dioxan (280 ml.) until precipitation ceased. The gummy ammonium salts were freed from dioxan by decantation, dissolved in water (105 ml), and acidified. The precipitate, a mixture of N benzoyl-DL-isoasparagine (LVIa) and N benzoyl-DL-asparagine (LVa) (6.79g) had

mp.195.5°. The mixture was dissolved in 0.2N-sodium carbonate (186 ml. 1.3 equiv), and portions of 1.15 N-hydrochloric acid (23.9 ml, 4.2ml, 5ml) were added to precipitate three fractions (4.04g, m.p.199°;0.93g; m.p. 194-196.5°; after sintering from 180°, and 1.01g,m.p. 188-189°, respectively). The last was almost pure N-benzoyl-DL-asparagine; Cocker (79) records m.p. 190-191°. The first fraction was again dissolved in aqueous sodium carbonate (1.25 equiv.)and hydrochloric acid (0.9 equiv.) was added; N benzoyl-DL-isoasparagine separated as needles; m.p. 208-209°, unchanged by recrystallisation from 10% aqueous ethanol. (Found, in material dried at 110°: C, 55.8;H,5.2;N,12.0%; equiv., 243 $C_{11}H_{12}O_4N_2$ requires C,55.9;H,5.1;N,11.9%; equiv., 236). This amide was recovered unchanged (90%) after being warmed for 3 hrs. at 50-60° with 0.28N-sodium carbonate (3 equiv.).

Mixtures of N-benzoyl-DL-asparagine (LVa) with up to 30% of N benzoyl-DL-isoasparagine (LVIa) melted at ca.190-192°, after sintering; higher proportions of the isoasparagine raised the m.p. e.g. 40% of the isoasparagine with 60% of the asparagine derivative melted at 192.5-193°.

Diethyl N-Benzoyl-DL-aspartate (LVIIIa).

N-Benzoyl-DL-aspartic acid (1g.) (Cocker, loc.cit) was treated in absolute ethanol (20 ml.) with dry hydrogen chloride, and then heated under reflux for 1 hour. The diester (1.04g.,84%) isolated in the usual way, crystallised from ether-light petroleum as rosettes of needles, m.p. 75-77° unchanged by recrystallisation. (Found, in sample dried at 65°: C,61.6;H,6.5; N,4.9. $C_{15}H_{19}O_5N$ requires C,61.4;H,6.5;N,4.8%).

α-Ethyl N-Benzoyl-DL-aspartate.(LIXa).

N-Benzoyl-DL-aspartic anhydride (2.1g.) in absolute ethanol (50 ml.) was heated under reflux for 3 hrs. The gum obtained by evaporation of the solution was dissolved in ether and extracted with 4 portions (3x9ml. and 20 ml.) of 0.28-N sodium carbonate. The ether was dried and evaporated to leave a gum (0.214g.) which crystallised from ether-light petroleum to give the diethyl ester (LVIIIa) m.p. 75-77° alone or in admixture with the foregoing product.

The four alkaline extracts on acidification yielded oils, all of which slowly crystallised except that from the first extract; the solids from the second, third and fourth extracts were collected separately (0.43g, m.p. 103-104°; 0.566g, m.p. 104-105°; 0.283g, m.p. 104-106°, respectively). The combined crystalline product was completely freed from the β-ethyl isomer (LIa) by countercurrent distribution (58 transfers) between ethyl acetate and phosphate buffer described on p.l. The distribution pattern showed one slightly skewed peak (K2.0), (graph III). The solutions from tubes 38-51 were combined, the separated aqueous layers was acidified and thoroughly extracted with ethyl acetate. The upper layers from these tubes and the extracts were dried and evaporated to a gum (1.0g). This crystallised from water to give the α-ethyl ester as prisms (0.785g.) m.p. 111-112.5° (Found: C,58.8; H,5.7; N,5.3. $C_{13}H_{15}O_5N$ requires C,58.9; H,5.7; N,5.3%); the mixed m.p. with the β-isomer (LIa) was 80-95°.

N-Benzoyl-DL-asparagine Ethyl Ester (LXa).

A stirred solution of the acid (LIXa) (0.26g.) in dioxan (20 ml.) and chloroform (4ml.) was treated at 0°

with triethylamine (0.14 ml.) and ethyl chloroformate (0.1 ml.). After 20 minutes an excess of aqueous ammonia (0.2 ml; d,0.880) was added and stirring was continued for a further 2 hours whilst the reaction mixture warmed to room temperature. After the addition of water, the solution was made just acid to litmus, and evaporated to 5 ml; the addition of water and evaporation were repeated. The neutral products were extracted into ethyl acetate (total volume 110 ml.), and the extract was washed with water, dried and evaporated to leave a white solid (0.208g, 80%). Recrystallised from water it yielded N-benzoyl-DL-asparagine ethyl ester as rosettes of long needles, m.p. 152-154°. (Found: C,59.3; H,6.2; N,10.4; $C_{13}H_{16}O_4N_2$ requires C,59.1; H,6.1; N,10.6%). In admixture with its isomer, N-benzoyl-DL-isoasparagine ethyl ester (LIIa) the m.p. was 132-149°.

Attempted Cyclisation of Benzoyl-DL-isoasparagine Ethyl Ester (LIIa)* under Acidic Conditions.

(a). A solution of anhydrous (LIIa) (0.1gm.) in anhydrous nitromethane (10ml.) was treated at 0° for one minute with a slow stream of dry hydrogen bromide. After standing

at room temperature in the dark for 16 hrs, the yellow solution had deposited crystals (71 mgm), shown to be the ammonium and /or methylamine hydrobromide derived from the nitromethane. (c.f. Albertson & McKay, (75).). The nitromethane was removed in vacuo and the residue partitioned between ethyl acetate and sodium bicarbonate solution. The neutral fraction (84 mgm.) was very soluble in ethanol (1 ml.) and no crystallisation occured on seeding with the authentic imide (LIVa).

(b). Dry hydrogen chloride was bubbled for 5 minutes into a solution of anhydrous (LIIa) (0.1g.)in absolute ethanol (35 ml.) at 0°. After standing at room temperature for 16 hrs, the solution was evaporated to a gum which was separated into two fractions by treatment with ether. One, readily soluble (40 mgm.) was the crude diethyl ester (LVIIIa) m.p. 70.5-72.5°, raised to 72.5-74.5° in admixture with an authentic sample. The second, sparingly soluble (39 mgm.) was a mixture of ammonium chloride and starting material.

Action of Bases on N-Benzoyl-DL-isoasparagine Ethyl Ester. (LIIa).

The ester (0.5g) was warmed with 0.28 N-sodium

carbonate (21 ml. 3 equiv.) at 50° for 3 hr. during which a complete solution was obtained. After warming for a further 3 hr. at 50°, the solution was adjusted to pH7, extracted with ethyl acetate (3x60 ml.), concentrated to 10 ml., and acidified. The product crystallised as needles (345 mgm.) m.p. 190-192°, after sintering at 175°. A portion (9.9 mgm.) was dissolved in water (10ml.), and run onto a column (1cm x 15cm) of IR-4B resin which had been washed thoroughly with N-hydrochloric acid and distilled water. The column was eluted with N-acetic acid (50 ml.), and then with N-hydrochloric acid (30 ml.). The latter eluted the asparagine derivative (LVa) (6.4mgm, 65%). Trial experiments with synthetic mixture of the two amides (LVa) and LVIa)had shown that complete separation was achieved under these conditions, the weaker acid (LVIa) being eluted with the N-acetic acid.

Glycine n-Hexylamide. (LXII; R=H).—n-Hexylamine (37.7g, 2 mol.) in dioxan (100 ml.) was added over 40 minutes to a stirred solution of phthaloylglycylchloride (36g.) (Sheehan and Frank (82)), in dioxan (600 ml.) and chloroform (300 ml.) at 0°. The solution was evaporated

to one-third bulk, and aqueous sodium bicarbonate solution (0.5%, 1l.) was added. The precipitated solid m.p. 157-161° was washed with water, and recrystallised from 50% aqueous ethanol (1l.) to give phthaloylglycine n-hexylamide as needles (34.2g, 73%) m.p. 165-166° raised by further recrystallisation from the same solvent to 166-167°. (Found: C,66.5; H,7.2; N,10.2. $C_{16}H_{20}O_3N_2$ requires C,66.6; H,7.0; N,9.7%).

