REVIEW ARTICLE

STRUCTURE AND SYNTHESIS OF NATURALLY-OCCURRING PEPTIDES

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Although proteins have long been recognised to be of paramount importance in nutrition, the investigation of proteins and the closely related polypeptides as substances that may possess specific biological activity is a comparatively recent development. Polypeptides, it should be stated, differ from proteins only in their lower molecular weight, the division being arbitrarily fixed at 10,000. One of the first proteins with biological activity to be recognised was the hormone insulin, which controls the blood sugar level of the body. Its molecular weight was originally thought to be about 24,000, but there is now general agreement[1–5] that it is of the order of 6000. Insulin should therefore be regarded as a polypeptide rather than as a protein. The various hormones produced by the pituitary gland also appear to be polypeptides. Vasopressin and oxytocin have indeed been shown to be polypeptides much simpler than insulin whilst the adrenocorticotrophic hormones have nearly the same molecular weight as insulin. Another group of polypeptides of biological importance are certain antibiotics, such as gramicidin, bacitracin and polymyxins, produced by bacteria.

Attempts to unravel the structure of polypeptides—to say nothing of the more complex proteins—would have been quite unthinkable a few years ago, but the application of paper chromatography has completely changed the picture, enabling the constituent amino-acids to be separated far more easily than was possible by the other techniques. At first the method was used merely to ascertain the nature and relative proportions of each amino-acid present. Progress became rapid when allied to the methods developed since 1945 which in the first place enabled the nature of the terminal amino-acid group in a polypeptide to be determined and in the second place indicated the sequence of amino-acids in the chain.

Identification of Terminal Amino-acid

In one of the more widely used methods[6,7] for identifying the terminal amino-acid, the polypeptide is reacted with dinitrofluorobenzene, the dinitrophenyl group attaching itself to the free amino group of the terminal amino-acid. On hydrolysis the corresponding dinitrophenyl-amino-acid is isolated and identified, thus indicating which amino-acid occupied the terminal position in the peptide chain. Another method[8,9] of characterising the terminal amino-acid involves reduction of the esterified polypeptide by means of lithium borohydride or of the polypeptide itself with lithium aluminium hydride. This converts the free carboxyl group of the polypeptide into a carbinol, and on subsequent hydrolysis to split the polypeptide into its component amino-acids, the liberated

amino-alcohol is identified. In a third method[10,11] the polypeptide is reacted with phenyl *iso*thiocyanate which combines with the free amino group, converting it into the phenyl thiocarbamyl derivative; this undergoes cleavage into a phenylthiohydantoin and a residual polypeptide containing one less amino-acid residue. The thiohydantoin is identified by paper chromatography thus indicating the nature of the terminal amino-acid. In a closely related method[12,13] the polypeptide is heated with acetic anhydride and ammonium thiocyanate which reacts with the free carboxyl group giving an acyl-thiohydantoin. Both these methods can be used for the step-wise degradation of polypeptides. In yet another method[14–18] the terminal amino-acid is removed by treatment with a selective enzyme and identified by means of paper chromatography. Thus carboxy peptidase removes the *C*-terminal amino-acid and amino-peptidase the *N*-terminal amino-acid. An advantage of this method is that like the preceding method it can be used to effect stepwise degradation of the polypeptide.

Partial Hydrolysis of Gramicidin S

A second big advance in the investigation of the structure of polypeptides was made in 1947 when Synge and his colleagues[19–21] used paper chromatography to separate the products of partial hydrolysis of the bacterial antibiotic gramicidin S. This has a much lower molecular weight than insulin, and was shown to consist of 10 amino-acid units—two molecules each of L-ornithine, L-valine, L-leucine, L-proline and D-phenylalanine. On partial hydrolysis it gave a mixture of di- and tripeptides, which were separated from one another by paper chromatography. Each spot on the paper was separately eluted and the individual di- and tri-peptides were then hydrolysed completely, and the component amino-acids identified by means of a second paper chromatogram. This enabled the constitution of the di- and tripeptides to be deduced and hence the way in which the amino-acids were linked together, giving a complete picture of the sequence of the amino-acids in the molecule. The complete structure of gramidin S is shown in Figure 1.

