SHORT TITLE: Solid Phase Synthesis of Some Analogues of Oxytocin

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Abstract

The Merrifield method of Solid Phase peptide synthesis has been applied to a study of the evolution of the neurohypophysial hormones. This work was carried out in an attempt to establish the identity of a principle designated EOP I (elasmobranch oxytocin-like principle I) which had been isolated from the pituitary gland of the spiny dogfish (Squalus acanthias) and to test hypotheses on possible evolutionary intermediates.

Three new analogues, 8-glutamine-oxytocin, 8-phenylalanine-oxytocin and 4-proline 8-glutamine-oxytocin were synthesized while a fourth, 8-valine-oxytocin, previously synthesized by classical methods was synthesized for the first time by solid phase. Each analogue was synthesized on the Merrifield resin by the stepwise addition of the appropriate BOC and CBZ protected amino acids. Cleavage from the resin, giving the protected nonapeptide amide intermediates was accomplished by ammonolysis. Reduction of the intermediates with sodium in liquid ammonia and subsequent oxidation of the sulfhydryl groups with ferricyanide to form cyclic disulfides yielded the crude peptides in each case. Each analogue was then isolated in highly purified form by gel filtration on Sephadex G-15. The peptides were then evaluated pharmacologically and the results compared with those of EOP I.

While none of the analogues synthesized appeared to be EOP I it is possible that one or more of them could exist in nature.

Solid Phase Synthesis of some possible Evolutionary Intermediates of the Neurohypophysial Hormones

by

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Dedication

to Georgiana and Robert

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INTRODUCTION

Part A : The Neurohypophysial Principles

I. First Evidence of Neurohypophysial Principles

The earliest recorded reports dealing with the neurohypophysial hormones oxytocin and vasopressin date from the end of the last century when Oliver and Schäfer (1895) noted that intravenous injections of bovine pituitary gland extracts caused an increase in the blood pressure in dogs. This was followed by a second report some three years later (Howell, 1898) indicating that the substance responsible for this pressor activity was obtained from the pituitary's posterior lobe, the neurohypophysis. These papers marked the beginning of over half a century of research which was to culminate in the 1950's with the syntheses of oxytocin and the vasopressins. This synthetic breakthrough in turn opened the way to further studies that are continuing to the present time.

As noted later by Dale (1957) the news that such an active substance should come from the posterior lobe, which consists almost entirely of nerve fibres was received with surprise at that time. The anterior lobe, quite glandular in nature, would have been considered the more likely candidate for elaboration of this activity. However, the ensuing years saw further activities assigned to these posterior pituitary extracts. The oxytocic or uterine contracting effect was discovered quite accidently by Dale (1906) and subsequently confirmed by him (Dale, 1909). The galactogenic or milk-ejecting effect of these extracts

was reported in 1910 (Ott and Scott, 1910) followed by that of the lowering of blood pressure in birds (Paton and Watson, 1912) and the antidiuretic effect (von den Velden, 1913). The finding of such widely diverse physiological effects from these extracts naturally gave rise to the question as to whether one or more than one active substance was present in the posterior lobe. The answer to this very intriguing question would of course have to await the separation and purification of the active principle or principles in question.

II. Early Attempts at Isolation, Unitary Theory, Binding Proteins

In 1919 Dudley (1919) reported the first partial separation of oxytocic and milk-ejecting activities from those of vasopressor and antidiuretic by means of a butanol extract of his dilute aqueous pituitary solutions. In the same paper he effectively disproved the hypothesis put forward by Abel and Kukota (1919) suggesting that the observed posterior pituitary effects were due to the presence of histamine. The number of principles responsible for the observed effects became a contentious point since Guggenheim (1914, 1917) had suggested on the basis of equal destruction of all activities on exposure to alkali that there was only one active principle. Abel and his co-workers (1923, 1930) became active exponents of this single principle or unitary theory claiming that there was a mother molecule composed of three or four active principles joined by something resembling a peptide linkage. They also contended that the separation of oxytocic and vasopressor activities achieved

by Dudley (1919) and later by Kamm and associates (1928) was actually due to a breakdown of the mother molecule during the isolation procedure. MacArthur (1931) reported a method for obtaining a single product containing both types of activities. However, details of the experimental procedure used were sketchy and the work was never repeated. Rosenfeld (1940) using press juice of pituitary glands obtained a product which by centrifugation studies gave a molecular weight of 20,000 - 30,000. When treated with acetic acid and the solution brought to the boiling point, biologically active substances of much lower molecular weights were obtained. Further, in 1942 van Dyke and his associates (van Dyke et al., 1942) isolated what appeared to be a pure protein, molecular weight 30,000, which contained equal amounts of oxytocic, vasopressor and antidiuretic activities. They claimed that it was not possible to separate these activities without hydrolysis. This van Dyke protein was later studied by Acher et al. (1956) who were able to separate the active hormones from the bulk of protein by electrodialysis under conditions which would not destroy covalent linkages. The biologically inactive binding protein thus separated has since been termed neurophysin (Chauvet et al., 1960) and several attempts have been made to obtain pure fractions of it (Ginsburg and Ireland, 1965; Hope and Hollenberg, 1966). Most resently Wuu and Saffran (1968) have reported isolating in pure form a peptide from the neurohypophysis which specifically binds neurohypophysial hormones. In contrast to other reported values of molecular weights ranging around 20,000 this peptide, containing 89 residues was found to have a

molecular weight just over 9,000.

III. Further Purification; Some Evidence of Chemical Nature

With regard to the active principles, the preparation of Kamm et al., (1928) resulted in a further resolution of the two sets of activities. Acetone dried bovine pituitary lobes were first extracted with hot 0.25% acetic acid followed by precipitation of the active principles with ammonium sulfate. The precipitate was then redissolved in glacial acetic acid and differentially reprecipitated using acetone, ether and finally petroleum ether. This resulted in the acquisition of two active fractions, one containing high pressor (80 i.u./mg) and low oxytocic (15 i.u./mg) activities and the other low pressor (6 i.u./mg) and high oxytocic (160 i.u./mg) activities.

During these early isolation and purification studies some knowledge had been accumulating as to the chemical nature of the active principle or principles present. Early studies had indicated that the principles were susceptible to destruction by proteolytic enzymes (Dale, 1906; Dudley, 1919) which led to the suggestion that some sort of peptide linkage might be involved in their structure (Thorpe, 1926). Stehle and Fraser (1935) were able to demonstrate with their partially purified preparations the presence of certain amino acids including tyrosine and cysteine which led them to suggest that the principles might be polypeptides of low molecular weight. Further

in this line was the finding by Potts and Gallagher (1942) that the oxytocin fraction prepared by them was relatively free of arginine (0.8%) while the pressor fraction had 12.3% arginine. This provided one of the first pieces of chemical evidence for differences between the two principles.

It was the reported presence of sulfur which first lead du Vigneaud to look at the neurohypophysial principles. His work on insulin up to that time had dealt largely with the effect of oxidation and reduction of sulfur on the biological activity of insulin and he was consequently interested in comparing this hormone with oxytocin and vasopressin (du Vigneaud, 1952). The presence of sulfur in the form of cysteine in both peptides was soon established (du Vigneaud et al., 1933) and further studies indicated that unlike in the case of insulin, reduction followed by re-oxidation did not lead to an inactive product (Sealock and du Vigneaud, 1935).

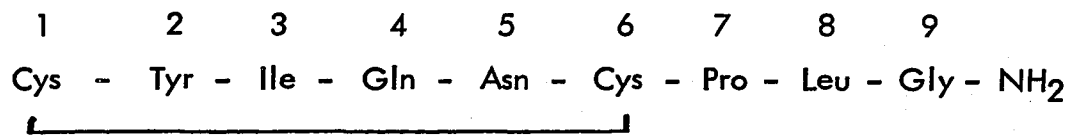
Before the war further progress was also made in purifying the principles by electrophoretic means (Irving and du Vigneaud, 1938) and this lead to the tentative establishment of isoelectric points of 8.5 for oxytocin and 10.8 for vasopressin (Cohn et al., 1941). The isoelectric point of oxytocin was subsequently adjusted to 7.7 (Kunkel et al., 1953). The final purification of the neurohypophysial principles, however, awaited the development of the technique of counter current distribution (Craig, 1944). The purification of oxytocin was carried out in a solvent system of sec-butanol and dilute acetic with two sets of 53 transfers giving a preparation

yielding 500 units of activity per milligram (Livermore and du Vigneaud, 1949). This was followed in 1951 with the purification of arginine vasopressin (Turner et al., 1951a) also using counter current distribution.

IV. Amino Acid Analyses and Sequence Determinations of Oxytocin and the Vasopressins

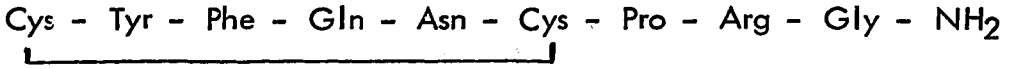
With supplies of the pure hormones being at last available it was possible to mount a serious attack on the hitherto unchartered territory of chemical structure. Amino acid analysis of the purified oxytocin preparation hydrolysate (Pierce and du Vigneaud, 1950a) by the Moore and Stein starch column chromatographic technique (1949) revealed the presence of eight amino acids; leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine and cystine present in a 1 : 1 molar ratio to each other. In addition three moles of ammonia per mole of amino acid were detected. The cystine present furthermore could account for all sulfur in the preparation (Pierce and du Vigneaud, 1950b). This amino acid analysis was confirmed by Privat de Garilhe (1951) using a different preparation of oxytocin. Amino acid analysis of bovine vasopressin revealed the presence of phenylalanine and arginine in place of leucine and isoleucine (Popenoe et al., 1952a; Acher et al., 1952). The vasopressin isolated from hog glands however was found to contain lysine in place of arginine (Popenoe et al., 1952b).

The primary sequence of oxytocin shown here was arrived at independently by du Vigneaud's group (du Vigneaud et al., 1953a) and by Tuppy (1953).



Performic acid oxidation (Mueller et al., 1951) and Raney nickel desulfurization studies (Turner et al., 1951b) led du Vigneaud to conclude that the compound contained a cyclic structure involving a disulfide bridge. Using the dinitrofluorobenzene end group technique of Sanger (1945) cysteine was shown to be present at the N-terminal (Davoll et al., 1951). The rest of the sequence was worked out using a combination of cleavage with bromine water (Ressler et al., 1953), sequential degradation using the Edman method (Edman, 1950) and partial acid hydrolysis (Turner et al., 1951b). Assignment of the ammonia to the carboxyl groups of glutamic acid, aspartic acid and glycine was based on isoelectric point studies (Kunkel et al., 1953) which showed oxytocin to have no free carboxyl groups.