A solution of the foregoing product (32.4g.) in ethanol (700 ml.) was heated under reflux for 1 hour with N-ethanolic hydrazine hydrate (113 ml.). The cooled solution was filtered, and evaporated to dryness. The residue was treated with warm N-hydrochloric acid (600ml.), the precipitated phthalhydrazide removed by filtration, and the solution evaporated to a gum. After being thoroughly dried (P_2O_5), the gum was crystallised from ethanol-ethyl acetate (1.10) to give glycine n-hexylamide hydrochloride as hygroscopic plates (11.35g, 52%), m.p. 249° (decomp; after considerable sintering at 95°) (Found, in material dried at 78°: C,49.3; H,10.0%; equiv., 199. $C_8H_{19}ON_2Cl$ requires C,49.3; H,9.8%; equiv., 194.5).

γ-Ethyl Benzyloxycarbonylglycylglycyl-L-glutamate.(LIe).

After many experiments in which the effect of temperature, reaction time, and solvent was studied, the following satisfactory procedure was used. A vigorously stirred solution of benzyloxycarbonylglycylglycine (21.7g) (Bergmann and Zervas (84)), and triethylamine (12 ml.) in chloroform (210 ml.) was treated at 0° with ethyl chloroformate (7.8 ml.). After 25 minutes a solution of γ-ethyl-L-glutamate (XL ,figl) (21.4g.) (Miller and Waelsch (56)) in N-sodium hydroxide (118 ml.) and water (220 ml.) was added, and stirring was continued for 2 hr at 0° and 2 hr at room temperature. After being filtered the aqueous and chloroform layers were separated, and the latter was washed with aqueous ammonia (1.5%, 2 x 100 ml.) and water (100 ml.). The dried chloroform solution was evaporated to a neutral gum which was not further examined. All the aqueous solutions were combined and acidified. The precipitated oil rapidly crystallised, and the solid was recrystallised from water (170 ml.) to give the tripeptide (LIe) (8.4g,24%) m.p. 144-146° unchanged by recrystallisation from water. (Found: C,53.9; H,6.1; N,10.0%; equiv., 424. $C_{19}H_{25}O_8N_3$ requires

C,53.9; H,6.0; N,9.9%; equiv., 423).

The tripeptide (LIe) (2g.) was fractionated by countercurrent distribution (100 transfers) between sec-butanol and aqueous phosphate buffer made from 0.5M-KH_2PO_4 (9 Vol.) and 0.5M-K_2HPO_4 (1 Vol.). A single peak was obtained (K 2.0), (graph VIII). The solid recovered from tubes 64-83 in the same way as before was crystallised from water to give the tripeptide (LIe) as needles (1.5g), m.p. 151-153°. When chromatographed on Whatman No. 1 paper with sec-butanol (100 vol.) and aqueous ammonia (3%, 40 vol.) as the solvent system, this product had R_F 0.61; benzyloxycarbonylglycylglycine had R_F 0.38 in the same solvent.

General Method for Preparation of Glycine n-Hexylamides of Acyl Aspartic and Glutamic Acid Mono-esters. - A solution of the mono-ester (5 mmol.) in dioxan (80 ml.) and chloroform (15 ml.) was stirred at 0° with triethylamine (0.7 ml.) and ethyl chloroformate (0.48 ml.). After 20 minutes a further portion (0.7 ml.) of triethylamine was added, followed immediately by a solution of glycine n-hexylamide hydrochloride (5mmol.) in dioxan (15 ml.) and water (1 ml.). The mixture was stirred for 2 hr at 0°

then 2 hr at
/room temperature, and finally adjusted to pH6 with hydrochloric acid. Water (50 ml.) was added and the chloroform and dioxan were evaporated. The oil which separated was extracted into ethyl acetate (3 x 100 ml.) and the extract was washed with 2-N hydrochloric acid (10 ml.), saturated aqueous sodium bicarbonate (15 ml.), and finally with water. Evaporation of the dried extract left the coupled product as a gum. Unchanged mono-ester (ca. 25%) was recovered from the alkaline extracts by acidification. The following peptides were prepared by this method:

(a). N-Benzoyl-α-DL-aspartylglycine n-hexylamide ethyl ester (LIIb)* - crystallised as long needles (80% based on unrecovered mono ester), m.p. 99-102° raised by recrystallisation thrice from aqueous ethanol to 105-107° (Found: C,62.5; H,7.6; N,10.4. $C_{21}H_{31}O_5N_3$ requires C,62.2; H,7.7; N,10.4%).

(b). N-Benzoyl-β-DL-aspartylglycine n-hexylamide ethyl ester (LXb) — (60%), crystallised as fine needles m.p. 82-87° after sintering. (Found in material dried at 56°: C,61.9; H,7.7; N,10.1. $C_{21}H_{31}O_5N_3$ requires C,62.2; H,7.7; N,10.4%).

This ester was also prepared by dissolving the corresponding acid (LVb) (0.116g.) in ethanol (10 ml.) containing dry hydrogen chloride (1.3g.) and allowing the solution to stand at room temperature for 21 hr. Crystallisation of the neutral matter from aqueous ethanol gave (LXb) (75 mgm; 60%) m.p. 82-86°.

(c). N-Benzyloxycarbonylglycylglycyl-α-L-glutamylglycine n-hexylamide ethyl ester (LIIe) - was prepared as above, save that the mono ester (LIe) (5 mmol.) was dissolved in dioxan (100 ml.) and toluene (70 ml.) to avoid racemisation (Vaughan (62)). The peptide crystallised from aqueous ethanol as rosettes of needles (76%), m.p. 142-146°, after sintering, $[\alpha]_D^{18}$ +3.6° (C,4.88 in ethanol). (Found: C,57.6; H,7.3; N,12.2. $C_{27}H_{41}O_8N_5$ requires C,57.5; H,7.3; N,12.4%).

N Benzoyl-α-and-β-DL-aspartylglycine n-Hexylamides. (LVIb) and (LVb). - A solution of glycine n-hexylamide hydrochloride (0.72g.) and triethylamine (1.05ml, 2 equiv.) in dioxan (35 ml.) and chloroform (10 ml.) was warmed at 40° for 4 hr. with N-benzoyl-DL-aspartic anhydride (0.82g.). The solvents were evaporated with occasional addition of

water (total 40 ml.), and the final aqueous solution was acidified and extracted with ethyl acetate (3 x 70 ml.). After being washed with water, the extract was shaken with an excess of 2 N-aqueous ammonia. The alkaline solution was acidified, and the oil which was precipitated gradually crystallised (1.09g, 77%), m.p. 150-162°. A portion (0.3g) was fractionated by countercurrent distribution (40 transfers) between ethyl acetate (8 Vol.), n-butanol (2 Vol.) and aqueous phosphate buffer (11.2 Vol.) made from 0.5M-KH_2PO_4(11 Vol.) and 0.5-MK_2HPO_4(1.6 Vol.). Two peaks which were almost completely separated were obtained. (K 1.05, 43%; K 5.15, 57%) (graph IV). The aqueous layer from tubes 13-27 (K1.05) was acidified, and then shaken again with the organic layer from these tubes, followed by two extractions with ethyl acetate. The combined extracts were shaken with an excess of sodium carbonate solution, and the separated aqueous phase acidified. N-Benzoyl-β-DL-aspartylglycine n-hexylamide (LVb) crystallised as needles (88 mgm.), m.p. 163-164° (decomp.), unchanged bt recrystallisation from water (Found: C,60.5; H,7.2; N,10.9%; equiv., 372. $C_{19}H_{27}O_5N_3$ requires C,60.5; H,7.2; N,11.1%; equiv., 377.). Isolation

of the material from tubes 30-42 (K5.15) in the same way yielded N-benzoyl-α-DL-aspartylglycine n-hexylamide (LVIb) (IIImgm.) as needles, m.p. 179-180° (decomp.), unchanged by recrystallisation from water (Found: C,60.3; H,7.1; N,10.9% equiv., 368).

The ester (LIIb) (1g.) was boiled with acetone (12.5ml) water (8.5ml.) and concentrated hydrochloric acid (3.8ml.) for 2 hr., and the products were separated into an acidic and a neutral fraction as usual. The former, crystallised from water, gave the weaker acid (LVIb) (0.384g,40%) m.p. 176-176.5° raised to 176.5-177° in admixture with the foregoing sample.

N-Benzoyl-DL-Asparaginylglycine n-Hexylamide (LXIb). - This was prepared on the 1 mmol. scale from the related acid (LVIb) as for the amide (LXa). The organic solvent was removed by evaporation in vacuo, and the resultant suspension adjusted to pH7 with sodium bicarbonate. After being thoroughly shaken the solid was filtered off and resuspended in 0.001 N-hydrochloric acid. After being washed with water the product crystallised from aqueous-ethanol to give the asparaginyl peptide (LXIb) ad fine needles (66%), m.p. 184-185.5° raised by one recrystallisa-

tion from the same solvent to 186-187° (Found: C,60.1; H,7.6; N,15.3. $C_{19}H_{28}O_4N_4$ requires C,60.6; H,7.5; N,14.9%).