Purification of Polypeptides

Gramicidin S is an exceptional polypeptide in that it is easily crystallised, giving a homogeneous product free from other peptide impurities. Most polypeptides, however, are not so easily purified and it is clear that before the structure of a polypeptide can be determined all traces of other peptides and proteins must be removed. A technique now widely adopted for the separation of polypeptides from one another is the method of counter-current distribution developed by Craig[22–24]. In this method the mixture of polypeptides is successively partitioned between a solvent phase and an aqueous phase in an apparatus consisting of a large number of tubes so arranged that the components with a high solvent/water partition coefficient accumulate at one end of the apparatus and the components with a low partition coefficient at the other end; by carrying out a large number of partitions it is possible to separate substances which

exhibit only very slight differences in partition coefficient. The method was used for example, to fractionate the first known bacterial antibiotic, tyrothricin, which after a preliminary separation by conventional methods into the two components, gramicidin and tyrocidine, was further fractionated by means of countercurrent distribution into several closely related polypeptides.

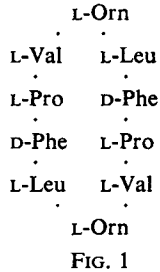


FIG. 1

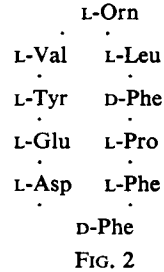


FIG. 2

Another technique that has been used to separate closely related polypeptides from one another is paper electrophoresis[25–30] in which the polypeptide mixture is applied to a strip of damp filter paper and a voltage is applied to the two ends of the paper; this causes the components of the mixture to migrate towards one electrode or the other. Generally the different components migrate at different rates forming separate spots on the paper which can be separately eluted.

Structure of the Tyrocidines

A brief description has been given above of the way in which the structure of gramicidin S (Fig. 1) was determined: precisely similar methods were used to determine the structures of tyrocidines A and B, and are at present being used to elucidate the structure of even more complex peptide antibiotics.

As already mentioned tyrothricin, which is produced by *Bacillus brevis* and was the first bacterial antibiotic to be discovered, was separated into gramicidin—a neutral substance, and tyrocidine—a basic substance; and each of these was in turn separated into several components. Some progress has been made in the study of the gramicidins[31,32] whilst the complete structure of tyrocidine A was established in 1954 by Paladini and Craig[33] and that of tyrocidine B in 1955 by King and Craig[34]. The two structures are given in Figures 2 and 3. What is particularly interesting is the close similarity between the two tyrocidines and gramicidin S. Each is a cyclic decapeptide, and five of the ten amino-acids are identical and are linked together in the same sequence. Moreover, each contains D-phenylalanine and not the L-isomer—the isomer commonly present in proteins. It is considered possible that the presence of this "unnatural" isomer may be one of the factors responsible for the antibacterial activity of these polypeptides. Although an open chain pentapeptide with the same sequence of amino-acids as in gramicidin S was found by Harris and Work[35] to have only limited antibacterial activity, a straight chain decapeptide with the same amino-acid sequence as that of gramicidin S

was synthesised (see below) and found to be strongly antibacterial[36,37] having an activity 1/10th to 1/40th that of gramicidin S. This suggests that peptides do not necessarily have to be cyclic in nature in order to possess antibacterial properties. The greater activity of the natural polypeptide may be due to its lower susceptibility to destruction by bacterial enzymes.

Structure of Other Peptide Antibiotics

Several antibiotics are produced by *Bacillus subtilis* and these, like the antibiotics produced by *B. brevis*, appear to be polypeptides. Bacitracin, perhaps the most clinically valuable of the bacterial antibiotics, was isolated in 1945 and resolved by countercurrent distribution into ten different polypeptides, three of them with antibacterial activity[38–40]. The major component, known as bacitracin A, was studied by the methods used for gramicidin S, and part of the molecule was thereby shown[41–43] to comprise the amino-acid sequence shown in Fig. 4. Further work

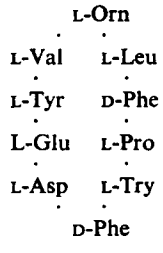


FIG. 3

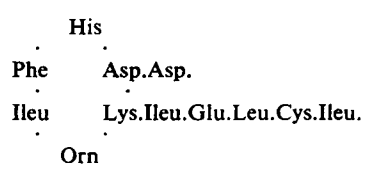


FIG. 4

showed that another part of the molecule contained a thiazoline ring formed by condensation of a cysteine and an *iso*leucine residue[44,45]. A crystalline fragment, thought to be 2-*iso*valeryl-thiazole-4-carboxylic acid was actually isolated from the hydrolysate of bacitracin F. The complete formula of the antibiotic is not yet known.