The structure of arginine vasopressin



was arrived at in the same manner (du Vigneaud et al., 1953c) and was subsequently confirmed by Acher and Chauvet (1953, 1954), while that of lysine vasopressin

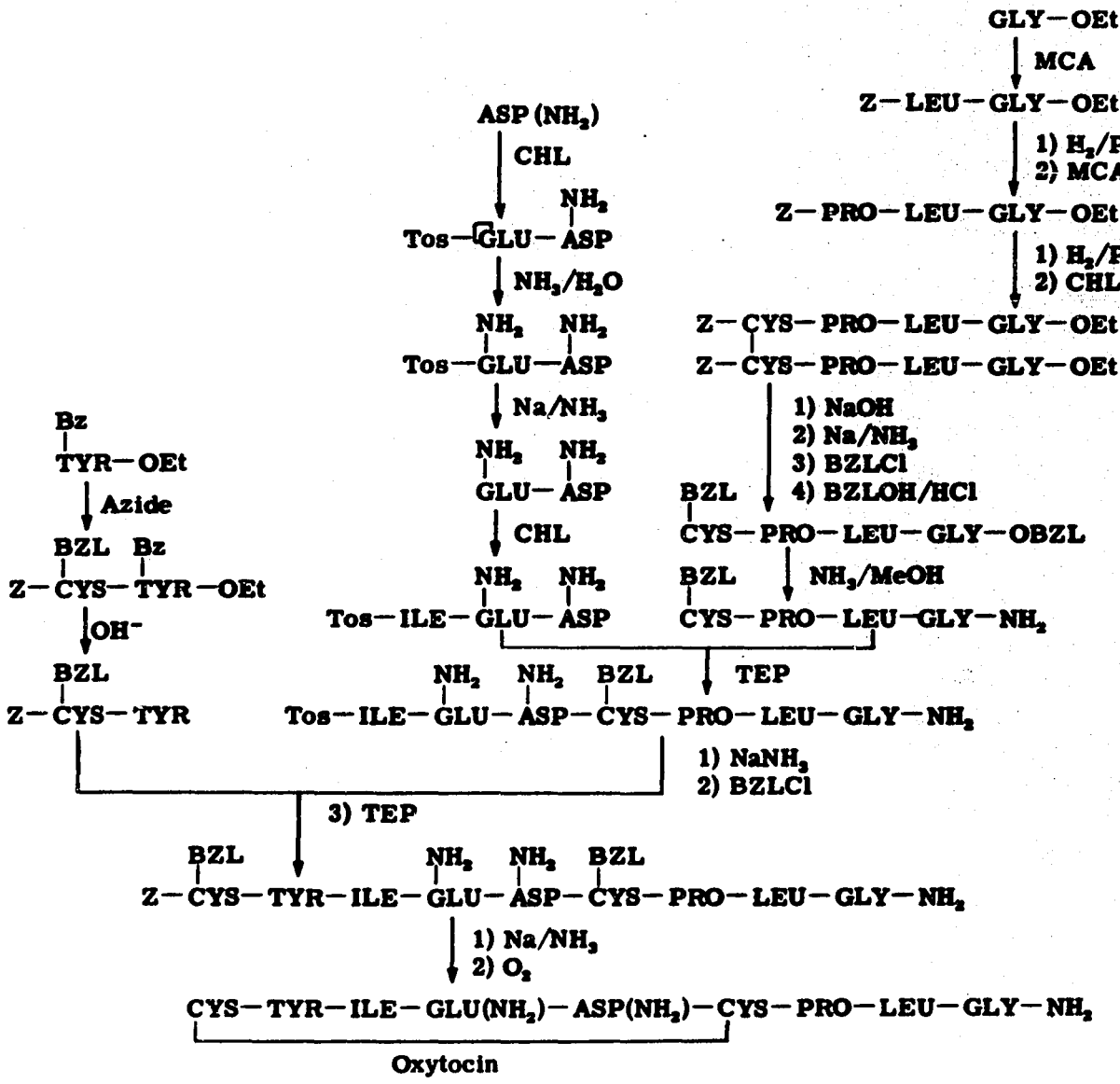
Cys - Tyr - Phe - Gln - Asn - Cys - Pro - Lys - Gly - NH_2

containing lysine in place of arginine was confimed by synthesis (du Vigneaud et al., 1957).

V. Chemical Synthesis of Oxytocin

The final confimation of the structure of these principles would have to await their chemical synthesis in the laboratory. The synthesis of oxytocin was therefore undertaken by du Vigneaud and his associates as a means of proving the structure put forward by them. Earlier work on the reduction-oxidation of the cysteine groups (Sealock and du Vigneaud, 1935) led them to believe that oxytocin could be generated from its protected nonapeptide intermediate N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide by means of sodium liquid ammonia reduction to remove the benzyloxy-carbonyl and benzyl groups followed by air oxidation to form the disulfide bond. Synthesis of this intermediate was therefore undertaken according to the scheme shown in Figure 1. The tetrapeptide amide S-benzylcysteinyl prolylleucylglycinamide was coupled with isoleucylglutaminylasparagine to form the heptapeptide amide which was in turn condensed with N-benzyl-oxycarbonyl-S-benzyl-cysteinyltyrosine thus forming the desired protected nonapeptide amide. The smaller fragments had likewise been built up through

Figure 1 The Original Synthesis of Oxytocin*Scheme used by du Vigneaud et al., (1954)



*For abbreviations see appendix.

condensation of appropriately protected amino acids.

Reductive deprotection of the protected peptide (Figure 2) and air oxidation of the disulfide followed by counter current distribution purification revealed a product that was physically, chemically and pharmacologically indistinguishable from the natural material (du Vigneaud et al., 1953b, 1954). The structure of oxytocin is shown in Figure 3. The synthesis of lysine vasopressin was likewise undertaken (Bartlett et al., 1956; du Vigneaud et al., 1957) followed by that of arginine vasopressin (du Vigneaud et al., 1958; Katsoyannis et al., 1958).

These syntheses of the neurohypophysial principles by du Vigneaud and his associates marked the first time that fully active peptides had been synthesized chemically in the laboratory. It thus opened the door to many avenues of research.

For those interested in the mechanism of action of the neurohypophysial hormones it raised the possibility of specifically modifying the chemical structure of the hormone and looking at the effect on activity. With a knowledge of structure activity relationships it might also be possible to design analogues for clinical therapeutic use which would have the unwanted side effects present in the naturally occurring hormones minimized. In addition to this, syntheses could be used as a model for the development and testing of new synthetic procedures and isolation techniques. The great interest in these areas is witnessed by the fact that since 1953 in the

Figure 2 Protected Nonapeptide Amide Intermediate used by du Vigneaud et al., (1954) in the Synthesis of Oxytocin

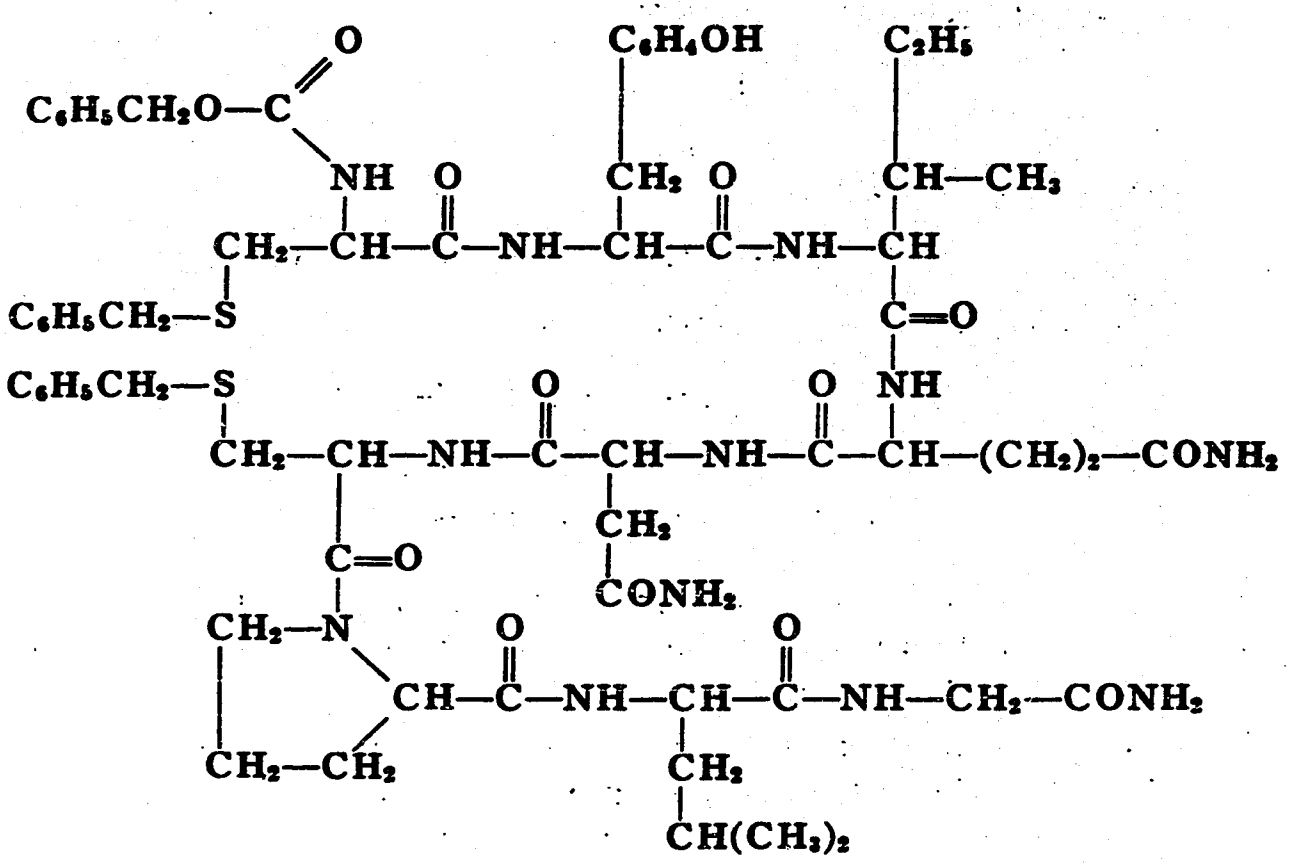
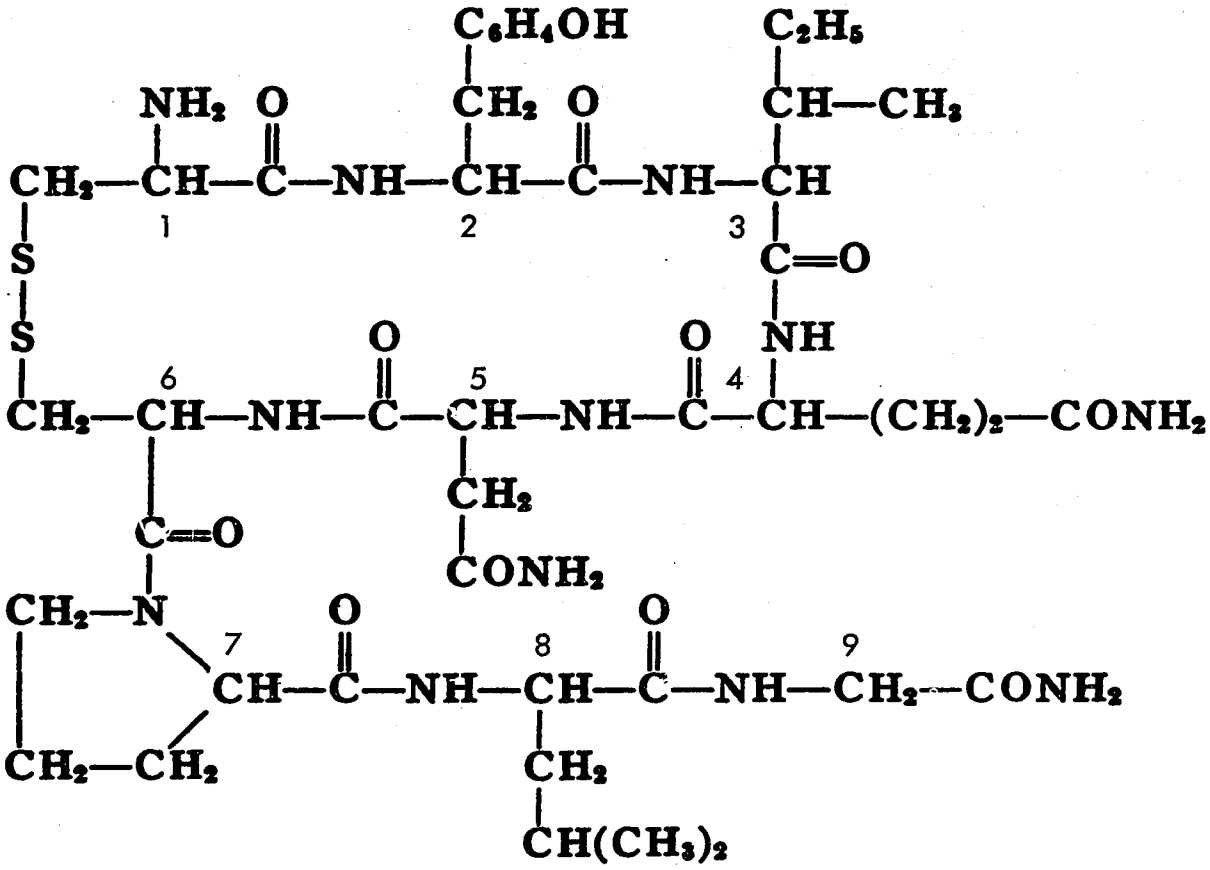


Figure 3 The Chemical Structure of Oxytocin



neighbourhood of 200 analogues of the neurohypophysial hormones have been synthesized and tested for biological activity (Schröder and Lübke, 1966; Wojciech and Margoliash, 1968). Three groups particularly active in this field have been du Vigneaud and associates at Cornell, Berde and Boissonnas and co-workers at Sandoz in Basel and Rudinger's group in Prague.