The ester (LIIb) when treated with saturated alcoholic ammonia at room temperature for 4 days yielded a neutral fraction from which the asparaginyl peptide (LXIb) was isolated (12%) by crystallisation from aqueous ethanol. The m.p. 183-184° was unchanged in admixture with the foregoing product.

N-Acetylglycylglycyl-α-L-glutamylglycine n-Hexylamide Ethyl Ester (LIIf). - A solution of the benzyloxycarbonyltetrapeptide (LIIe) (6g.) in methanol (250ml.) and containing glacial acetic acid (0.8ml.) was shaken with hydrogen and palladium oxide (0.4g.) until the evolution of carbon dioxide ceased. The filtered solution was evaporated to a gum, and treated with water (200ml.). The insoluble matter (1.71g.) was filtered off and crystallised from aqueous ethanol to afford unchanged starting material (1.55g.) m.p. 138-142° (sintering at 120°), raised to 140-144° (after sintering) in admixture with authentic (XLVIIe). The aqueous filtrate above was shaken with acetic anhydride (160 ml.) at a temperature below 45° for 20 minutes. After

adding a further portion (30ml.) of acetic anhydride shaking was continued for a further 5 minutes, and the solution was allowed to stand at room temperature for 3.5 hr. The gum obtained by evaporation of the solution was crystallised from water (70ml.) to yield the acetyltetrapeptide (LIIf) (1.2g.), m.p. 190-194°. Treatment of the mother-liquor from this crop with acetic anhydride (60ml.) in the same way yielded a second crop of (LIIf) (0.6g.) m.p. 188-195°, and concentration of the final mother-liquor yielded a third crop (0.64g.) m.p. 187-189°(total yield 2.44g.,(68%), based on unrecovered starting material). The first crop, recrystallised twice from water gave needles, m.p. 193.5-194.5°. (Found: C,53.9; H,8.0; N,15.1. $C_{21}H_{37}O_7N_5$ requires C,53.5; H,7.9; N,14.9%).

Action of Bases on the Esters and Amides of Aspartyl Peptides. - (a) On N-benzoyl-α-DL- aspartylglycine n-hexylamide ethyl ester (LIIb). This ester (0.71g.) was stirred with 0.28 N-sodium carbonate (25ml., 4 equiv.) at 40°. After 2 hr. the undissolved oil was removed, dissolved in ethanol (0.5ml.), and returned to the reaction mixture. A cloudy solution was obtained

which rapidly became clear. After 4 hr., the cooled solution was adjusted to pH7, freed from neutral matter (12mgm.) by extraction with ethyl acetate, and acidified. The precipitated oil rapidly crystallised (0.63g.,95%) m.p.155-157° (decomp; after sintering). A sample (0.3g.) was fractionated by countercurrent distribution (40 transfers) (graph V) using the same solvent system as used previously for the separation of the acidic aspartyl peptides (LVb) and (LVIb). The same two peaks were obtained. From one (K1.05, 75%) the stronger acid (LVb) was isolated as before, m.p. 160-162°(decomp.) alone or in admixture with the earlier sample. The other peak (K5.15 25%) yielded the weaker acid (LVIb) m.p. 177-179°(decomp.) raised to 178-180° in admixture with the earlier sample.

In a second experiment on the same scale, the material (0.15g) undissolved after 2 hr. was collected and washed with water. It crystallised from aqueous ethanol to afford DL-benzamido-succinoyl-glycine n-hexyl-amide (LIVb) as stout prisms, m.p. 149-150°. (Found: C,63.1; H,7.1; $C_{19}H_{25}O_4N_3$ requires C,63.5; H,7.0%).

(b). On N-benzoyl-β-DL-aspartylglycine n-hexylamide ethyl ester.(LXb). This was treated as above on 1/5 scale, the material remaining undissolved after 3.5hr. was removed and crystallised from aqueous ethanol to give the imide (LIVb)(37mgm.) m.p.148-149° alone or in admixture with the foregoing product. The mother-liquors from the imide were added to the main alkaline solution, which was then warmed at 50° for a further hour. The acidic products (91mgm.) isolated as before, were fractionated by countercurrent distribution (40 transfers) (graph VI) in the solvent system used in the previous experiment. Two peaks (K.1.05,77%;K 5.15,23%) were obtained corresponding to the isomeric acids (LVb) and (LVIb). It was confirmed that the latter band contained the weaker acid (LVIb) by isolation of the solute from tubes 29-38 as earlier; m.p.175.5-176° raised to 176-177° in admixture with an authentic sample.

(c). On N-benzoyl-DL-asparaginylglycine n-hexylamide(LXIb). Ammonia was slowly evolved when this peptide (0.1g.) was shaken vigorously with 0.1 N-sodium hydroxide (8ml., 3 equiv.) for 6 hr. at room temperature. Unchanged

starting material (82mgm.) was filtered off, and the acidic products (14mgm.) m.p. 143-145.5° were recovered from the filtrate as before.

A second portion of the peptide (LXIb) (0.1g.) was heated for 6hr. at 50° with 0.25 N-sodium carbonate (3.2ml., 3 equiv.). The products were worked up as before to yield unchanged starting material (79 mgm.) and an acidic fraction (14mgm.) m.p. 149-151°.

The two portions of acidic material were combined and fractionated by countercurrent distribution (40 transfers) (graph VII) in the solvent system used in the previous experiment. The areas under the two peaks obtained (K.1.05 and 5.15) showed that the two components were present approximately in the ratio 3:1 respectively. The solute recovered from tubes 15-26 (K 1.05) was the stronger acid (LVb) m.p. 157.5-158° alone or in admixture with an authentic sample.

Action of Bases on the Esters of Glutamyl Peptides. - On N-acetylglycylglycyl-α-L-glutamylglycine n-hexylamide ethyl ester (LIIf). This ester (2.31g.) was shaken for 6 hr. at room temperature with 0.1-N sodium hydroxide (62ml., 1.2 equiv.). The alkaline solution was extracted

with ethyl acetate (2 x 50ml.), adjusted to pH5 and concentrated to 15 ml. On acidification of the solution N-acetylglycylglycyl-γ-DL-glutamylglycine n-hexylamide (LVf) separated as plates (1.22g., 56%), m.p. 186-190° raised to 194.5-195.5° by recrystallisation from water, $[\alpha]_D^{18} = 0° \pm 0.1°$ (C,12.4 in 1.2 equiv. of aqueous Naoh) Found: C,51.5; H,7.4; N,15.6%; equiv., 439. $C_{19}H_{33}O_7N_5$ requires C,51.5; H,7.5; N,15.8%; equiv; 443); it had pKa, 3.45 by potentiometric titration in water. (Graph XI).

The mother-liquor from (LVf) was concentrated to yield a second crop of acidic material (0.508g.), m.p. 185-187° (after sintering), and the final mother-liquor was evaporated to a gum. This was worked through dimethylformamide, ethanol, and ethyl acetate to remove inorganic salts, and afforded an amorphous solid (182 mgm.) which contained an appreciable amount of chloride ion, but no sodium ion. The second crop above, recrystallised from water, gave needles (293 mgm.) (Found: equiv., 446) m.p. 194-195.5° depressed in admixture with (LVf). A portion of this material was completely hydrolysed by being heated with 6N-hydrochloric

acid for 20 hr. at 110°. The presence in the hydrolysate of glutamic acid glycine and n-hexylamine was shown by paper chromatography.

N-Benzoyl-α-and-γ-DL-glutamylglycine n-Hexylamides (LVId) and (LVd). - These were prepared from glycine n-hexylamide hydrochloride (LXII; R H) (9.8g.) and N-benzoyl-DL-glutamic anhydride (LVIIc) (II.7g.) (Battersby(74)), in the way used above for the aspartyl analogues. During the isolation of the acidic products as before a portion (8.91g.) failed to dissolve in the volume (1.5 l.) of ethyl acetate used in the extraction. Recrystallisation from 30% aqueous ethanol (1.3 l.) gave (LVId) (7.79g.) m.p. 184°. (Battersby (74) records m.p. 184-186°). After shaking the aqueous phase again with ethyl acetate (2 x 100 ml.), the organic layers were combined and extracted with 2 N-sodium hydroxide (3 x 25 ml.). Acidification of the alkaline solution afforded a mixture of (LVId) and (LVd) (9.5g.), which was dissolved in 0.25 N-sodium carbonate (170ml.1.76 equiv.) and N-hydrochloric acid (24.5ml; 1.01 equiv.) added to give a further crop of (LVId) (1.6g.) m.p. 183.5°-184.5°. The mother liquor was acidified, and the

product crystallised from 30% aqueous ethanol (120ml.) to give (LVd) (5.8g.) m.p. 128-132° (Battersby (74) records m.p. 129-131°). Total yield of acidic products, 15.19g. (77%), made up of (LVId) (62%), and (LVd) (38%).