Another group of peptide antibiotics, the structure of which is partly known, is the group of substances known as the polymyxins, derived from *B. polymyxa*. In 1947, Ainsworth, Brown and Brownlee[46,47] in this country described a new antibiotic which they called aerosporin, and a similar substance obtained at about the same time by Stansly, Shepherd and White[48,49] in the U.S.A. was termined polymyxin. By countercurrent distribution the substance originally termed polymyxin was separated into five components and the main constituent, polymyxin A, was shown to be identical with aerosporin. All five polymyxins were found[50] on analysis to contain a branched chain fatty acid, identified as (+)-6-methyl-octanoic acid, and $\alpha\gamma$-diaminobutyric acid. Polymyxin B_1 has recently been shown[51] to contain 6 moles of the latter and one mole of the former, together with 2 moles of L-threonine, one mole of D-phenylalanine and one mole of L-leucine.

Nisin, originally isolated in 1947 by Hirsch and Mattick[52] from *Streptococcus lactis*, is another peptide now under investigation. It was separated

into four active polypeptides by countercurrent distribution and all four substances yielded on hydrolysis two sulphur-containing amino-acids not normally found in proteins[53,54] These appear to be lanthionine and β-methyl-lanthionine and these new amino-acids have also been detected in two bacterial antibiotics, subtilin and cynnamycin. Results have been published on the structure of other antibiotic peptides but work has not advanced sufficiently to justify inclusion in this review.

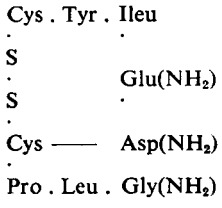


FIG. 5

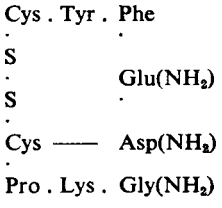


FIG. 6

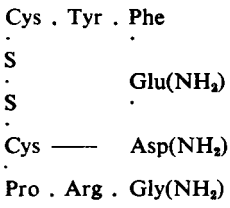


FIG. 7

Structure of Oxytocin and Vasopressin

In parallel with these investigations into the structure of the polypeptide antibiotics, similar work has been proceeding with the object of determining the constitution of the polypeptide active principles of the pituitary gland. Two of these, oxytocin and vasopressin, proved to be octapeptides, and so have a somewhat simpler structure than the tyrocidines and gramicidin S. These two posterior pituitary principles were separated from one another by electrophoresis and countercurrent distribution. Oxytocin, isolated as the crystalline flavinate, was hydrolysed and the constituent amino-acids were separated by partition chromatography on a starch column. The molecule was found to be made up of eight different amino-acids[55–56]. The structure of the molecule was investigated by du Vigneaud and his colleagues[57–63] at Cornell University, using a variety of methods—oxidation with performic acid, desulphurisation with Raney nickel, determination of the terminal groups, and degradation with bromine water. Finally the amino-acid sequence was established by the application of Edman's method and by partial hydrolysis with acid. These investigations led to the structure shown in Figure 5 being postulated for oxytocin. The same structure was arrived at independently by Tuppy[64,65], working in Vienna, and has since been confirmed by synthesis—one of the most important landmarks in the recent history of organic chemistry.

The other posterior pituitary principle vasopressin, was investigated by

similar methods[66–70]. The active substance from pig glands was found to have the same amino-acid composition as the hormone of ox glands, except that the former contained lysine and the latter arginine. The structures of the two hormones—lysine-vasopressin and arginine-vasopressin—are shown respectively in Figures 6 and 7. Lysine-vasopressin has been synthesized.