VI. Structure-Activity Relationships

There have been a great many papers dealing with the question of the structure-activity relationships of these peptide hormones (Acher, 1960; Boissonnas et al., 1961; Sawyer, 1961a; Schwartz and Livingston, 1964; Rudinger and Jost, 1964a; Walter et al., 1967; Berde and Boissonnas, 1968). It will suffice here to point out a few of the more salient conclusions arrived at by the various studies.

In addressing oneself to the problem of correlating the various structural alterations instituted, to changes in biological activity, it has been noted that even using in vitro assay systems it is not possible to assess such factors as transport across cellular or subcellular barriers nor the effects of antagonists or of enzymatic degradation on activity (Walter et al., 1967). With in vivo assay systems the complications become even greater. Nevertheless certain conclusions as to what parts of the molecule are necessary for activity have been drawn.

The hormones, described as cyclic octapeptides containing a disulfide linkage between cysteine residues in positions 1 and 6 can be looked upon as structures having a 20-membered ring with a three amino acid residue side chain.

(i) Ring Size

Both ring size and integrity would appear to be critical to activity of these hormones. Absence of the ring structure either by reduction or by complete removal of the disulfide bridge results in loss of all activity (Huguenin and Guttmann, 1965; Jost et al., 1964). Similarly it has been found that increasing the ring size to either 21, 22 or 23 members from the original 20 effectively destroys activity (Ressler and du Vigneaud, 1957; Guttmann et al., 1957; Lutz et al., 1959; Manning and du Vigneaud, 1965).

(ii) Disulfide Bridge

While it had been suggested by some (Fong et al., 1960; Rasmussen et al., 1963; Schwartz et al., 1960) that these hormones initiated their action by attachment to the receptor site on the target organ through a disulfide bond, subsequent work with analogues lacking the disulfide bridge but with the ring structure intact have shown this not to be the case. The sulfur in position 1 has been replaced by a methylene group in one analogue of oxytocin (Rudinger and Jost, 1964), in position 6 by selenium in another

(Walter and du Vigneaud, 1965) and most recently both sulfurs have been replaced with selenium (Walter and du Vigneaud, 1966). All of these analogues retained substantial amounts of oxytocin activity.

(iii) Tripeptide Side Chain

Shortening of the tripeptide side chain of oxytocin either by deletion of glycine, leucine or proline residues (Jaquenoud and Boissonnas, 1962) results in a significant decrease in activities in mammalian assay systems. Interesting enough, however, is the fact that even complete elimination of the side chain leaving only the pentapeptide ring causes no loss of intrinsic toad bladder activity although there is a large reduction in affinity (Ressler, 1956). This serves to illustrate the possible difference in function of the tripeptide side chain in mammalian and non-mammalian systems.

(iv) Amino Terminal

Addition of extra residues to the amino terminal cysteine residue of the hormones results in the production of hormonogens. A hormonogen is acquired as a derivative whose biological effects, particularly *in vivo*, are wholly or largely due to its conversion to the active hormone by enzyme action (Walter *et al.*, 1967). Both hormonogens of oxytocin (Jost *et al.*, 1963; du Vigneaud *et al.*, 1960) and lysine vasopressin (Kasafirek *et al.*, 1966) have been synthesized.

The replacement of the terminal amino group of oxytocin by a

hydrogen to produce deamino oxytocin (Hope et al., 1962; Ferrier et al., 1965) resulted in an increase in oxytocin activities. Despite the apparent lack of need for the amino group for oxytocin activity it is needed for binding to neurophysin as deamino oxytocin does not bind appreciably to neurophysin (Stouffer et al., 1963; Breslow and Abrash, 1966). The corresponding change in lysine-vasopressin (Kimbrough et al., 1963) increased antidiuretic but lowered the pressure activity.

(v) Diastereoisomers

Replacement of the naturally occurring L-amino acids by their D-isomers generally results in a drastic reduction in activity. However, it is interesting to note that while inversions in positions 4, 5 and 6 result in complete inactivation some activities are retained when these inversions are made in positions 1, 2 or 8 (Berde and Boissonnas, 1968). The enantiomer of oxytocin containing all D-amino acids was found to have no oxytocic or avain depressor activity (Flouret and du Vigneaud, 1965).

(vi) Positional changes

A great number of modifications have been carried out at each of the 9 positions of the hormone structure with greater or lesser effect on activity. Oxytocin and vasopressin however, differ from each other only in positions 3 and 8 and consequently it is of interest to see which positions affect the different activities to the greatest extent. It would appear that

position 3 is of greater relative importance to oxytocin activities than is position 8. Replacement of isoleucine for leucine in position 3 for example (Boissonnas et al., 1956; Rudinger et al., 1956) results in a much greater loss in activity than does the corresponding leucine to isoleucine transition in position 8 (Berde and Konzett, 1960). In contrast it is the basic residue in position 8 of the vasopressins that is more crucial for activity, as vasotocin (3-isoleucine 8-arginine vasopressin) (Katsoyannis and du Vigneaud, 1958) is very much more active than is the corresponding oxypressin (8-leucine vasopressin) (Katsoyannis, 1957).

While therefore as can be seen a great deal of work has been done with respect to structure activity relationships very little remains known about the actual three-dimensional structure of these peptide hormones and consequently the topochemistry of hormone-receptor interaction. One of the chief tools of this trade, X-ray crystallography, has not yet been brought successfully to bear on the problem because none of the native hormonal peptides has yielded to crystallization, a prerequisite for successful application of this technique. Recent progress in this area with the successful crystallization of several analogues including that of deamino-oxytocin (Jarvis and du Vigneaud, 1964) is, however, beginning to bear results (Low and Chen, 1966). In addition the technique of circular dichroism is also being brought into use with these peptides (Urry et al., 1968) for elucidation of their three-dimensional structure.

VII. Bioassays

Biological assays are necessary for the detection, identification and characterization of biologically active substances. The ideal bioassay should be precise, specific, sensitive, economical, simple and reliable (Sawyer, 1961b). Work on the neurohypophysial hormones has necessitated the development of assay systems as well as the setting of standards so that results obtained by different laboratories can be compared.

The International Standard for the neurohypophysial hormones is a dry powder prepared from fresh bovine pituitary glands by acetone extraction. The International Unit is the amount of oxytocin and vasopressin contained in 0.5 mg of the International Standard (Stürmer, 1968).

The types and methods of bioassay for both oxytocin and vasopressin activities have been well reviewed (Fitzpatrick, 1961; Sawyer, 1961a; Thorpe, 1962; Sawyer, 1966a; Berde and Saameli, 1966; Stürmer, 1968). Of the assays for oxytocin, contraction of isolated guinea pig uterus by the hormone served as the basis for one of the first assays developed (Dale and Laidlaw, 1912). This oxytocic or uterine contracting assay has since been replaced by one using isolated rat uterus strips (Holton, 1948). The measurement of pressure within the lactating mammary gland of the rabbit (van Dyke et al., 1955) serves as the basis for the Rabbit Milk-ejecting assay. In 1939 Coon (1939) described a quantitative method for the determination of oxytocin based on the decrease of chicken blood pressure. Further work on this method was

reported by Thompson (1944) and it now serves as the method of standarization for oxytocin. The increase in blood pressure in the rat on intravenous injection of pituitary extracts has been used to measure vasopressor. The method of Dekanski (1952) using anaesthetized rats is commonly used. The antidiuretic assay (Burn, 1931) measuring the effect of vasopressin on increased water reabsorption in the kidney while difficult to perform is extremely specific and highly sensitive.

VIII. Non-Mammalian Neurohypophysial Principles, Early Work and Difficulties

The syntheses of oxytocin and of the vasopressins by du Vigneaud climaxed the work on the active mammalian neurohypophysial principles establishing their molecular structures beyond doubt. It left unanswered, however, the question as to the nature of the principles from the non-mammalian vertebrates. Very early on Herring (1908, 1913) had reported finding vasopressor and milk-ejecting activities in pituitary extracts from cyclostomes, teleosts, reptiles and birds. By 1941 Heller (1941, 1942) had demonstrated that the biological activities of pituitary extracts from these lower vertebrates differed from those of mammals. They were, for example, much more active in promoting water uptake in frogs than were mammalian extracts containing equivalent amounts of oxytocic and antidiuretic activities. This led Heller to suggest that there was a 'water balance principle' in

these lower vertebrates distinct from anything found in mammals.

Unlike with mammalian systems however, little more was done in isolating and characterizing neurohypophysial principles from non-mammalian species until the late 1950's. Part of the reason for this can be attributed to the fact that while large amounts of pituitary tissue were available from mammalian sources, namely cattle and swine, it was difficult to get even smaller amounts from species of the lower vertebrates. Consequently the identification of uncharacterized principle or principles awaited the development of new and refined techniques.

The past ten years has, however, seen a rapid expansion of knowledge in this field with over a hundred different non-mammalian species being surveyed for their neurohypophysial principles. At present, the number of characterized principles stands at seven with at least one more known to be present but as yet uncharacterized.

IX. Isolation and Characterization Techniques

This advance has been due at least in part to the development of new techniques which have allowed for the successful processing of much smaller amounts of tissue. Coupled with this of course has been acquisition of the capability of synthesizing these principles chemically as an easy and rapid method of confirming their structures.

A rough separation of the active principles from non-active material in the extracts is often obtained by gel filtration using Sephadex G-25 (Rasmussen and Craig, 1961; Sawyer and van Dyke, 1963).

Another method of achieving this preliminary separation has been by binding the hormones to neurophysin (Acher et al.,1958;1965b). As a first step one precipitates the complex from posterior pituitary extract by sodium chloride and eliminates all the peptides and the small contaminating molecules by dialysis. The complex can then be split by means of trichloroacetic acid which precipitates only neurophysin leaving the active principles in solution (Acher, 1968). The basic principles, the vasopressins and arginine vasotocin are readily separated from the neutral oxytocic principles by a variety of solvent systems on thin-layer or paper chromatography (Heller and Lederis, 1958; Pickering and Heller, 1959; Ferguson, 1965) on which they have characteristic mobilities. Ion exchange columns such as amberlite IRC-50 (Taylor, 1954) carboxymethylcellulose (Ward et al., 1957) CM-Sephadex (Perks and Sawyer, 1965) have also been used for this purpose. Neutral oxytocin-like principles absorb only weakly on these ion exchange columns and mixtures of them cannot therefore be separated by this method. The system of partition chromatography on Sephadex G-25 developed by Yamashiro (1964) (Yamashiro et al., 1966) will, however, separate closely related synthetic peptides and its use has therefore been suggested for purification of naturally occurring neutral peptides (Sawyer, 1968).

Once the active principles have thus been isolated they can be characterized pharmacologically. Pharmacological methods are useful in this type of comparative study because of their extreme sensitivity. They can, for example, be used to indicate the presence of active principles using much smaller amounts of tissue than would be feasible using chemical methods. The activities of the various peptides are usually quoted as ratios with respect to the activity on the rat uterus or oxytocic assay.

While the basic principles, the vasopressins and vasotocin have distinct biological activity profiles, the differences between oxytocin and its neutral analogues tends to be much less pronounced (Sawyer, 1968). One method of distinguishing oxytocin from the other naturally occurring neutral principles is based on the fact that the activity of the other neutral principles on the rat uterus assay is enhanced by the presence of magnesium (0.5 mM) while the activity of oxytocin is not (Munsick, 1960). This test has been used to characterize principles from many non-mammalian species (Heller and Roy, 1965a; Chauvet et al., 1965; Sawyer, 1966a; Munsick, 1966). This potentiation effect with magnesium has however been found to give somewhat variable results, the ratios being apparently dependent on other factors as well as the type of peptide analogue and the state of estrus of the rat as was first thought (Munsick and Jeronimus, 1965). The variability has recently led to the suggestion that the magnesium potentiation effect is not a suitable criterion for distinguishing between different elasmobranch principles (Heinicke

and Perks, 1969).

X. Phyletic Distribution of the Neurohypophysial Principles

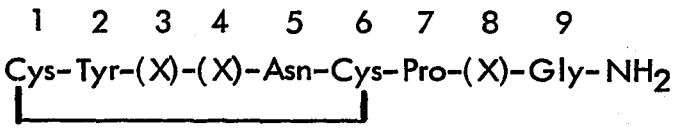
The pharmacological properties of the neurohypophysial extracts indicate that at least eight active principles are present among the vertebrates. Seven of these have been chemically identified and are all found to be cyclic octapeptide amides differing from each other in only positions 3, 4 and 8 (See Table I).