(V). (b)(ii). The specific fission of γ-glutamyl peptides.

(a) Anodic oxidation. (Boissonnas (53)). (cf.p 50). - Ice cold solutions of N-benzoyl-α-and-γ-DL-glutamyl-glycine n-hexylamide (LVId) and (LVd) (10 mgm.) in anhydrous methanol (3ml.) were anodically oxidised for 6-7 hours, (100V; 75mA). An ice-cold solution of N-acetyl-glycylglycyl-γ-DL-glutamylglycine n-hexyl-amide (LVf) (5mgm.)in anhydrous methanol (3ml.) was similarly oxidised for 12 hours, (100V; 10-20 mA). The solutions were evaporated to dryness in vacuo, and the residues hydrolysed with constant boiling hydrochloric acid (5ml.) in sealed tubes at 110° for 16-20 hours. The hydrolysates were chromatographed on Whatman No.1 filter papers in the system formic acid/sec-butanol/water. The chromatogram from N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd) showed that the spot corresponding to glutamic acid had disappeared, whereas that from (LVId) showed that glutamic acid had been almost completely destroyed with the production of a new amino acid, probably α-amino-γ-methoxybutyric acid. In the case of (LVf) the glutamic acid residue had been almost completely destroyed, but no new amino acid had been produced.

(b) By Bergmann-Zervas Degradation. (45) (cf. p.45).

A solution of N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd) (0.5 g.) in anhydrous methanol (15ml.) containing dry hydrogen chloride (0.6g.) was heated at 50° for 5 hours. The residue obtained by evaporation was dissolved in ethyl acetate (30ml.) and the solution extracted successively with aqueous sodium bicarbonate (5%, 10ml.), hydrochloric acid (0.2N, 5ml.), and finally with water. After being dried, the ethyl acetate solution was evaporated to a gum (0.3g., 85%), which crystallised from aqueous ethanol to give dimethyl N-benzoyl-DL-glutamate monohydrate (LXIV) as needles (0.17g., 48%), m.p. 82-83.5°, raised to 80-85° by further recrystallisation from the same solvent. (Found, in sample dried at 56°: C,56.1; H,6.0; N,5.0. $C_{14}H_{17}O_5N.H_2O$ requires C,56.6; H,6.4; N,4.7) A solution of the dimethyl ester (LXIV) (0.5 g.) in ethanol (5.5ml.) was added dropwise with shaking to hydrazine hydrate (0.6 ml., 100%, 10 equiv.), during 5-10 minutes (cf. Le Quesne and Young (86)). After standing for 20 hours at room temperature, the solution deposited N-benzoyl-DL-glutamyldihydrazide (LXV) (0.47 g., 94%) m.p. 171-173°,

raised to 172-173°
/by recrystallisation from ethanol. (Found: C,51.6; H,6.2. $C_{12}H_{17}O_3N_5$ requires C,51.6;H,6.1%).

A portion of the dihydrazide was hydrolysed with constant boiling hydrochloric acid in a sealed tube at 110° for 20 hours. The hydrolysate was chromatographed in the system formic acid/sec-butanol/water, and the chromatogram showed two spots corresponding to glutamic acid and hydrazine.

(V) (b)(iii). The structure and attempted rearrangement of tyrocidine A methyl ester. -

(a) Hofmann degration of the methyl ester. -

Tyrocidine A methyl ester was prepared in the way described by Battersby and Craig (25). A portion (0.05g.) of the methyl ester was subjected to Hofmann degradation with sodium hypobromite in exactly the same way as described above (p.120) for the degradation of tyrocidine A. The final product was hydrolysed, and examined by two dimensional paper chromatography in the same systems used on p.114. The chromatogram showed the distinct spot of α,β-diaminopropionic acid; no evidence was obtained for the presence of α,γ-diaminobutyric acid in the hydrolysate.

(b) Attempted rearrangement. Tyrocidine A methyl ester (0.5g.) was shaken at room temperature with 0.1N-sodium hydroxide (12.1 ml., 3.2equiv.), water (2 ml.) being added after 1 hour. The clear solution obtained after 3 hours was allowed to stand for a further 3.5 hours, and then acidified with hydrochloric acid. After standing at 0° for 15 hours the acidic products were collected,

dried in vacuo, and then fractionated by countercurrent distribution (90 transfers) between ethyl acetate and aqueous phosphate buffer, made from 0.5MKH_2PO_4(11 Vol.) and 0.5 MK_2HPO_4(1.6 Vol.). The distribution pattern was a broad band indicating the presence of at least two components.

(V). (b)(iv). The preparation and alkaline cleavage of 1-acyl-2-pyrrolidone derivatives from α-glutamyl peptides.

The preparation of 5-carbethoxy-1-benzoyl-DL-pyrrolid-2-one. (LXVI).

α-ethyl N-benzoyl-DL-glutamate (LIXc) - N-benzoyl-DL-glutamic anhydride (LVIIc) (3g.) (Battersby (74)), was suspended in dry ethanol (60ml.), and the mixture refluxed on a water bath; a clear solution was obtained after 1 hour. After a further 2 hours the solution was evaporated in vacuo to a gum (3.89g.), which was dissolved in ether (20ml.) and the solution extracted successively with 0.25 N-sodium carbonate (3 x 11ml, 25ml). The ethereal solution was washed, dried and evaporated, and the residue (0.19g.) crystallised from petroleum ether (60/80°) to give diethyl N-benzoyl-DL-glutamate (LVIIIc) (0.102g.) m.p. 72.5-73°, raised to 76-79° by recrystallisation from aqueous ethanol. (Abderhalden and Rossner (87) record m.p. 77-78°).

The four alkaline extracts were acidified, and all gave oils save the last one. The first three extracts were combined, and the mixture was extracted with ethyl acetate (total Vol, 116 ml.). After being dried the

ethyl acetate solution was evaporated to a gum (2.78g.). The gum was fractionated by countercurrent distribution (37 transfers) (graph IX) between ethyl acetate and aqueous phosphate buffer made from 0.5 M-KH_2PO_4 (11 Vol.) and 0.5 M-K_2HPO_4 (1.6 Vol.). The combined lower layers from tubes 20-40 were acidified, shaken again with the upper layers from these tubes, and then extracted with a further portion of ethyl acetate. The upper layers from the tubes and the extract were combined, the solution washed with a little water, and evaporated in vacuo. The product, α -ethyl N-benzoyl-DL-glutamate (LIXc), was obtained as a gum (1.81g, 50.5%), which could not be induced to crystallise. It was purified for analysis by short path distillation (Found: N,5.0. $C_{14}H_{17}O_5N$ requires N,5.3%). The solute from tubes 2-18 was isolated in the same way, and gave a gum (0.45g.,12.5%) which crystallised from aqueous ethanol to give γ -ethyl N-benzoyl-DL-glutamate (LIc) m.p. 110-112°, raised to 112-114° in admixture with an authentic sample of (LIc) m.p. 115-115.5°.

The fourth extract above was shaken with ethyl acetate (3 x 25 ml.). After being washed and dried

the solution in ethyl acetate was evaporated to gum, which crystallised from water to give N-benzoyl-DL-glutamic acid monohydrate (0.302g., 8.5%), m.p. 97.5-99.5°.

5-Carbethoxy-1-benzoyl-DL-pyrrolid-2-one (LXVI) - A portion of the α-ethyl ester (LIXC) (0.28g.) was shaken in a stoppered vessel with thionyl chloride (1ml., 14 equiv.) at 0°. (cf. King and Spensley (88)). After several minutes a clear solution was obtained, and this was allowed to stand at 0-1° for 15 hours. The thionyl chloride was removed by evaporation in vacuo, firstly at 0° and finally at 30°. The residue, an oil, was dissolved in ether (7ml.) and the solution extracted with aqueous sodium bicarbonate (3%., 5ml.). After being washed with water the ethereal solution was dried and evaporated in vacuo to a colourless gum (0.218g., 84%). The gum crystallised from ether/petroleum ether (60/80°) (4:7, 11 ml.) to give 5-carbethoxy-1-benzoyl-DL-pyrrolid-2-one (LXVI) (0.148g.) m.p. 54-59°. After being recrystallised three times from aqueous ethanol the product had m.p. 55.5-58°(platelets).(Found, in sample dried at 35°: C,64.8; H,5.6; N,5.6. $C_{14}H_{15}O_4N$

requires C,64.4; H,5.8; N,5.4%).