Structure of α-Corticotrophin

The hormones of the anterior lobe of the pituitary gland appear to be more complex than those of the posterior lobe, and the adrenocorticotrophic hormones—the group about which most is known—appear to contain about forty amino-acid residues. So far, four corticotrophins have been isolated and one of these, α-corticotrophin, from sheep pituitaries, was purified by zone electrophoresis on starch, chromatography on an ion exchange resin and countercurrent distribution. Investigation of its amino-acid composition and the terminal amino-acids and a study of the peptides obtained on partial hydrolysis by means of carboxy peptidase and acid led to the following structure for α-corticotrophin being advanced[71–75]:

Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys. Pro.
Val.Gly.Lys.Lys. Arg.Arg.Pro.Val.Lys. Val. Tyr.
Pro.Ala.Gly.Glu.Asp.Asp.Glu.Ala.Ser.Glu.Ala.
Phe.Pro.Leu.Glu.Phe.

Structure of Insulin

Even more complicated is the hormone insulin, which contains 48 amino-acid residues. Its structure was recently described by Sanger and his colleagues[76,77]. The methods employed were similar in principle to those already outlined, but obviously with such a complex molecule the difficulties were very much greater and the work took considerably longer. Insulin consists of two polypeptide chains joined at two points by disulphide linkages. These were broken by oxidation with performic acid, and the two chains were then investigated separately. The sequence of amino-acids in each was elucidated by partial hydrolysis and identification of the peptide fragments so formed. By piecing together the information so obtained, the whole insulin molecule was reconstructed. The structure of cattle insulin is given in Figure 8. It can be seen that the molecule consists of one large ring containing four half-cystine residues and a very much smaller ring containing two half-cystine residues. It is interesting to note that the nature of this second and smaller ring is different in insulin derived from sheep and pig pancreas. In sheep insulin the amino-acid sequence is Cys.Cys.Ala.Gly.Val.Cys. and in pig insulin Cys.Cys.Thr.Ser. Ileu.Cys. It may be significant that this smaller ring in insulin happens to be the same size as the molecules of oxytocin and vasopressin.

Synthesis of Polypeptides

The first two polypeptides ever to be synthesised were prepared by Fischer and Fourneau[78] as far back as 1901, but the method used and others developed during the next few years are quite unsuitable for the synthesis

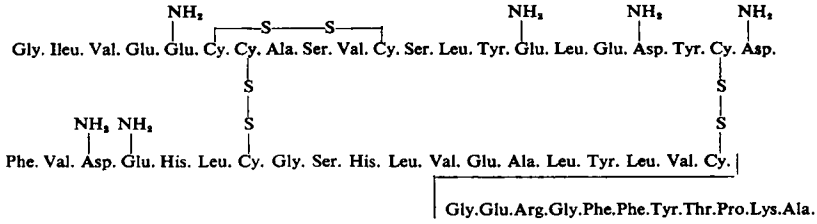


FIG. 8

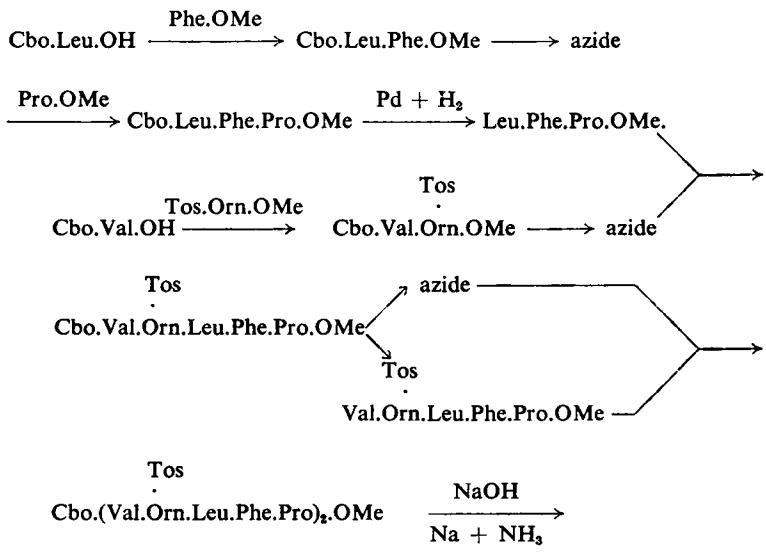
of complex polypeptides such as the bacterial antibiotics and pituitary hormones. All the practical methods, including those recently used for the synthesis of vasopressin and oxytocin, depend on protecting the amino group of one amino-acid, converting this into a reactive derivative such as the acid chloride or azide and then reacting this with the amino group of a second amino-acid. The groups that have been employed in this way to protect the amino group include the benzoyl, carbethoxy, *p*-toluene-sulphonyl (tosyl), haloacyl, carbobenzoxy (benzyloxycarbonyl), and phthalyl radicals. Of these the two last named are the most important. The first two radicals can be removed only by hydrolysis which also splits the peptide bond, whilst the haloacyl group is converted by treatment with ammonia into the corresponding amino acyl group. The tosyl group proved to be more useful than either of these other two radicals, for Fischer and Lipschitz[79] showed that it could be removed without affecting the peptide bond by treatment with phosphonium iodide and hydriodic acid, the tosyl group being split off as tolylmercaptan. The method has been used for preparing several di- and tri-peptides. In its more modern version sodium in liquid ammonia is used in place of phosphonium iodide.