(i) Vasotocin

After arginine vasotocin, an analogue containing the ring structure of oxytocin and the side chain of arginine vasopressin was synthesized by Katsoyannis and du Vigneaud (1958) and evaluated pharmacologically it was found that its spectrum of biological activities closely resembled that of a principle isolated from the chicken pituitary (Munsick et al., 1959). Further studies, both on chromatographic mobilities and enzymatic inactivation studies as well as biological activities, supported this finding (Munsick et al., 1960) and positive identification of the natural principle by chemical analysis was provided by Chauvet et al. (1960). Since then vasotocin has been pharmacologically identified in lungfish (Follett and Heller, 1964a; Sawyer, 1966b), elasmobranchs (Chauvet et al., 1965), a holocephalian (Sawyer, 1965a) and chemically identified in frogs

TABLE I Amino Acid Sequences of Known Natural Neurohypophysial Principles

Common structure with variable amino acids in positions 3, 4 & 8 denoted by X:



	Amino acids in positions 3	4	8	Probable phyletic distribution
Basic principles:				
Arginine vasopressin	Phe	Gln	Arg	Most mammals
Lysine vasopressin	Phe	Gln	Lys	Some mammals (pigs, etc.)
Arginine vasotocin	Ile	Gln	Arg	All nonmammalian vertebrates
Oxytocin-like ("neutral") principles:				
Oxytocin	Ile	Gln	Leu	Mammals, birds (reptiles, amphibians, lungfishes, holocephalians)
Mesotocin	Ile	Gln	Ile	Reptiles, amphibians, lungfishes
Isotocin	Ile	Ser	Ile	Actinopterygian fishes
Glumitocin	Ile	Ser	Gln	Some elasmobranchs

(Acher et al., 1960b) and teleost fishes (Heller and Pickering, 1961; Acher et al., 1965b) as well as previously mentioned in chickens. Its presence in the most primitive of vertebrates, the cyclostomes (Sawyer et al., 1961; Sawyer, 1965a) suggests that this principle appeared very early in evolution and is perhaps the original neurohypophysial vertebrate principle.

(ii) Arginine Vasopressin

Whereas arginine vasotocin has been found in all the non-mammalian vertebrates studied, its place has been pre-empted by the vasopressin in mammalian systems. Arginine vasopressin was first conclusively shown to be present in cattle by du Vigneaud et al., (1953c) and this has now been extended monotremes, marsupials and placental mammals with some exceptions.

(iii) Lysine-Vasopressin

Lysine vasopressin has found exclusive of arginine vasopressin in certain species of suina (Ferguson and Heller, 1965) and recently its presence has been demonstrated in a strain of mice (Stewart, 1968). Some species of suina however, such as the wart hog and peccaries contain either one or the other principles and quite often both together in the same animal (Ferguson et al., 1962; Ferguson, 1969).

(iv) Oxytocin

Oxytocin was first chemically identified in cattle and pig

pituitaries by du Vigneaud et al. (1953a) and has since been likewise identified in man, whale, horse and sheep. It is now generally assumed to be present in all mammals (Sawyer, 1965b). Oxytocin has also been chemically identified in chickens (Acher et al., 1960a). The neutral principles isolated from some lower vertebrates including reptiles, anuran amphibians and teleost fish (Pickering and Heller, 1959; Sawyer et al., 1959) were first believed to be oxytocin. More recently however, the discovery of oxytocin-like principles with similar chromatographic and pharmacological properties has cast doubt on this hypothesis (Sawyer, 1968).

(v) Isotocin (4-Serine 8-Isoleucine-Oxytocin)

Isotocin was first discovered in pollack pituitaries (Heller et al., 1961) by virtue of its having its activity on the rat uterus assay potentiated by the presence of magnesium, thus distinguishing it from oxytocin. The principle was then isolated from pollack and two other teleost fishes and chemically characterized (Acher et al., 1962) using amino acid analysis and partial enzyme degradation. The analogue was synthesized (Guttmann, 1962) and direct pharmacological comparison with the natural principle confirmed the structure (Sawyer and van Dyke, 1963). Pharmacological studies (Follett and Heller, 1964b; Acher et al., 1968) now indicate that isotocin is probably the neutral oxytocin-like principle in two of the three groups of the actinopterygran fish, the teleosts and the holosteans.

(vi) Mesotocin (8-Isoleucine-Oxytocin)

8-isoleucine-oxytocin was synthesized and evaluated pharmacologically (Berde and Konzett, 1960; Jaquenoud and Boissonnas, 1961) before it was recognized as occurring naturally. In 1964 Follett and Heller (1964a) demonstrated the presence of 'fast moving' oxytocin-like principle from amphibians by paper chromatography and on the basis of biological activities suggested that it might be 8-isoleucine-oxytocin.

This was confirmed shortly thereafter by Acher et al., (1964) who determined the structure by amino acid analysis and partial acid hydrolysis. Mesotocin has also been found in some species of the primitive bony fish polypterus (Sawyer, 1964) a position it apparently shares with oxytocin (Follett and Heller, 1964a). Some species of amphibians also apparently contain mesotocin as well as oxytocin.

(vii) Glumitocin (4-Serine 8-Glutamine-Oxytocin)

4-serine 8-glutamine-oxytocin is the most recent of the neurohypophysial principles to be isolated and characterized (Acher et al., 1965a). It has been found in several species of ray belonging to the family of cartilaginous fish, Elasmobranchii. There would appear to be a certain amount of heterogeneity of principles in the elasmobranchs. Sawyer has reported the isolation of two oxytocin-like principles, EOP I (elasmobranch oxytocin-like principle I) from Squalus acanthias (Sawyer, 1965a, 1967) and EOP II from Raia ocellata (Perks and Sawyer, 1965). Heller and Roy (1965a,b)

found two principles which they termed E_1 and E_2 in Raia clavata and recently Roy (1969) has reported finding at least two oxytocin-like principles in a wide variety of elasmobranch fish. Sawyer (1967) has pointed out that Heller's E_1 and glumitocin may be identical since they both occur in the same species R. clavata and are both apparently absent in S. acanthias. Furthermore he suggests that EOP I and E_2 might be the same principle. The recent chemical synthesis of glumitocin (Kleiger, 1968; Manning et al., 1968a) has enabled pharmacological comparison between the EOP's and glumitocin and it would now appear (Manning et al., 1968a; Sawyer et al., 1969a) that the second of these principles, EOP II, is in fact glumitocin.

XI. Evolution of the Neurohypophysial Hormones

At the present time therefore there are seven chemically identified neurohypophysial principles and at least one and possibly more principles yet to be characterized. Partial knowledge of the distribution of these principles throughout the vertebrates and the advent of the genetic code has made it tempting to construct an evolutionary scheme for these peptides. The neurohypophysial hormones lend themselves to such a study because of their comparatively small size and because they differ from each other in their common cyclic octapeptide structures in the identities of their amino acid moieties only in positions 3, 4 and 8. These peptide hormones have therefore served

as sort of a model for studying the evolution of peptides in general.

In attempting such an evolutionary analysis Heller (1963) points out that "so few orders and species within each vertebrate class have as yet been investigated that any statement on the distribution of the neurohypophysial hormones must at present be regarded as tentative." Furthermore, as suggested by Sawyer (1968), it is quite possible that some evolutionary intermediates with important physiological functions may be present in vertebrates but have as yet gone undetected because they do not affect standard assay preparations. It is also possible that peptides which existed during some stage of evolution are not present in any living species while some others are present in such small amounts that have gone undetected. Such missing principles might fill important gaps in the evolutionary scheme of the neurohypophysial principles.

Despite these limitations several attempts have been made to construct evolutionary schemes for these peptides (Acher et al., 1965c; Sawyer, 1965b; Vliegenthart and Versteeg, 1965; Geschwind, 1967). The evolution of the peptides is believed to have occurred through a combination of two distinct genetic mechanisms (a) gene duplication and (b) single base mutations. Evolution of peptide hormones, proteolytic enzymes and other proteins by these mechanisms has been the subject of numerous studies (Acher, 1966; Neurath et al., 1967; Smith, 1967; Nolan and Margoliash, 1968).

The evolution of the vasopressor-antidiuretic principles would appear

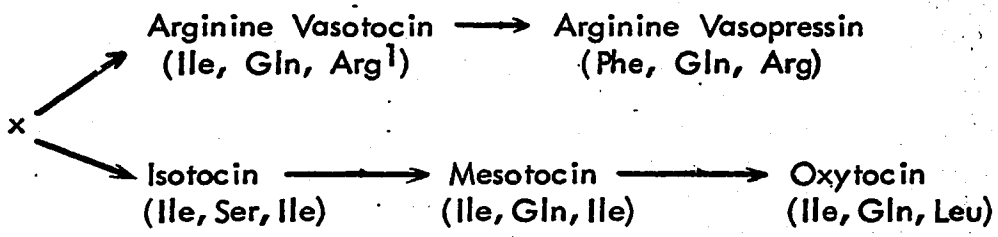
to lead toward rather straight forward and simple hypothesis (Sawyer, 1968), since only three such principles have been discovered.

Arginine vasotocin, the principle present in all non-mammalian systems and the principle present in cyclostomes is believed to have evolved into arginine vasopressin, present in many mammals, through a single point mutation. The substitution of isoleucine in position 3 of arginine vasotocin by phenylalanine to give arginine vasopressin possibly occurred by a single point mutation in one of the AUC/AUU codons for isoleucine to one of the UUC/UUU codons for phenylalanine. Similarly the replacement of arginine in position 8 of arginine vasopressin by lysine to give lysine vasopressin could also have come about by a single point mutation in the appropriate codon.

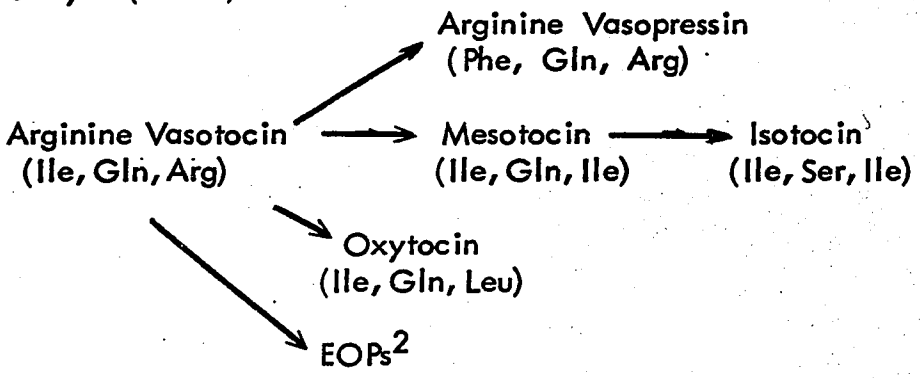
The construction of an evolutionary scheme for the neutral oxytocin-like principles however would appear at the present more difficult. The confusion which exists as to the distribution of the various neutral principles reflects in part the lack of discrimination in the existing assay systems. Several schemes for the evolution of these principles (Figure 4) have been suggested; none of which can be at this time taken as the final word.

Figure 4 Some Suggested Schemes for the Evolution of the Neurohypophysial Principles

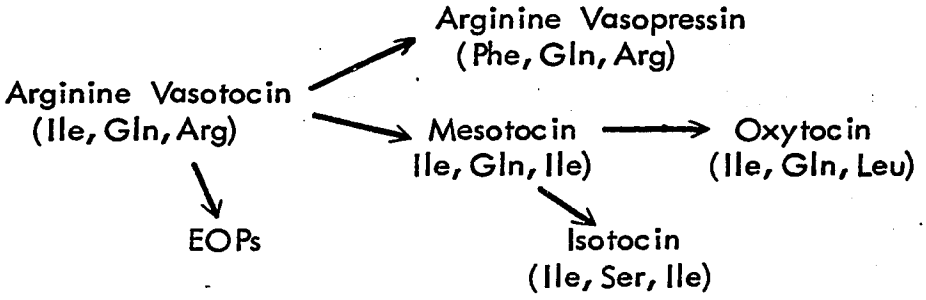
I. Acher et al. (1965d)



II. Sawyer (1965b)



III. Vliengenthart and Versteeg(1965)



[1]Amino acids in positions 3, 4 and 8 respectively.

[2]EOP - Elasmobranch Oxytocin-Like Principle

Part B : Peptide Synthesis

I. Historical

Peptides, like proteins, can be viewed chemically as polymers of amino acids (Finar, 1968). The primary structure of peptides was first postulated by Fischer (1906) as amino acids joined in linear fashion by peptide linkages, the carboxyl group of one amino acid forming an amide bond with the amino group of the next.