Alkaline hydrolysis of 5-carbethoxy-1-benzoyl-DL-pyrrolid-2-one (LXVI). - A solution of the acylpyrrolidone (LXVI) (0.13g.) in ethanol (3.5 ml.) was titrated with 0.1N-sodium hydroxide (5.01 ml, 1 equiv.) over 20 minutes, phenolphthalein being used as indicator. 0.4 equiv. of alkali was consumed quickly, but thereafter the rate of alkali consumption decreased. The ethanol was removed by evaporation in vacuo, and the neutral product extracted into ether (2 x 5 ml.). The ethereal solution was washed and dried, and evaporated to a gum (0.041g., 26%), which crystallised from aqueous ethanol to give diethyl N-benzoyl-DL-glutamate (LVIIIc, fig.2.) m.p. 77-79°, undepressed in admixture with an authentic sample.

In a further experiment the acylpyrrolidone (LXVI) (0.292g.) was dissolved in ethanol (8ml.), and the solution titrated with 0.1N-sodium hydroxide (0.64 equiv. 7.2 ml.) over 15 minutes. The ethanol was removed in vacuo, and the oily aqueous mixture extracted with ether (15 ml., 10 ml.). The aqueous solution was concentrated slightly by evaporation in vacuo, and acidified; benzoic

acid (29.7 mgm, 34%) crystallised, m.p. 112-116°. After being recrystallised from water the m.p. rose to 119.5-121°, undepressed in admixture with an authentic sample.

The ethereal extract above was washed with water, dried, and evaporated in vacuo to a gum, (0.15g.). The latter was dissolved in ethanol, and titrated as above with 0.1N-sodium hydroxide (4.0 ml., 0.36 equiv.), using phenolphohalein as indicator. Alkali was consumed only very slowly; in consequence the last portion (2.6 ml.) was added altogether; and the solution allowed to stand at room temperature for 3.5 hours. The ethanol was removed by evaporation in vacuo, and the aqueous solution extracted with ether (10ml., 5 ml.). After being washed with water and dried the ethereal solution was evaporated to dryness; the residue (33 mgm.) crystallised from aqueous ethanol to give diethyl N-benzoyl-DL-glutamate (LVIIIc), m.p. 76.5-78°.

In a separate experiment a solution of the acyl-pyrrolidone (LXVI) (20.4 mgm) in purified dioxan (64) (0.5 ml.) and water (1.0 ml.), was titrated with 0.1N-sodium hydroxide (0.8 ml., 1 equiv.) over 10 minutes. The dioxan was removed by evaporation in

vacuo, and the aqueous suspension was extracted with ethyl acetate (2 x 10 ml.); each extract was washed with a little water, and the washings were added to the aqueous solution. The latter was concentrated slightly by evaporation in vacuo, acidified, and extracted with petroleum ether (40/60°) (3 x 30 ml.). The extracts were combined, dried, and evaporated in vacuo; benzoic acid (4.4 mgm., 46%) was obtained, m.p. 115-121°. The recovery of benzoic acid by this procedure was checked by dissolving a portion (10.4mgm.) in water (6 ml.), and extracting the solution with petroleum ether in the way described above, 8.4 mgm. (80%) of benzoic acid was recovered in this way. The yield of benzoic acid recorded in this experiment has been corrected in accordance with this recovery.

The 1-benzoyl-DL-pyrrolid-2-one derivative (LXVIII) formed from N-benzoyl-α-DL-glutamylglycine n-hexylamide (LVId) - N-benzoyl-α-DL-glutamylglycine n-hexylamide (LVId) (2g.) was shaken for several minutes with thionyl-chloride (7.3 ml. 20 equiv.) in a stoppered vessel, at 0° (89). Almost complete solution was obtained at the end of this period, and this was allowed to stand

at 0-1° for 20 hours. The reddish-brown solution was evaporated as described on p.157, and the residue partitioned between ethyl acetate (150 ml.) and aqueous sodium bicarbonate (2%, 50ml.). The ethyl acetate was washed with water, dried, and evaporated to dryness in vacuo; the residue (1.83g, 96%) was crystallised from ethyl acetate (50 ml.) to give a mauve coloured product (1.01g.) m.p. 158-160°, unaltered on a further recrystallisation from ethyl acetate. The product was recrystallised from 35% aqueous ethanol (charcoal), and was obtained as almost colourless needles. m.p. 156-157°, raised to 160.5-161.5° by further recrystallisation from ethyl acetate. (Found: C,64.7; H,7.2; N,11.5. $C_{20}H_{27}O_4N_3$ requires C,64.3; H,7.3; N,11.3%).

Three further experiments were carried out in which portions (0.04g., 0.5g., 0.5g.,) of (LVId) were treated separately with thionyl chloride (20 equiv.) at 0-1° for 2, 10 and 15 hours respectively. The thionyl chloride was removed as described above, and the neutral products were extracted into ethyl acetate. Evaporation of the solutions in ethyl acetate gave 13.3 mgm., (34%); 0.45g., (94%); and 0.45g.,(94%) of

the neutral products in the three cases respectively.

Finally a portion (1g.) of (LVId) was treated as above with thionyl chloride (3.7 ml, 20 equiv.) at -20° for 19 hours. After removal of the thionyl chloride, the neutral products were extracted into ethyl acetate, and obtained by evaporation of the solution (0.85g., 90%).

Alkaline hydrolyses of the neutral products obtained by the action of thionyl chloride on N-benzoyl-α-DL-glutamylglycine n-hexylamide (LVId)

(a) - Of the products obtained by using thionyl chloride (20 equiv.) at 0° for 20 hours.

A solution of the neutral products (0.5g. m.p. 158-160°) in purified dioxan (15 ml.) and water (10 ml.) was titrated with 0.1N-sodium hydroxide (13.8 ml., 1.02 equiv.) over 20 minutes. The dioxan was removed by evaporation in vacuo, and the aqueous alkaline solution was extracted with ethyl acetate (2 x 100 ml.). After being washed with water and dried, the solution in ethyl acetate was evaporated to a white crystalline residue (0.166g.,46%), which was recrystallised from ethyl acetate/petroleum ether

(60/80°) to give the pyrrolid-2-one derivative (LXIX) m.p. 109-110°, as needles. (Found; in sample dried at 76°: C,56.5; H,9.1; N,14 .8. $C_{13}H_{23}O_3N_3 \cdot \frac{1}{2}H_2O$ requires C,56.1; H,8.7; N,15.1%., m.p. 90-91° which all flowed at 103-104°).

The aqueous alkaline solution was concentrated slightly by evaporation in vacuo and acidified. The acidic products were collected by filtration, dried, and extracted with warm petroleum ether (3 x 40 ml.); the insoluble fraction (0.18g.) had m.p. 140-151°. After extracting the aqueous filtrate with petroleum ether (2 x 40 ml.), the extracts were combined, dried and evaporated in vacuo; benzoic acid (59 mgm. 36%), m.p. 120.5-121°, was obtained. The aqueous filtrate was extracted again with ethyl acetate; (100 ml, 80 ml.) the extracts were combined, dried and evaporated to a gum (51 mgm.). The latter was combined with the acidic products (0.18g.) which were insoluble in petroleum ether, and the mixture was fractionated by countercurrent distribution (50 transfers) (graph X) between ethyl acetate (4 Vol.), n-butanol (1 Vol.) and aqueous phosphate buffer (5.6.Vol.) made from

0.5 M-KH_2PO_4 (4 Vol.) and 0.5 M-K_2HPO_4 (2.8 Vol.). Two completely separated peaks were obtained; the solute recovered from tubes 8-25 (K 0.47, 49%) (88mgm; theor. 113 mgm from area under curve) in the way described on p.156 was crystallised from 30% aqueous ethanol (3 ml.) to give N-benzoyl-γ-DL-glutamylglycine n-hexylamide (LVd) m.p. 118.5-123°, raised to 126-132° by one recrystallisation from the same solvent; the m.p. was unaltered in admixture with an authentic sample of (LVd), m.p. 128-132°.

The solute recovered from tubes 29-45 (K 2.7, 51%) (99 mgm.) in the same way was recrystallised from 30% aqueous ethanol to give N-benzoyl-α-DL-glutamyl-glycine n-hexylamide (LVIa) m.p. 182.5-183° raised to 183-183.5° in admixture with an authentic sample of (LVId) m.p. 184-184.5° (The preparation of (LVd) and (LVId) is described on p.148).

(b) - Of the products obtained by using thionyl chloride (20 equiv) at 0° for 10, and 15 hours respectively.

Solutions in aqueous dioxan of the total neutral products obtained in both experiments were titrated with 0.1N-sodium hydroxide (1 equiv.), and the benzoic acid

isolated in the way described above under (a). The weights of benzoic acid obtained in the two cases were: 49.3 mgm., (33.6%); and 50.8mgm., (35%), respectively.