By far the most important method, however, is the carbobenzoxy method introduced by Bergmann and Zervas[80,81] The carbobenzoxy or benzyloxycarbonyl group, which has the formula $C_6H_5CH_2OCO$, is introduced into an amino-acid by reaction with benzylchloroformate, $C_6H_5CH_2OCO{\cdot}Cl$, which is prepared by the action of phosgene on benzyl alcohol. The carbobenzoxy-amino-acid is converted into its azide and then reacted with a second amino-acid. To remove the carbobenzoxy group from the peptide thus formed it is catalytically hydrogenated, a method that avoids any danger of hydrolysing the peptide bond.

Synthesis of Antibacterial Decapeptide

This was the method used by Erlanger *et al.*[36,37] in synthesising the straight-chain decapeptide containing the same amino-acid sequence as gramicidin S. Carbobenzoxy-L-leucine was coupled with D-phenylalanine ester and the product was converted to the corresponding azide, carbobenzoxy-L-leucyl-D-phenylalanyl azide. This was coupled with L-proline ester and the product catalytically hydrogenated to remove the carbobenzoxy group, giving L-leucyl-D-phenylalanyl-L-proline ester. This was reacted with carbobenzoxy-L-valyl-*p*-tosyl-L-ornithine azide, obtained from carbobenzoxy-L-valine and *p*-tosyl-L-ornithine ester, giving

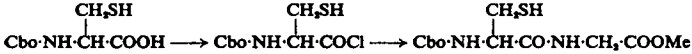
carbobenzoxy-L-valyl-*p*-tosyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline ester. One portion of this was converted to the corresponding azide and another portion was catalytically hydrogenated to remove the carbobenzoxy group. The two products were combined with one another, giving the decapeptide ester in which the end amino group carried a carbobenzoxy group and the ω-amino group of the ornithyl residue a *p*-tosyl group. After removing the ester group by hydrolysis, the carbobenzoxy and tosyl groups were removed at the same time by treatment with sodium in liquid ammonia. These reactions can be represented in abbreviated form as follows (Cbo = the carbobenzoxy radical $C_6H_5{\cdot}CH_2{\cdot}O{\cdot}CO$ and Tos = the *p*-tosyl radical):—

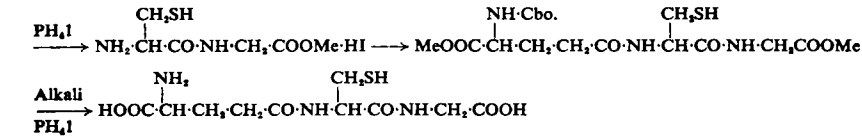


Val.Orn.Leu.Phe.Pro.Val.Orn.Leu.Phe.Pro.OH.