The first synthesis of a peptide, a dipeptide glycylglycine, was accomplished in 1901 by Fischer and Fourneau (1901). Using the α-haloacid procedure Fischer (1907) then synthesized an octadecapeptide, leucyl (triglycyl) leucyl (triglycyl) leucyl (octaglycyl) glycine. This was, as Hoffmann (1963) points out, an achievement of major proportions considering the techniques available at that time. The intervening years saw the synthesis of several more peptides, for example the synthesis of an nonadecapeptide by Abderhalden and Fodor (1916) but little in the way of development of new synthetic methods. The next great advance came in 1932 with the development of the readily removable amino protecting benzyloxycarbonyl group by Bergmann and Zervas (1932). The synthesis of the small peptides carnosine (Sifferd and du Vigneaud, 1935) and glutathione (Harrington and Mead, 1935) soon followed as a consequence of this discovery. Introduction of the sodium liquid ammonia reduction technique by Sifferd and du Vigneaud (1935) allowed for the easy removal of several protecting groups including the p-toluenesulfonyl group and

especially the benzyl group from the sulfur of cysteine, a procedure which was subsequently to play an important role in the synthesis of oxytocin. The 1950's saw the development of several important new coupling methods including those of the mixed anhydrides (Wieland and Bernhard, 1951; Boissonnas, 1951) and active esters (Schwyzer et al., 1955; Bodanszky, 1955). The discovery, also at that time, of diimides as coupling reagents especially that of dicyclohexylcarbodiimide by Sheehan and Hess (1955) has proved to immeasurable importance to the field. Several new protecting groups were also introduced, two of the most important of which were the tert-butyloxycarbonyl for protection of the amino groups (Anderson and McGregor, 1957) and tert-butylesters for the carboxyl function (Roeske, 1959).

The advances made in the various aspects of peptide chemistry to 1953 culminated in a very real sense with the synthesis of oxytocin by du Vigneaud and co-workers (1953b, 1954) as it marked the first time that such a biologically active peptide had been synthesized by chemical means. It has since been followed by the synthesis of many other biologically active peptides. These include the syntheses of angiotensin II (Schwyzer et al., 1957, Bumpus et al., 1957) α-MSH (Guttman and Boissonnas, 1959), bradykinin (Boissonnas et al., 1960), gastrin (Anderson et al., 1964), ACTH (Schwyzer and Sieber, 1963) and insulin (Katsoyannis et al., 1964; Kung et al., 1963; Meienhofer et al., 1963).

II. Classical Methods of Synthesis

These peptides were all synthesized by the same general approach used in the chemical synthesis of other organic compounds. This classical method of synthesis can be envisioned in the following way. Two monomer units, amino acids suitably protected, are coupled in solution followed by extraction and purification of the resulting product dimer. This product can then be placed in solution and coupled to a third amino acid and the product of this reaction likewise isolated, purified and characterized and so on. A peptide of desired sequence can thereby be synthesized, either by adding one amino acid at a time to the end of the growing chain or alternatively by forming several smaller sequences separately and then joining these small peptides together to form larger ones. In each case, however, the reaction is carried out in solution and the resulting product at each stage must be isolated from unreacted material and purified before continuing with the next step.

Since amino acids are di- and often polyfunctional the ordered coupling of an amino acid either to a peptide chain or to another amino acid requires protective blocking of functional groups not taking part in the coupling reaction. This has entailed the development of a whole series of groups which serve to protect these reactive functions and which can then be readily and selectively removed without damage to the peptide. Formation of the peptide bond requires activation of one of the participating groups, usually the carboxyl function.

It is of course desirable to have the reaction at each step go as far as possible to completion without forming side products or causing racemization. In any synthesis it is necessary to take into account the limitations of the protecting groups and coupling reagents and design a scheme such that the desired product can be obtained in good yield with a minimum of contaminating side products.

Detailed approaches to the methods and strategies employed in peptide synthesis can be found in monographs by Greenstein and Winitz (1961), Schröder and Lübke, (1965) and Bodanszky and Ondetti (1966).

III. Problems with the Classical Approach

The main problem with the classical approach to synthesizing a peptide is that the method becomes increasingly unwieldly as the length of the peptide increases. Since the loss at each stage is multiplied, in order to get sufficient amounts of the final product it is necessary to use increasing amounts of the starting materials as the length of the projected peptide increases. Isolation and purification of the product at each stage of the synthesis results in immense expenditures of time and energy and with the separation of the product from reactants becoming increasingly more difficult as length of the peptide increases.

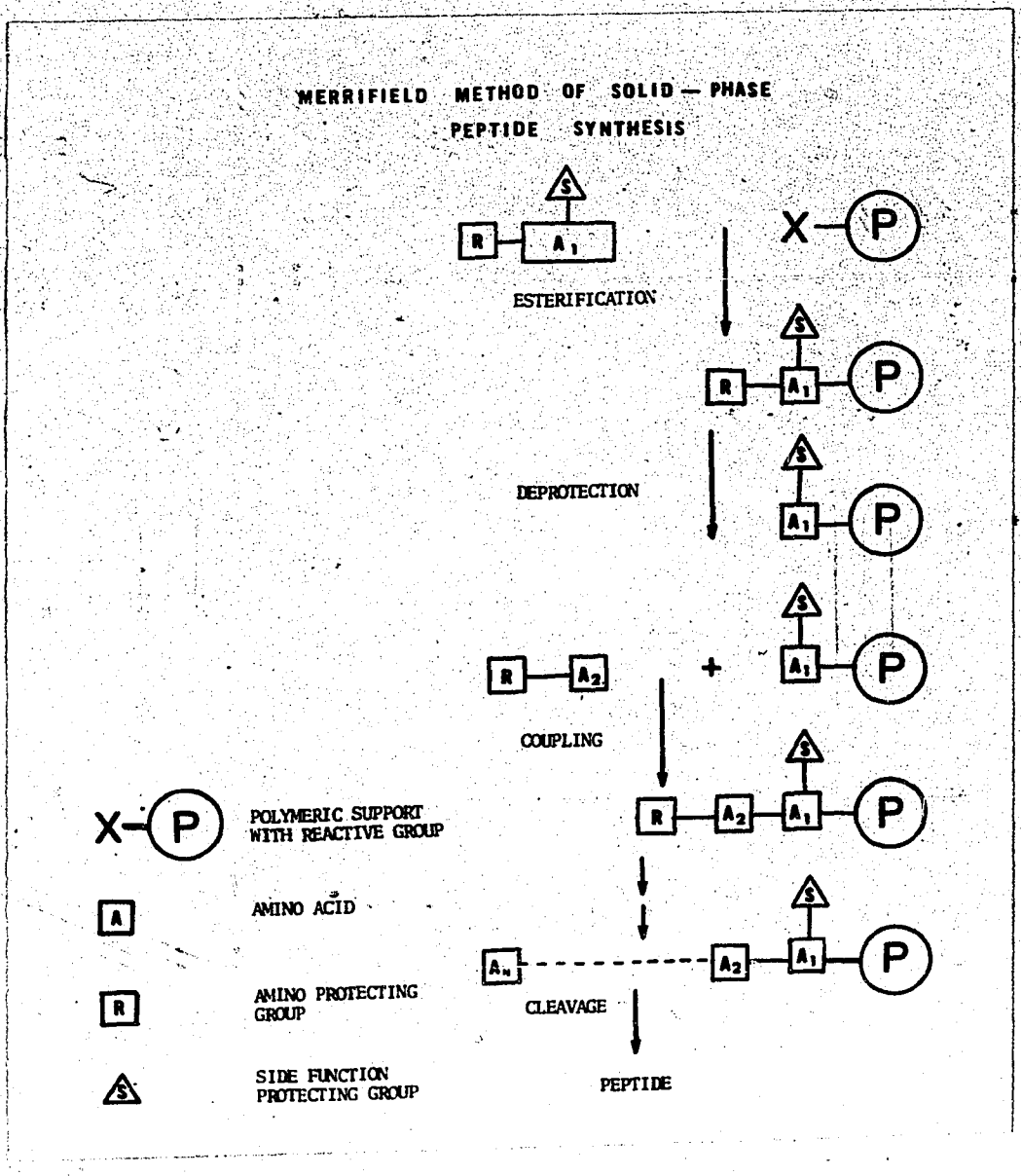
IV. The Merrifield Method of Solid Phase Peptide Synthesis

(i) General Procedure

These problems inherent in the classical approach become evident in the synthesis of insulin which required some 200 steps with the isolation and purification of the resulting product at each step being necessary. These problems led Dr. R.B. Merrifield of Rockefeller University to design an entirely new approach to peptide synthesis. It involved quite simply the attachment of an amino acid to an insoluble support through a covalent bond and then addition of subsequent amino acids one at a time to the first. The growing peptide chain remains at all time firmly attached to the insoluble polymeric support, while unreacted material remains in solution. The advantage of such a procedure over classical methods is immediately obvious. Whereas the product in the classical procedure must be isolated and purified at each step, the intermediate in the Merrifield method is freed of unwanted materials merely by washing the insoluble support with appropriate solvents. The technique is now commonly known as the Solid Phase Method of peptide synthesis, or more simply the Merrifield Method. The method as described by Merrifield (1965a, 1965b, 1968) can be briefly outlined as follows (Figure 5).

The solid support, a synthetic polymer designated here as P contains reactive groups X. The first amino acid A_1, the C terminal residue of the desired peptide, with its amino group masked with protecting group R

Figure 5 Merrifield Method of Solid Phase Peptide Synthesis



and side chain functional group with protecting group S reacts with X to form a covalent bond to the polymer through its carboxyl group. The amino protecting group R is then selectively removed and the resulting free amino group is reacted with a second appropriately protected amino acid A_2 to form the first peptide bond. This cycle of steps is repeated until the desired sequence of amino acids is assembled on the resin. The bond between the first residue and the resin is then selectively cleaved and the peptide liberated for the first time into solution. In order to achieve a homogeneous product using this method it is evident that at each step in the synthesis the reaction must go to completion. Since the only method of purifying intermediates during the synthesis is by washing the resin, failure to react the growing peptide on the resin with the protected amino acid in solution results in the production of side products on the resin which differ from the desired product by only a few amino acids. These of course make it extremely difficult to obtain the product in pure form.

(ii) Practical Aspects

A detailed approach to the theory as well as to the practical aspects of the Merrifield method has recently been published (Stewart and Young, 1969) and the reader is referred to it. A few of the more important points concerning the method will however be mentioned here.

(a) Solid Support: The solid support first chosen by Merrifield

and still used extensively in solid phase is a synthetic resin in bead form (20-70 microns in diameter) composed of polystyrene with 2% divinylbenzene crosslinking. This degree of crosslinking has been found to result in sufficient swelling of the resin in certain solvents to allow penetration of reagents to the inside of the resin while at the same time permitting easy filtration. The polymer itself fulfills the basic requirement for such a support in that while remaining inert to all reagents used in the synthesis it can be suitably modified so that it will form a covalent bond with the carboxyl group of an amino acid.

(b) Covalent Linkage: The divinylbenzene styrene resin is generally modified to provide a point of attachment for the first amino acid by reacting it with chloromethyl ether and stannic chloride in a Friedel Crafts type reaction. A chloromethyl group is in this way substituted on the benzene ring and will react with the triethylammonium salt of the first protected amino acid. The substituted benzyl ester linkage formed is stable to conditions used to remove most amino protecting groups. Esterification of the first amino acid in the presence of the base triethylamine results in the side reaction of the triethylamine with the chloromethyl groups to form quaternary ammonium groups on the resin which can act as anion exchanges. One way to avert this problem is to convert the chloromethyl group to hydroxymethyl groups and then couple the first amino acid to the resin using dicyclohexylcarbodiimide. This procedure has been used to esterify the BOC-im-benzylhistidine to the resin after failure

to obtain the right product by regular means (Stewart et al., 1969).

(c) Amino Protection: There is a great variety of amino protecting groups now available (Schröder and Lubke, 1965). The one used most extensively at the moment for protection of the α-amino function is the tertiary-butyloxycarbonyl group or the BOC group as it is more commonly called (Anderson and McGregor, 1957). It can be readily removed under mild acid conditions which normally include 1 N HCl in acetic acid or 4 N HCl in dioxane for 30 minutes or trifluoroacetic acid for 15 minutes, all at room temperature. While none of these treatments are supposed to touch the benzyl ester linkage of the peptide to the resin there is a growing feeling that the last of these treatments using trifluoroacetic acid does in fact cleave the benzyl ester linkage to a certain extent (Gutte and Merrifield, 1969). In contrast to the BOC group however, removal of the benzyloxycarbonyl group necessitates the use of HBr in acetic acid, a treatment which will cleave the benzyl ester linkage unless the resin is either brominated or nitrated.