(c)- Of the products obtained by using thionyl chloride (20 equiv) at -20° for 19 hours.

The total neutral products obtained were dissolved in aqueous dioxan and the solution titrated with 0.1N-sodium hydroxide, (1 equiv.); benzoic acid (94 mgm.33%) was isolated as above; m.p. 119-120.5°.

To ensure that benzoic acid was not lost in the above experiments during the evaporation of the solutions in petroleum ether a portion of benzoic acid (15.5 mgm.) was dissolved in petroleum ether (90 ml.) and the solution was evaporated in vacuo. Benzoic acid (15.3 mgm., 99%) remained after evaporation.

5-Diethylcarbamoyl-1-benzoylglycyl-DL-pyrrolid-2-one.(LXXVI) Dimethyl N-benzoylglycyl-DL-glutamate.(LXXII) - A stirred solution of benzoylglycine (LXX) (91) (0,179g.) and triethylamine (0.14 ml.) in dioxan (15 ml.) and toluene (7 ml.) was treated at 0° with ethyl chloroformate (0.1ml.).

After 20 minutes, a solution of dimethyl DL-glutamate hydrochloride (LXXI) (92) (0.211g.) and triethylamine (0.14 ml.) in dioxan (10 ml.) and water (0.6 ml.) was added, and stirring was continued for a further 3 hours whilst the bath warmed to room temperature. The organic solvent was removed by evaporation in vacuo; water was added and the evaporation was repeated. The aqueous suspension was extracted with ethyl acetate (2 x 10 ml.), and the solution in ethyl acetate was extracted with hydrochloric acid (0.1N-, 2 ml.), followed by aqueous sodium bicarbonate. The solution was washed with water, dried, and evaporated to a gum (0.219g., 65%), which crystallised on standing as needles. The product was recrystallised from ethyl acetate/petroleum ether (60/80°) (3:4,7 ml.) to give dimethyl N-benzoylglycyl-DL-glutamate (LXXII) (0.143g.) m.p. 109-113° raised to 117-118° by further recrystallisation from the same solvent. (Found: C,57.0; H,6.1; N,8.0. $C_{16}H_{20}O_6N_2$ requires C,57.1; H,6.0; N,8.3%).

N-benzoylglycyl-DL-glutamic acid (LXXIII). - To a solution of the foregoing product (LXXII) (0.5g.) in methanol (5 ml.) was added aqueous sodium hydroxide (approx N-)

(8.9 ml., ca. 6 equiv.), and the solution was allowed to stand at room temperature for 48 hours. The methanol was removed by evaporation in vacuo, and the aqueous phase acidified; N-benzoylglycyl-DL-glutamic acid (LXXIII) crystallised as needles (0.38g., 84%), m.p. 219°, unchanged by recrystallisation from water. (Found: C,54.4; H,5.1; N,9.4; equiv., 159.5. $C_{14}H_{16}O_6N_2$ requires C,54.5; H,5.2; N,9.1%; equiv. 154.1).

N-Benzoylglycyl-α- and -γ-DL-glutamyldiethylamide. (LXXIV) and (LXXV). - To a stirred solution of N-benzoylglycyl -DL-glutamic acid (LXXIII) (6.16g.) and triethylamine (2.8 ml.) in chloroform (200 ml.) and dimethylformamide (110 ml.) at 0° was added ethyl chloroformate (2.0 ml.). After 20 minutes a solution of diethylamine hydrochloride (2.18g.) and triethylamine (2.8 ml.) in chloroform (90 ml.) was added, and stirring was continued for a further 3 hours at 0°. The solution was evaporated to dryness in vacuo; water (25 ml.) was added and the evaporation repeated. Water (20-30 ml.) was added to the residue, and the aqueous suspension extracted with ethyl acetate (200 ml.). The undissolved solid (1.77g., m.p. 179-182°) was filtered off, and crystallised from ethanol to give

N-benzoylglycyl-α-DL-glutamyldiethylamide (LXXIV) (1.54g.) m.p. 182-184°, raised to 183.5-184.5° by further recrystallisation from the same solvent. (Found: C,59.4; H,6.9; N,11.7; equiv., 356. $C_{18}H_{25}O_5N_3$ requires C,59.5; H,6.9; N,11.6%; equiv., 363).

The aqueous phase was extracted with a further portion (50 ml.) of ethyl acetate, the latter was combined with the first extract (total Vol. 200 ml.), and the solution in ethyl acetate was extracted with aqueous potassium carbonate (5%, 30 ml.). The aqueous phase on acidification precipitated an oil which crystallised on standing at 0° for 2 days, (3.29g.) m.p. 87-92°, equiv., 388. This was treated with hot ethyl acetate (250 ml.), the undissolved solid (0.94g. m.p. 114-130°) was filtered off as before, and the solution in ethyl acetate was extracted with three portions (3.1 ml.(+5.0 ml. water); 6.2ml., 5.2 ml.) of 0.5N-sodium hydroxide. The aqueous alkaline extracts on acidification all yielded oils which crystallised on standing at 0°, (0.298g. m.p. 85-88°; 0.970g. m.p. 90-91°; 0.322g. m.p. 84-86°) respectively. A portion (0.756g.) of the crystalline product, m.p. 90-91°, from the second

extract was dissolved in 0.5 N-sodium hydroxide (4.4ml., 1.05 equiv.) and portions (0.52 ml. and 1.7 ml.) of N-hydrochloric acid were added two precipitate two fractions (0.132g., m.p. 90-95°; 0.437g., m.p. 87-91°). The latter crystallised from water to give N-benzoylglycyl-γ-DL-glutamyl-diethylamide (LXXV) as hygroscopic needles, m.p. 108-111° (Found, in sample dried at 76°: C, 59.9 ; H, 6.9 ; N, 11.4 %; ~~equiv. 367.~~).

The portion of the product (0.94g. m.p. 114-130°) above was shown to be a mixture of (LXXIV) and (LXXV) by adding to a solution of the mixture in 0.5 N-sodium hydroxide (5.2 ml., 1 equiv) portions of N-hydrochloric acid (1.0 ml., 0.5 ml., 0.56 ml.) to precipitate three fractions, (0.199g., m.p. 154-164°; 0.214g., m.p. 90-96°; 0.203g., m.p. 90-94°)respectively. The first fraction was redissolved in 0.5 N-sodium hydroxide (1.1 ml., 1 equiv.) and N-hydrochloric acid (3 x 0.15 ml.) added to precipitate three fractions as before. The first two fractions were almost pure samples of (LXXIV), (0.04g., m.p. 182-184°; 0.057g., m.p. 181-181.5°). The last fraction (0.056g.) was shown to be largely N-benzoylglycyl-γ-DL-glutamyldiethylamide

(LXXV) by its m.p. 92-95°. (Total yield of (LXXVI) and (LXXVII), 5.06g., 70%).

5-Diethylcarbamoyl-1-benzoylglycyl-DL-pyrrolid-2-one(LXXVI)