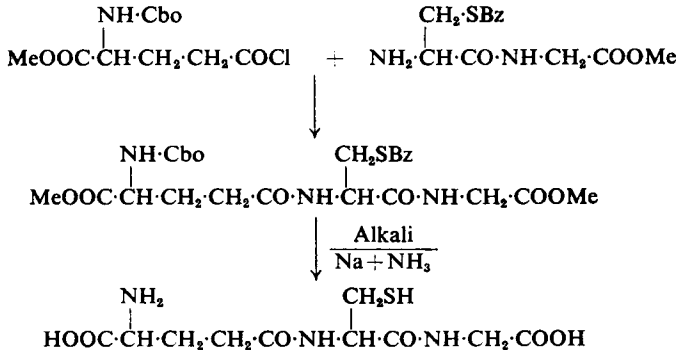
Synthesis of Glutathione

This method in its original form has one serious limitation: it cannot be used for producing polypeptides containing cysteine, because the catalyst used in the reduction is poisoned by the sulphur. Harrington and Mead[82], however, in 1935 prepared the tripeptide, glutathione (γ-glutamylcysteinyl-glycine) by using phosphonium iodide in acetic acid instead of catalytic hydrogenation to remove the carbobenzoxy group. *N*-Carbobenzoxy-cysteine was converted to the acid chloride and coupled with glycine ester. The product was reduced by means of phosphonium iodide to give cysteinylglycine ester hydriodide, which was then coupled with *N*-carbobenzoxyglutamyl chloride; the ester groups were removed by hydrolysis and the carbobenzoxy group was split off by reduction with phosphonium iodide:





A further improvement was made by du Vigneaud and his colleagues in their work on the synthesis of oxytocin. They had observed that the carbobenzoxy group could be removed just as readily by reduction with sodium in liquid ammonia as by catalytic hydrogenation, and it then occurred to them that a benzyl group used to protect a mercapto group could also be removed from *S*-benzylcysteine or from *S*-benzylcysteinyl-glycine by reduction with sodium in liquid ammonia, so that this one reaction might be used to remove the two protecting groups from the amino and the mercapto groups. They were able to synthesise glutathione by the following series of reactions (Bz = benzyl):—



Synthesis of Oxytocin

This synthesis of glutathione formed the basis of du Vigneaud's synthetic approach to oxytocin. When the structure of oxytocin was deduced from degradation experiments it was considered possible that if the protected nonapeptide amide could be synthesised reduction with sodium in liquid ammonia ought to give the reduced form of oxytocin. Air-oxidation should convert this into oxytocin. Accordingly, the reduced form of oxytocin was prepared from the natural hormone by reduction with sodium in liquid ammonia and the *SS'*-dibenzyl derivative was prepared from it in order to find out if the principle could be regenerated from its benzylated derivative. It was in fact found[83] that the biologically inactive *SS'*-dibenzyl derivative of oxytocin on treatment with sodium in liquid ammonia regenerated biologically active material.

The synthesis of oxytocin[84–88] was achieved by combining the tetrapeptide amide, *S*-benzylcysteinyl-prolyl-leucyl-glycyl amide with *p*-tosyl-*iso*leucyl-glutaminyl-asparagine and condensing the heptapeptide amide so formed with *S*-benzyl-*N*-carbobenzoxy-cysteinyl-tyrosine thus forming the desired protected nonapeptide amide. The tetrapeptide amide was prepared as follows:—

Carbobenzoxy-L-leucyl-glycine ethyl ester was synthesised by the mixed

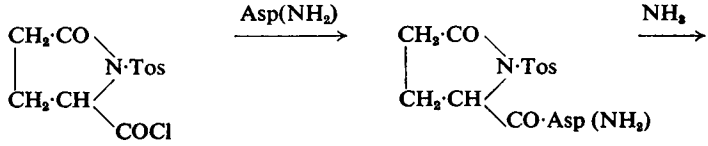
anhydride procedure of Vaughan and Osato[89]. After catalytic removal of the carbobenzoxy group, the L-leucyl-glycine ethyl ester was condensed with carbobenzoxy-proline. The protected tripeptide thus obtained was catalytically hydrogenated to remove the carbobenzoxy group and the resulting L-prolyl-L-leucyl-glycine ethyl ester was converted into di-carbobenzoxy-L-cystinyl-L-prolyl-L-leucylglycine ester by condensation with di-carbobenzoxy-L-cystine dichloride. The ester was hydrolysed and the carbobenzoxy groups were removed by means of sodium in liquid ammonia, and the sulphur of the reduced compound was then benzylated in the same medium. The product was converted into the benzyl ester and thence into the amide.