Amino blocking groups with stability similar to that of the BOC group have also been used on occasion. These include the formyl group (Sheehan and Yang, 1958) and the t-amyloxycarbonyl group (Sakakibara et al., 1965).

There has been a tendency in some instances to employ even more labile amino-protecting groups which can be removed under even milder

conditions. The O-nitrophenylsulfenyl group which can be cleaved in 0.1 N HCl has been used in the synthesis of bradykinylbradykinin (Najjar and Merrifield, 1966). The use of such mild conditions is particularly attractive to peptide chemists and there is every reason to expect the more wide spread use of such groups in the future.

(d) Side-Chain Functional Group Protection: As in classical synthesis it is necessary to protect the functional groups on the side chain in order to prevent the occurrence of unwanted side products in the form of branched peptides. The hydroxyls of serine, threonine and tyrosine, the ω-carboxyl groups of glutamic and aspartic acids, the sulfhydral of cysteine, the ϵ-amino of lysine, the guanido of arginine and the imidazole of histidine all generally require protection. The great variety of protecting groups and their suitability to various types of synthesis can be found in the literature. As a general rule however, the side function protecting group must be stable to conditions used to remove the amino terminal protecting groups during the synthesis.

(e) Coupling Reagents: As mentioned previously the first step in the coupling comes through activation of the carboxyl group.

1. Dicyclohexylcarbodiimide, first introduced by Sheehan and Hess (1955), has proved to be the most widely used coupling reagent in solid phase. It has been found to give rapid and efficient coupling for all the common amino acids with exception of glutamine and asparagine. These latter

amino acids undergo dehydration of their amino groups to form nitriles in the presence of dicyclohexylcarbodiimide which therefore precludes its use in coupling these residues. One undesireable side reaction which the reagent can undergo, rearrangement of the active intermediate to form unreactive acyl urea can be largely overcome by equilibrating the amino acid with the resin before adding the diimide and by using a non-polar solvent, usually methylene chloride, in the coupling procedure. To date no instances of racemization in solid phase have been reported using this coupling reagent.

2. P-nitrophenyl esters with the solvent dimethylformamide are used routinely in solid phase for glutamine and asparagine residues. The synthesis of several peptides has been carried out using this reagent exclusively as the coupling reagent (Bodanszky and Sheehan, 1966; Hörnle, 1967).

3. Other coupling reagents including N-hydroxysuccinimide esters (Weygand and Ragnarsson, 1966) and Woodwards reagent (N-ethyl-5-phenylisoxazolium-3'-sulfonate) (Bumpus et al., 1967) have also been used in solid phase.

(f) Cleavage of Peptide from Resin: The peptide, once completed can be cleaved from the resin using a variety of methods. Nucleophilic displacement of the peptide by hydrogen bromide in anhydrous trifluoroacetic acid medium yielding the peptide with a free carboxyl group has been most frequently used. This treatment will, in addition, remove a great many of the side chain protecting groups. Anhydrous liquid hydrogen fluoride (Lenard

and Robinson, 1967) has recently applied to the cleavage of peptide resins with the added advantage that although it removes nearly all side functions (Sakakibana et al., 1967) it does not cause destruction of tryptophan (Marshall, 1968). Saponification was used to remove the peptide from the resin in the original synthesis by Merrifield (1963) but is no longer in general use. Ammonolysis which yields the peptide amide on bubbling ammonia through a methanolic suspension of the resin has been used successfully with the nitro resin (Takashima et al., 1968) as well as regular resin (Manning, 1968). In addition to these methods the peptide has also been successfully removed from the resin by hydrazinolysis (Bodanszky and Sheehan, 1964; Ohno and Anfinsen, 1967) and transesterified with alcohol in the presence of base or a strong anion exchange resin (Halpern et al., 1968).

V. Other Types of Solid Phase

It is worthy of note that several other schemes utilizing a solid support for the synthesis of peptides have been suggested and tried (Merrifield, 1967). Shemyakin has made use of a soluble polymer for the synthesis of a tetrapeptide, glycyl-glycyl-L-leucyl-glycine (Shemyakin et al., 1965). With this type of approach all reactions are carried out in solution thus avoiding the problems of permeability and diffusion. Letsinger and Kornet have suggested building the peptide on an insoluble support like Merrifield but starting with

the amino instead of the carboxyl group attached to the resin. The support used furthermore was of the popcorn type contained a lower degree of cross-linking 0.1 - 0.5%. The method has been applied to the synthesis of a dipeptide (Letsinger and Kornet, 1963; Letsinger et al., 1964). The use of polymer supported active esters of acids have also been suggested (Fridkin et al., 1965) and recently successfully applied to the synthesis of bradykinin (Fridkin et al., 1968). Each of the above mentioned methods contain certain inherent advantages. The polymer supported active esters for example are especially suited to the synthesis of cyclic peptides. None, however, have achieved the wide spread use to date experienced by the Merrifield method.

VI. Accomplishments of the Merrifield Method

Since the original reports were published on the Solid Phase (Merrifield, 1962, 1963) the method has been successfully applied to the synthesis of many peptides including those of bradykinin (Merrifield, 1964), angiotensin II and analogues (Khosla et al., 1967; Marshall and Merrifield, 1965; Park et al., 1967), oxytocin (Manning, 1968; Bayer and Hagenmaier, 1968; Ives, 1968; Beyerman et al., 1968), deamino-oxytocin (Takashima et al., 1968), lysine-vasopressin (Meienhofer and Sano, 1968), ferredoxin (Bayer et al., 1968) and insulin (Marglin and Merrifield, 1966). The rapidity of the Merrifield method is particularly evident in the synthesis of insulin which required on 19 days

for the synthesis of both α and ß chains.

One of the very real advantages of the Merrifield method is its capability of being automated (Merrifield, 1965b). To this end instruments for the automated synthesis of peptides have been described and used (Merrifield, 1966; Brunfeldt et al., 1968). These developments have culminated in the recent synthesis of the first enzyme ribonuclease (Gutte and Merrifield, 1969).

PURPOSE AND SCOPE OF PRESENT INVESTIGATION

In the present investigation the solid phase technique of peptide synthesis has been applied to the study of the evolution of the neurohypophysial hormones. Chemical synthesis has already played a major role in this type of study with these peptides. Arginine vasotocin, for example, was synthesized and evaluated pharmacologically (Katsoyannis and du Vigneaud, 1958) before it was realized that it existed in nature. In much the same way mesotocin, 8-isoleucine-oxytocin, had been synthesized (Berde and Konzett, 1960) three years before being isolated from the posterior pituitary of the frog and characterized (Acher et al., 1964). The vasopressin isolated from pigs was found to differ on amino acid analysis from the previously sequenced arginine vasopressin from cattle only in that it contained lysine instead of arginine. This led to the suggestion that lysine vasopressin differed from arginine vasopressin only that in position 8 it contained lysine in place of arginine. This hypothesis was first shown to be correct by synthesizing the sequence and comparing it with the natural material. The structures of all the other known characterized principles have also had their structure confirmed by synthesis.

The value of this chemical synthetic approach to the study of the neurohypophysial hormones has recently further been enhanced by the use of a solid phase. This method greatly increases the speed at which analogues can be made while at the same time still giving a pure product in good yield

as has been shown by the recent syntheses of oxytocin (Manning, 1968) and glumitocin (Manning et al., 1968a). In further applying solid phase to the synthesis of analogues of the neurohypophysial hormones four analogues of oxytocin have been synthesized utilizing this method. Three of the analogues, 8-glutamine-oxytocin, 8-phenylalanine-oxytocin and 4-proline 8-glutamine-oxytocin were synthesized for the first time while the fourth, 8-valine-oxytocin had been previously reported synthesized by classical methods (Jaquenoud and Boissonnas, 1961). Their synthesis was undertaken as part of a continuing study of the evolution of the neurohypophysial hormones in an attempt to elucidate the structure of the naturally occurring but as yet uncharacterized principle EOP I as well as because of a general interest in the structure-activity relationships of these hormones.

The first analogue attempted, 8-glutamine-oxytocin, had been suggested as a possible intermediate in the evolution of the neurohypophysial hormones (Vliegenthart and Versteeg, 1965) even before Acher et al. (1965a) first isolated the 8-glutamine substituted analogue glumitocin from ray fish. This analogue has now been isolated from several elasmobranchs including Raia clavata, R. batis, R. fullonica and R. naevus (Acher et al., 1965a, Acher, 1966) lending further credance to the suggestion that 8-glutamine-oxytocin might indeed exist as an evolutionary intermediate. The presence of glumitocin, together with indications of the presence of both vasotocin and oxytocin in cartilaginous fishes (Vliegenthart and Versteeg, 1967) makes this analogue a particularly

attractive possibility. It can easily be seen that 8-glutamine-oxytocin could be an intermediate in going either from oxytocin or vasotocin to glumitocin. Thus it had been suggested that the unidentified oxytocic principle, designated EOP I (elasmobranch oxytocin-like principle I) (Sawyer, 1965a) which has recently been shown not to be glumitocin (Sawyer et al., 1969b) might in fact be 8-glutamine-oxytocin (Sawyer, 1967). In order to determine whether or not this was the case therefore, the synthesis of this oxytocin analogue was undertaken in the laboratory.

The principle termed EOP I, as previously mentioned which has been isolated from the pituitary gland of the spiny dogfish, Squalus acanthias (Sawyer, 1967) has as yet not been obtained in sufficient quantities to permit characterization by studies which normally include amino acid analysis followed by sequence determination. Pharmacological studies however have shown that it differs from all the known naturally occurring principles as well as synthetic analogues with which it has been compared. It appeared in the first instance that 8-glutamine-oxytocin was the most likely candidate for this principle. However, subsequently elimination of this analogue from contention caused the search for its structure to continue along other lines of reasoning.

The knowledge that EOP I had a chromatographic mobility slightly greater than oxytocin on a hydrophobic system coupled with the fact that a great many substitutions in the naturally occurring principles are located in position 8 gave rise to the suggestion that EOP I might be an analogue of

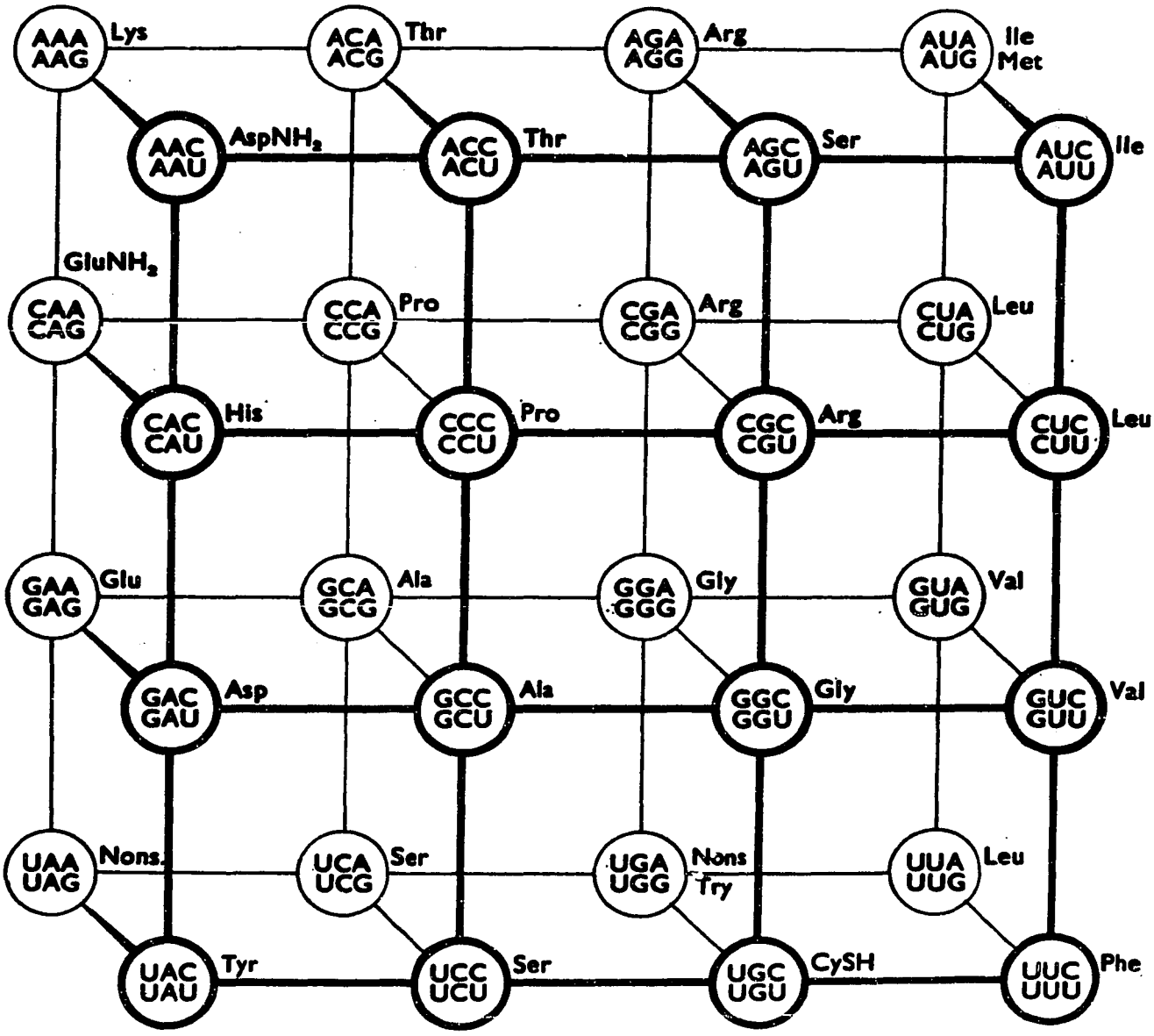
oxytocin containing a lipophilic residue in that position.