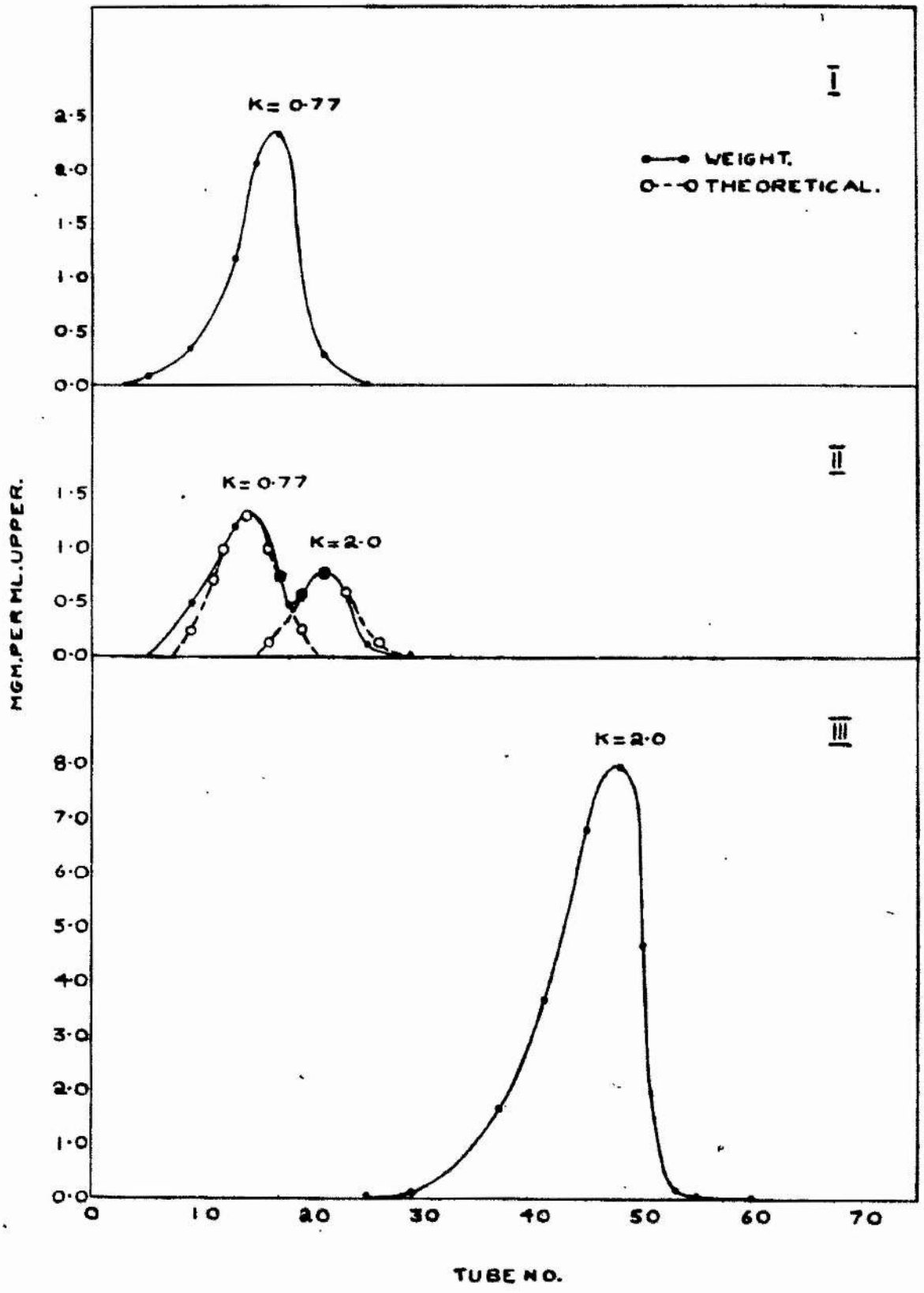
N-benzoylglycyl-α-DL-glutamyldiethylamide (LXXIV)(1.28g.) was shaken with thionyl chloride (10ml., ca. 40 equiv.) in a well stoppered vessel at 0° for several minutes during which almost completion solution was obtained. The solution was allowed to stand at 0-1° for 17 hours, the thionyl chloride then being removed by evaporation in vacuo at 0°. The residue was partitioned between ethyl acetate (60 ml.) and saturated aqueous sodium bicarbonate (30 ml.). After being washed with water, and dried the solution in ethyl acetate was evaporated to a brown resin (1.1g., 90%), which crystallised from ethyl acetate to give 5-diethylcarbamoyl-1-benzoylglycyl -DL-pyrrolid-2-one (LXXVI) as prisms, m.p.141-143° raised to 144.5-145.5° by one recrystallisation from ethyl acetate. The m.p. was unaltered by further recrystallisation from the same solvent. (Found: C,62.7; H,6.6; N,12.5. $C_{18}H_{23}O_4N_3$ requires C,62.6; H,6.7; N,12.2%)

Alkaline hydrolysis of 5-diethylcarbamoyl-1-benzoylglycyl -DL-pyrrolid-2-one.(LXXVI)

A solution of the foregoing product (LXXVI) (0.836g.) in purified dioxan (5 ml.) and water (2 ml.) was titrated with 0.1N-sodium hydroxide (27.2 ml., 1.1 equiv.) over 30 minutes. The solution was evaporated to dryness in vacuo, and the residue was extracted with hot ethyl acetate (60 ml.), followed by ether (30 ml.). The undissolved solid was dissolved in water (5ml.), and the solution extracted with ethyl acetate (3 x 40 ml.), each extract being thoroughly backwashed with small portions of water. The washings and aqueous phase were combined and the solution (now about 10 ml. in volume) was partially acidified by adding N-hydrochloric acid (0.9 ml, 0.33 equiv.). After standing at 0° for 15 hours N-benzoylglycyl-α-DL-glutamyl-diethylamide (LXXIV) (0.202g. 23%) was collected m.p. 178.5-181°, raised to 182-183° in admixture with an authentic sample of (LXXIV). After recrystallisation from ethanol the m.p. was 182-183°.

To the mother liquor, which was partially acidified was added more N-hydrochloric acid (0.9ml., 0.33 equiv);

benzoylglycine (LXX) (0.137g.,) was obtained m.p. 182-183°, depressed in admixture with (LXXIV), but raised to 184-186° in admixture with an authentic sample of benzoylglycine m.p. 186-188°. The mother liquor from this crop was reduced to half volume by evaporation in vacuo but despite seeding with (LXXIV) no crystallisation occured. The mother liquor was acidified and a further crop (0.06g.) of rather impure benzoylglycine was obtained, m.p. 165-172°, raised to 183-185° in admixture with an authentic sample. After being recrystallised once from water the m.p. of the specimen was 173±5°. Total yield of benzoylglycine, 0.197g. (43%).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II
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III
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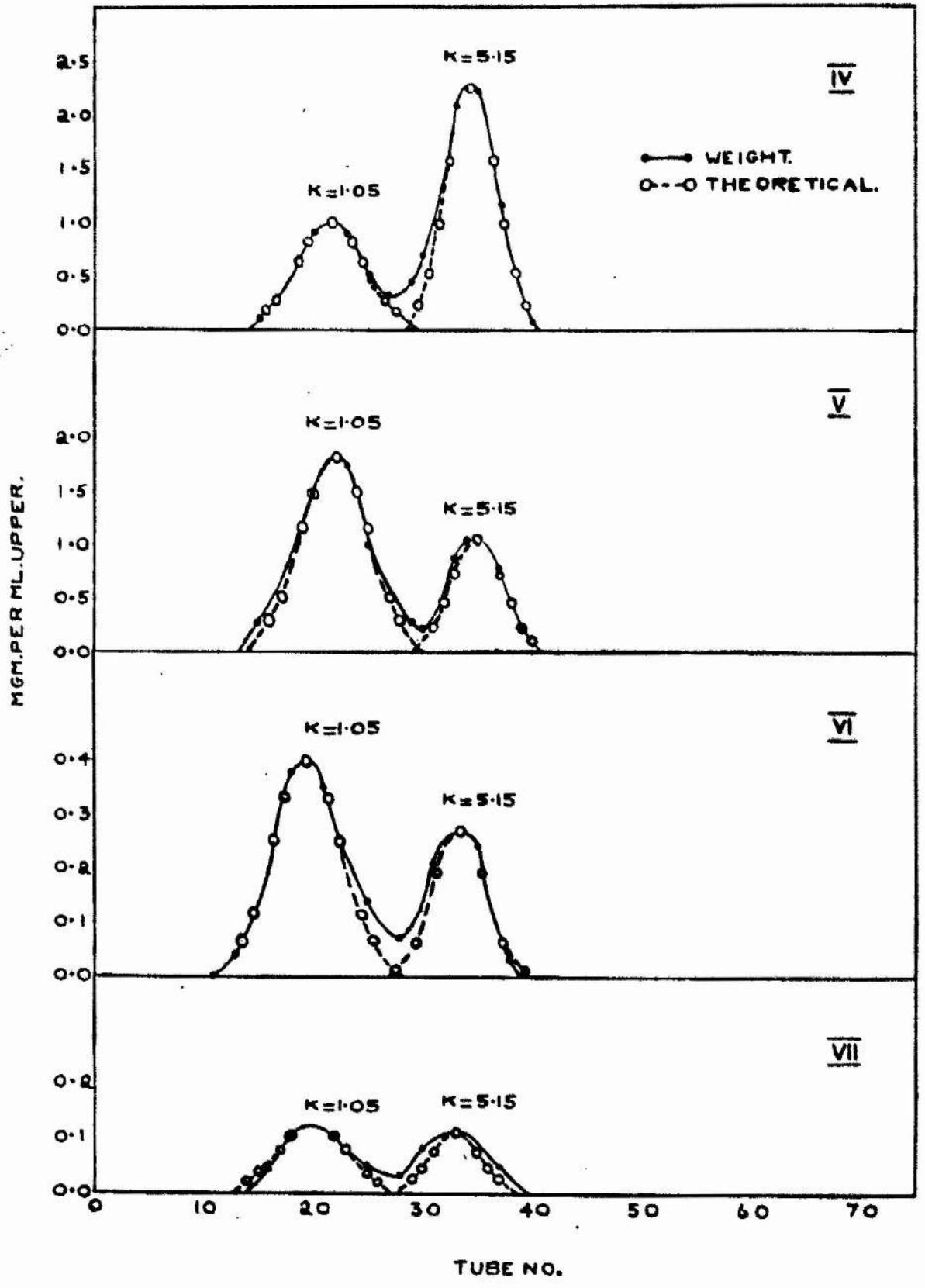
MGM. PER ML. UPPER.
TUBE NO.