A novel method was used for the synthesis of glutaminyl-asparagine, the key intermediate being 5-oxo-L-*p*-tosyl-pyrrolidine-2-carbonyl chloride. This was coupled with L-asparagine to give 5-oxo-1-*p*-tosyl-pyrrolidine-2-carbonyl-L-asparagine which in strong ammonia yielded *p*-tosyl-L-glutaminyl-L-asparagine, the pyrrolidine ring having been opened by the addition of the elements of ammonia. Treatment with sodium in liquid ammonia gave L-glutaminyl-L-asparagine in high yield. For the preparation of *p*-tosyl-L-*iso*leucyl-L-glutaminyl-L-asparagine, *p*-tosyl-L-*iso*leucine was converted into the corresponding acid chloride and the latter was then coupled with L-glutaminyl-L-asparagine giving the tosyl-tripeptide in good yield. Condensation of the tripeptide with the tetrapeptide amide was effected by the use of tetraethyl pyrophosphite, and the tosyl group was removed from the heptapeptide amide by means of sodium in liquid ammonia which also removed the benzyl group. The resulting reduced compound was then benzylated with benzyl chloride in the same medium, and the *S*-benzyl heptapeptide amide was condensed with *S*-benzyl-*N*-carbobenzoxy-L-cysteinyl-L-tyrosine in presence of tetraethyl pyrophosphite to yield the nonapeptide amide. Sufficient synthetic material was prepared to enable it to be tried out in the induction of labour in the human, and its activity was found to be identical with that of natural oxytocin. These reactions can be represented in abbreviated form as follows:—

$$\text{Cbo.Pro} + \text{Leu.Gly.OEt} \longrightarrow \text{Cbo.Pro.Leu.Gly.OEt} \xrightarrow{\text{Pd} + \text{H}_2}$$

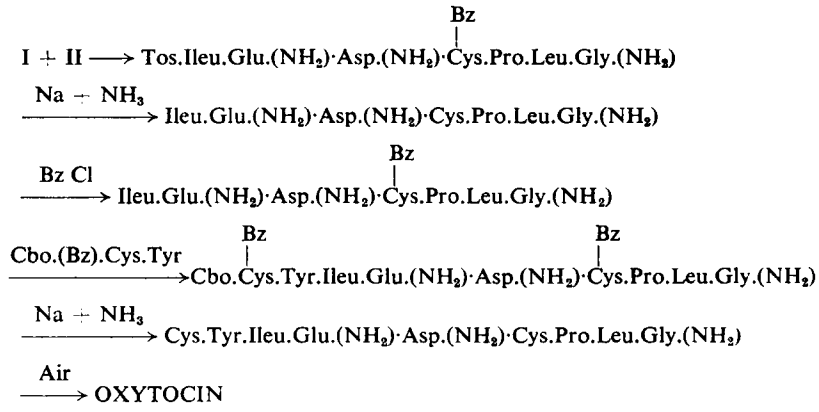
$$\text{Pro.Leu.Gly.OEt} \xrightarrow{\text{Cbo}_2\text{Cys.Cl}_2} \text{Cbo}_2\text{.Cys.(Pro.Leu.Gly.OEt)}_2 \xrightarrow[\text{Na} + \text{NH}_3]{\text{NaOH}}$$

$$\text{Cys.Pro.Leu.Gly.OH} \xrightarrow{\text{Bz Cl}} \text{Bz.Cys.Pro.Leu.Gly.OBz} \longrightarrow \text{amide} \quad \text{(I)}$$



$$\text{Tos.Glu.(NH}_2\text{)·Asp.(NH}_2\text{)} \xrightarrow{\text{Na} + \text{NH}_3} \text{Glu.(NH}_2\text{)·Asp.(NH}_2\text{)}$$

$$\xrightarrow{\text{Tos.Ileu.Cl}} \text{Tos.Ileu.Glu.(NH}_2\text{)·Asp.(NH}_2\text{)} \quad \text{(II)}$$



Lysine-vasopressin was synthesised by a method identical with that used for oxytocin except that leucine was replaced by ϵ *p*-tosyl-lysine and *iso*-leucine by phenylalanine at the appropriate stages.

The synthesis of these pituitary active principles undoubtedly sets a pattern for the synthesis of other polypeptides of comparable complexity and it is probably only a matter of time before more complex polypeptides are synthesised by methods similar to those used by du Vigneaud and his colleagues, who appear to have solved most of the basic problems. The synthesis of insulin, would, of course, involve a repetition of the steps involved in the synthesis of oxytocin many times over and would be a tedious operation resulting in extremely small yields of the final product.

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