It can be seen from Figure 6 that phenylalanine UUC/UUU can be obtained from either leucine UUA/UUG/CUC/CUU or isoleucine AUC/AUU by a single point mutation in their respective codons. Furthermore an analogue of oxytocin containing phenylalanine in position 8 would be expected to have chromatographic properties on a hydrophobic system similar to those found for EOP I. Thus the synthesis of 8-phenylalanine-oxytocin was undertaken in the hope that it would turn out to have the same pharmacological properties as EOP I.

Much the same reasoning was followed for the synthesis of 8-valine-oxytocin. Valine also contains a hydrophobic side chain as well as occupying a position in the genetic code in relation to both leucine and isoleucine similar to that of phenylalanine. While, as has been mentioned, 8-valine-oxytocin had been previously synthesized by conventional methods (Jaquenoud and Boissonnas, 1961) none was available for these studies. Consequently synthesis of this analogue was undertaken by the method of solid phase.

As has been noted previously the various schemes put forward for the evolution of the neurohypophysial hormones are at best incomplete, as they all contain gaps which cannot be filled by the known principles. One of the most obvious of these gaps exists between the principles containing glutamine in position 4, oxytocin and mesotocin as well as the pressor principles and those containing serine in the same position namely glumitocin and isotocin.

Figure 6 Spatial Diagram of the Genetic Code



The consequence of single space conversion in a codon, with respect to amino acid codon can be read off on three lines crossing the codon. Codons = Crick, 1966, except UGA = Brenner et al., 1967, from Vliegenthart and Versteeg, 1967.

An inspection of the genetic code (Figure 6) shows that it is impossible to go from the codons for serine to those for glutamine by a single base change. Consequently an intermediate or intermediates were probably involved in this evolutionary transition. The simplest way for this to occur involving the smallest number of base changes would be through a proline residue. One of the codons CAA/CAG for glutamine could first change to CCA/CCG for proline and this to UCA/UCG for serine. All other schemes would require at least one additional base change to achieve this serine-glutamine transition. This led Geschwind (1967) to postulate the existence of such a 4-proline intermediate. As a consequence it was decided to attempt the synthesis of some analogues containing proline in the 4th position. Since glutamine had been found in position 8 in the principle glumitocin it was considered possible that such a 4 position analogue of glumitocin might well be an evolutionary intermediate. The synthesis of 4-proline 8-glutamine-oxytocin was therefore undertaken along with the syntheses of two other 4-proline analogues, 4-proline-oxytocin and 4-proline-mesotocin (Manning and Wuu, unpublished data) in an concerted effort to find this hypothesized evolutionary 'missing link' of the neurohypophysial principles.

The key intermediates of these analogues of oxytocin, the protected nonapeptide intermediates, were synthesized by the solid phase method (Merrifield, 1963, 1964) following essentially the procedure outlined in the synthesis of oxytocin (Manning, 1968) and glumitocin (Manning et al., 1968a).

The BOC glycine was first esterified to the chloromethylcopolystyrene 2% divinylbenzene resin and the stepwise syntheses were then each carried out through eight cycles of deprotection, neutralization and coupling with the appropriate BOC amino acid derivatives to give the fully protected peptides on the resin. The BOC amino acids with protected side chains were; S-benzyl-L-cysteine and O-benzyl-L-tyrosine. The final cysteine residue was added as the N-benzyloxycarbonyl-S-benzyl derivative.

All coupling reactions to form peptide bonds were mediated by dicyclohexylcarbodiimide (Sheehan and Hess, 1955) in methylene chloride except those involving the carboxyl groups of asparagine and glutamine. These were coupled in dimethylformamide as their nitrophenyl esters (Bodanszky and du Vigneaud, 1959). Anhydrous trifluoroacetic acid was used to remove the BOC group from glutamine as the use of 1 N hydrochloric acid in acetic acid has been shown to cause termination of the peptide chain (Takashima et al., 1968) probably through cyclization of the N-terminal glutamine to form a pyroglutamate residue. All other BOC groups were removed by treatment with 1 N HCl in glacial acetic acid for 30 minutes.

Since the terminal amide was desired, the protected peptide chain was cleaved from the resin by ammonolysis (Bodanszky and Sheehan, 1964) following the procedure utilized in the solid phase synthesis of oxytocin (Manning, 1968). Conversion of the resulting protected nonapeptide amide intermediates to their respective cyclic octapeptides was accomplished by

reduction with sodium in liquid ammonia (Sifferd and du Vigneaud, 1935; du Vigneaud et al., 1953b) followed by oxidation in dilute acetic acid solution at pH 6.5 with potassium ferricyanide (Hope et al., 1962). The product was purified by gel filtration on Sephadex G-15 in a two-step procedure using 50% acetic acid and 0.2 N acetic acid respectively for elution at each step (Manning et al., 1968b).

The resulting products were tested for purity by standard physical-chemical techniques and then pharmacologically evaluated.

MATERIALS

The t-butyloxycarbonyl and benzyloxycarbonyl amino acids: BOC-glycine, BOC-L-isoleucine, BOC-L-leucine, BOC-L-phenylalanine, BOC-L-valine, BOC-L-proline, BOC-L-glutamine-p-nitrophenylester, BOC-L-asparagine-p-nitrophenyl-ester, BOC-O-benzyl-L-tyrosine, BOC-S-benzyl-L-cysteine and CBZ-S-benzyl-L-cysteine were purchased from Schwarz Bio-research Inc. and Cyclo Chemical Corp. and their purity was checked by thin-layer chromatography before use. Dicyclohexylcarbodiimide was obtained from Distillation Products Industries. The Merrifield resin (chloromethylated copolystyrene with 2% divinylbenzene cross linking, containing 1.40 mmoles Cl/g) was purchased from Cyclo Chemical Corp. The glass synthesis vessel as described by Merrifield (1963) came from Scientific Glass Blowing Inc. and the mechanical shaker used for rocking the synthesis vessel was obtained from Mann Research Laboratories.

Dimethylformamide, trifluoroacetic acid and triethylamine, all reagent grade, were each further purified by fractional distillation before use, the dimethylformamide under reduced pressure to prevent its decomposition. Dry methanol was prepared by standard methods (Vogel, 1968) and dry acetic acid was prepared by the method of Pictet and Gelegnaff (1903) as cited in Fieser (1955). The 1 N HCl in glacial acetic acid solution was prepared by bubbling glacial acetic acid (500 ml) with hydrogen chloride under anhydrous

conditions for 30 minutes at room temperature and then stored in a well sealed flask. The chloride content of the solution was estimated by the Volhard method (Stewart and Young, 1969) and adjusted to 1 N with respect to HCl by dilution with glacial acetic acid. Peroxide-free dioxane was obtained by passing dioxane through a column of activated alumina immediately before use. The dioxane could then be tested for the presence of peroxides with a potassium iodide solution. The anion exchange resin AG3X4 (Cl^- form) was purchased from Calbiochem and washed successively several times each with water, methanol and water before use. All other solvents and reagents used in the synthesis were reagent grade.

METHODS

1. Removal of 'fines': The following procedure was used to remove fines from the Merrifield resin. The resin (100 g) was suspended in 500 ml of methylene chloride in a 1 litre separatory funnel. As the resin settled out on the top of the solvent the suspended fines were removed by draining off the solvent. The procedure was repeated and the resin collected by filtration, air dried for several hours and finally dried in vacuo overnight.

2. Esterification: All peptides synthesized required glycine as their carboxyl terminal residue. Esterification of BOC-glycine to the Merrifield resin was carried out in the following way. The resin (10 g) was suspended in 15 ml of ethanol in a 250 ml round bottom flask and allowed to swell for several hours. A solution of 1.0 g (5.7 mmoles) of BOC-glycine and an equivalent amount of triethylamine (0.78 ml) in 3 ml of ethanol was then added to the resin suspension. The flask was connected to a water condensor containing a soda-lime drying tube on top and the esterification was allowed to proceed under anhydrous conditions with slow stirring in an oil bath at 80°C for 48 hours using a magnetic stirrer. At the end of this time the resin was removed to a filter funnel where it was washed successively with ethanol, water and methanol before being allowed to dry in vacuo over P_2O_5. An aliquot of the resin was then taken and processed for amino acid analysis. These esterification conditions resulted in the incorporation of BOC-glycine to the resin in the range of 0.2 mmoles/g.

3. Amino Acid Analysis: (i) Amino acid analysis of the BOC-glycine resin was carried out in the following manner: a 50 mg sample of the substituted resin was treated with 1 N HCl in glacial acetic acid (30 ml) for 30 minutes at room temperature to remove all BOC glycine ion exchangeably bound to quaternary ammonium groups on the resin. The deprotected resin was filtered, washed with glacial acetic acid and water and dried under high vacuum over P_2O_5. A 10 mg aliquot of the dried glycine resin was hydrolyzed by refluxing in a 10 ml solution of a 1 : 1 mixture of 12 N HCl : dioxane (peroxide-free) under an air condensor for 20 hours. The resin was removed by filtration and washed with 1 N HCl (3 x 10 ml). The combined filtrate and washings were evaporated to dryness under high vacuum using a rotary evaporator. An aliquot of the residue was used for estimation of glycine on the automatic analyzer (Spackman et al., 1958). (ii) Amino acid analyses of free and protected peptide were carried out using the following procedure: Samples (1-2 mg) were weighed directly into a Pyrex hydrolysis tube to which was then added 1 ml of twice glass distilled hydrochloric acid (constant boiling, 5.7 N). After freezing the contents in a dry-ice acetone bath the tube was evacuated, sealed with Bunsen burner flame under vacuum and stored in the oven at 110°C for 20 hours. The hydrochloric acid was removed under high vacuum on the rotary evaporator and an aliquot of the residue used for amino acid analysis. The analyses were performed according to the single column gradient elution procedure (Piez and Morris, 1960) on a Technicon automatic amino acid

analyzer using a 4 1/2 hour run.

4. Thin-Layer Chromatography: Thin-layer chromatography (Stahl, 1961) was carried out using glass plates (20 x 20 cm) to which a uniform 250 micron layer of Silica gel H had been applied using an adjustable spreader (Desaga, Heidelberg). Tanks were routinely equilibrated overnight with filter paper saturated with solvent lining the sides. With two phase systems the second phase was placed in the bottom of the tank in small beakers and the tanks equilibrated in the same way. Plates were spotted 1.5 cm from the bottom using 1 mm glass capillary tubing, 0.5 cm containing 1 µl, and run in the manner of ascending chromatography, the solvent being allowed to run at least 12 cm past the point of application of the sample. Plates were removed from the tanks and with the solvent fronts marked were dried in the air at room temperature. The BOC and CBZ amino acids were routinely checked for purity using two solvent systems. 10 to 40 µg of each sample dissolved in methylene chloride were applied to the chromatograms. The amino acids were visualized with ninhydrin spray after removal of the BOC groups by heating the plates for 15 minutes at 110°C. In addition the cysteine derivatives were detected with platinic spray (Toennies and Kolb, 1951).