IV
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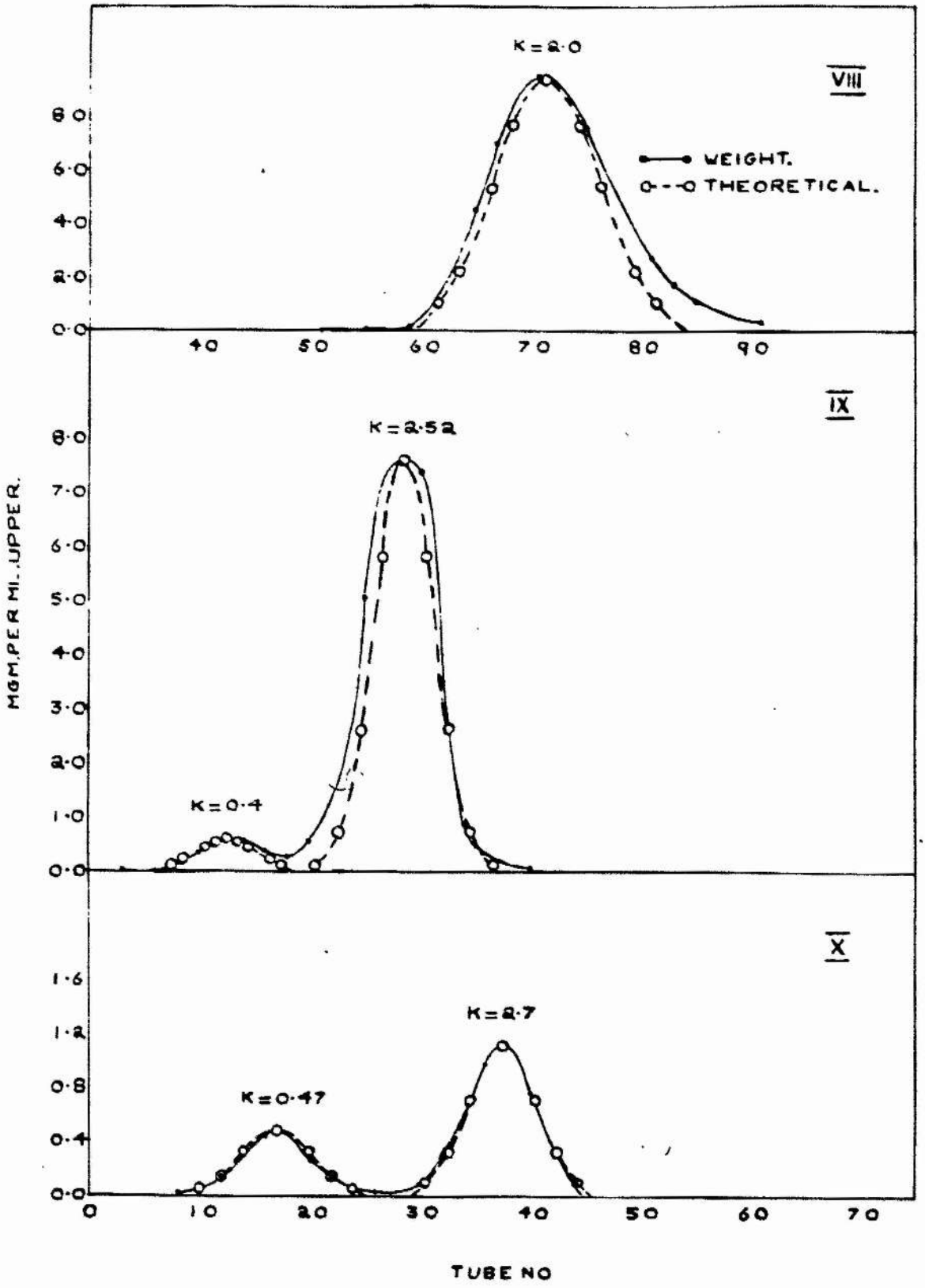
K=1·05
WEIGHT.
THEORETICAL.
V
K=1·05
K=5·15
VI
K=1·05
K=5·15
VII
K=1·05
K=5·15
MGM. PER ML. UPPER.
TUBE NO.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VIII
WEIGHT.
THEORETICAL.
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6·0
4·0
2·0
0·0
40
50
60
70
80
90
IX
K=2·52
K=0·4
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
MGM. PER ML. UPPER.
X
K=2·7
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0·4
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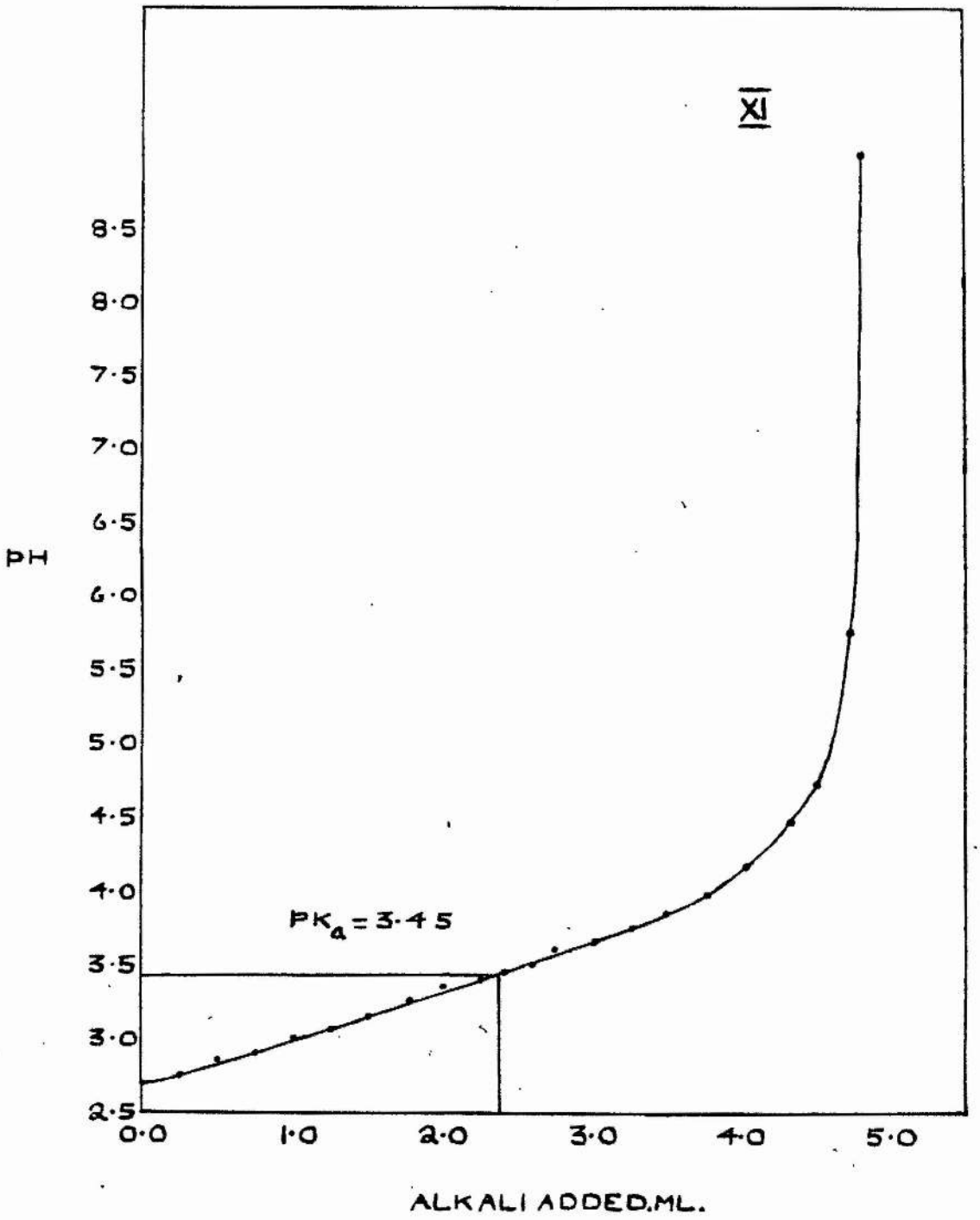
20
30
40
50
60
70
TUBE NO


XI
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7·5
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6·0
5·5
5·0
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3·0
2·5
PH
$PK_a = 3.45$
0·0
1·0
2·0
3·0
4·0
5·0
ALKALI ADDED.ML.

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