The R_f's of the amino acid derivatives on the two solvent systems used are listed on the following page.

Amino Acid	Solvent System A	B
BOC - Gly*	.20	.61
BOC - Ile	.35	.70
BOC - Leu	.32	.72
BOC - Phe	.28	.66
BOC - Pro	.25	.54
BOC - Val	.36	.71
BOC - Gln - ONP	-	.65
BOC - Asn - ONP	.18	.65
BOC - O - BZL - Tyr	.34	.62
BOC - S - BZL - Cys	.31	.66
CBZ - S - BZL - Cys	.30	.63

A - Chloroform (95), acetic acid (5)

B - Acetone (98), acetic acid (2)

*For abbreviations see the appendix.

The protected peptides (5–25 µg in DMF) and final peptides (20–60 µg in H_2O) were checked for purity routinely using three solvent systems.

(a) Butanol : acetic acid : water : : 4 : 1 : 5 (2 phases)

(b) Propanol : water : : 2 : 1

(c) Isoamyl alcohol : pyridine : water : : 7 : 7 : 6

Chromatograms were developed with ninhydrin and platinic reagents in the same manner mentioned above.

5. Melting Points: Melting point determinations on the protected peptides were carried out on an electrothermal melting point apparatus using glass capillary tubes sealed at one end for holding the samples. All temperature ranges quoted for the protected peptides were uncorrected.

6. Paper Electrophoresis: Paper electrophoresis was carried out routinely on the final peptides as a means of checking their purity. Whatman 3 MM paper cut in 5" x 21.5" strips was lined, placed on a rack and saturated with the buffer used in the run. 40 µl samples containing 200 µg of the peptide were lined using a microppipetteon the centre of the strip after it was blotted dry. Dinitrophenolethanolamine was used as a neutral marker. Two buffers were routinely used. (a) Buffer pH 3.5, pyridine : acetic acid : water : : 20 : 200 : 1780, diluted 3 : 1 with distilled water, (b) pH 6.42, pyridine : acetic acid : water : : 200 : 8 : 1800. The samples were run in a Precision Scientific Ionograph at constant voltage (800 v) for runs of 1.5 to 2.0 hours at which time the papers were removed and dried. The spots were detected by spraying

with platinum reagent (Toennies and Kolb, 1951) or with a solution of ninhydrin (335 mg) in ethanol (250 ml), acetic acid (75 ml), collidine (10 ml).

7. Optical Rotation: A Bellingham Stanley model A polarimeter was used for optical rotation measurements. Samples were weighed directly into a 3 ml volumetric flask and the appropriate solvent added to the mark. The solution was transferred using a pasteur pipette to a polarimeter tube of 2.4 ml capacity and one decimeter in length. The values reported were the average of ten separate readings on one sample.

8. Microchemical Analysis : All elemental analyses of C, H and N were carried out by Dr. C. Daessle, Organic Microanalysis, Montreal.

Before analysis the protected nonapeptides were weighed into a glass vial and dried for 24 hours at 100°C over P_2O_5 in a drying pistol. The final peptides were dried in the same manner but without heating.

9. Sodium Stick Preparation: Pieces of freshly cut sodium were added to a beaker and the level covered with toluene. The beaker was then carefully heated until the toluene was boiling (110.6°C) and the sodium became molten. The beaker was removed from the heat and a 1 ml pipette with the tapered end removed was placed below the level of the molten sodium and sodium was drawn up into the pipette using a rubber bulb. The sodium stick was stored in a graduated cylinder containing toluene. Immediately before use the pipette was filed around near the bottom and the piece near the bottom of the tube carefully removed to reveal a fresh surface of sodium.

10. Cleaning (Synthesis) Vessels: Vessels coming in contact with the protected peptide or peptide resin were routinely cleaned by heating them in a heated solution of Aqua Regia (3 parts conc. HCl : 1 part con. HNO_3) before proceeding with the regular washing. This treatment was needed to insure the removal of any residual peptide present.

11. Handling Peptide Material: The protected peptides in the form of dry off-white powders posed no problems in handling and were generally stored over P_2O_5 in a vacuum desiccator. The final peptides, however, when free of salt were light and fluffy and contained much static electricity which made handling difficult and necessitated the use of a static remover brush (Chemical Rubber Co.). In the dry state the peptides were extremely hydroscopic and were for this reason stored over P_2O_5 in a vacuum desiccator usually in the freezer.

EXPERIMENTAL AND RESULTS

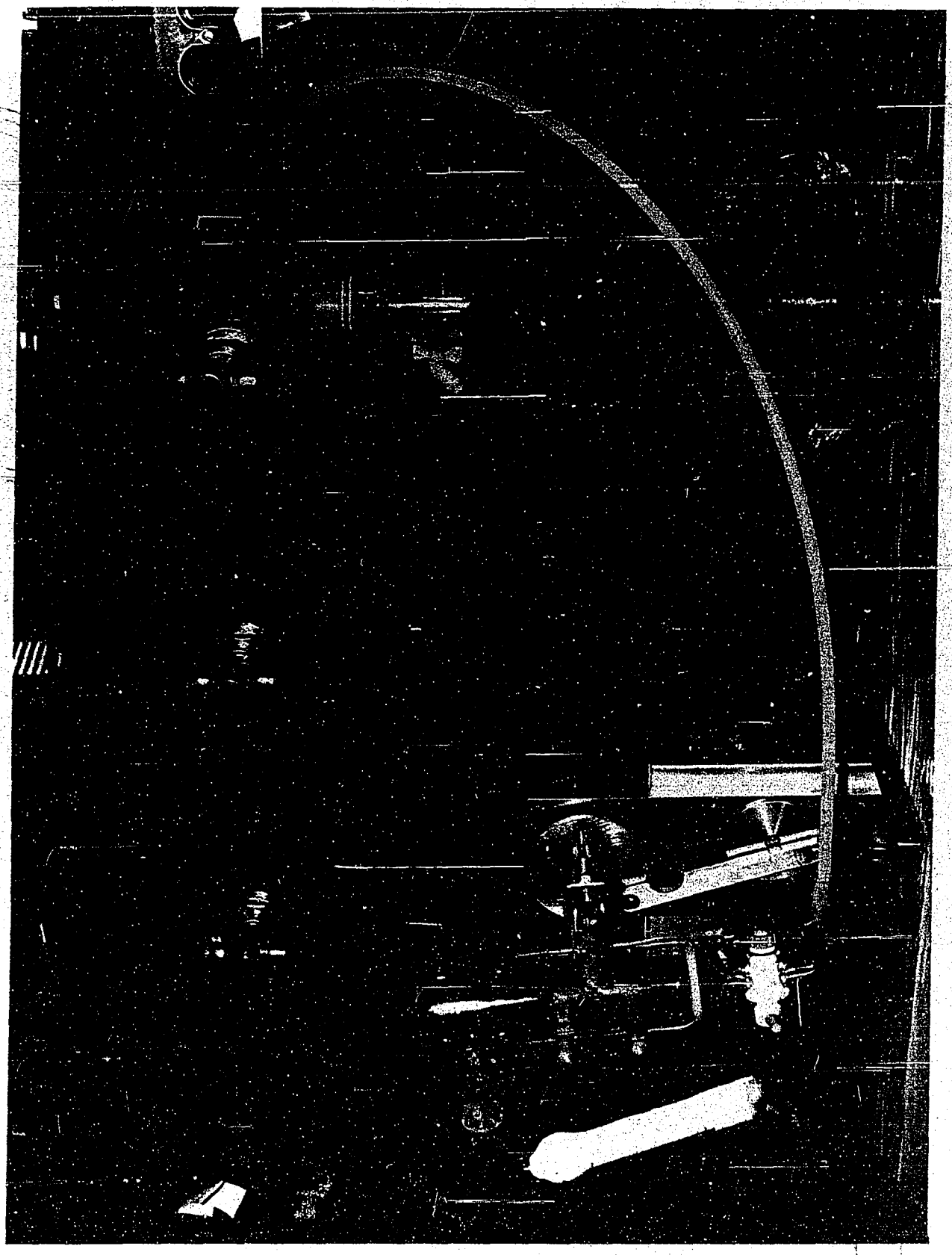
I. 8-glutamine oxytocin: (Manning et al., 1968c & d, Baxter et al., 1969b) Synthesis of N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-glutaminyl-glycylresin.

Three grams of the BOC glycine resin containing 0.591 mmole of glycine (.197 mmole gly/g substituted resin) was introduced into the synthesis vessel (see Figure 7) washed successively with methanol and methylene chloride and allowed to swell in 30 ml of methylene chloride overnight.

The following general procedure was used for all washing steps during the synthesis: the resin was washed three times with 30 ml aliquots of each solvent. Each 30 ml aliquot was allowed to mix with the resin on the mechanical rocker for 3 to 5 minutes before being filtered and replaced by the next 30 ml aliquot, the time for washing with one solvent requiring about 15 minutes. When changing from one solvent to another an intermediate washing (3-5 minutes) with a 1 : 1 mixture (30 ml total) of each of the two solvents was employed.

The following cycle of deprotection, neutralization and coupling, which incorporates small variations from the original procedure of Merrifield (1964) was used to introduce each new residue into the growing peptide chain.

Figure 7. Apparatus used in Solid Phase Peptide Synthesis



(1) The resin was washed successively with methylene chloride, ethanol and glacial acetic acid in the manner prescribed above. (2) The amino protecting BOC group was removed by treatment with 1 N HCl in glacial acetic acid using the following procedure: 30 ml of 1N HCl in glacial acetic acid were added to the synthesis vessel and allowed to equilibrate with the resin by rocking the vessel for 5 minutes. The resin was filtered and a second 30 ml aliquot of 1N HCl in glacial acetic acid was added and allowed to react in the rocked vessel for a further 25 minutes. During this time the vessel was occasionally removed from the mechanical rocker and shaken manually for a few moments to insure complete mixing and to prevent caking of the resin on the sides of the vessel. For deprotection of the BOC glutaminyl residues the procedure outlined previously (Manning, 1968) using trifluoro-acetic acid (Takashima et al., 1968) was employed as follows: the resin was washed with dry acetic acid and then briefly shaken with a 30 ml portion of trifluoroacetic acid. This was removed by filtration and a second 30 ml portion of trifluoroacetic acid was added and the suspension rocked for 15 minutes occasionally, as before, manually. The resin was filtered and washed with dry acetic acid before proceeding. (3) The resin was washed successively with acetic acid, ethanol and chloroform. (4) The free amino group was liberated from the hydrochloride or trifluoroacetate by treatment with tri-ethylamine as follows: to a suspension of the resin in chloroform (10 ml) a solution of triethylamine (1 ml) in 5 ml of chloroform was added and allowed

to rock with intermittent manual shaking for 15 minutes. (5) The resin was washed with chloroform followed by methylene chloride. (6) The resin was suspended in methylene chloride (10 ml) and a three-fold excess (1.77 mmoles) of the appropriate BOC amino acid, dissolved in 3 ml of methylene chloride, was added and allowed to equilibrate with the resin with rocking for 20 minutes. (7) An equivalent amount of dicyclohexylcarbodiimide (1.77 mmoles) in a 50% (w/v) solution of methylene chloride was added and the resulting mixture was allowed to react on the mechanical rocker for 4 hours. The reaction vessel was removed from the rocker and agitated manually at intervals during this period to ensure adequate mixing within all parts of the vessel. All of the amino acid derivatives were coupled in this way with the exception of BOC-glutamine and BOC-asparagine which were coupled as their nitrophenyl ester derivatives as follows: Step (7) was eliminated in the procedure and dimethylformamide was substituted for methylene chloride in steps (5) and (6). In this manner eight cycles of deprotection, neutralization and coupling were carried out successively with the following amino acids. BOC-L-glutamine-p-nitrophenylester, BOC-L-proline, BOC-S-benzyl-L-cysteine, BOC-L-asparagine-p-nitrophenylester, BOC-L-glutamine-p-nitrophenylester, BOC-L-isoleucine, BOC-O-benzyl-L-tyrosine and N-benzyloxycarbonyl-S-benzyl-L-cysteine to produce the desired protected nonapeptide on the resin. After coupling the final residue, steps 1-5 were repeated to remove all of the N-CBZ-S-Bzl-L-cysteine which had been bound by ion exchange during

the final coupling step to the quaternary ammonium groups on the resin which were formed during esterification and subsequent neutralization steps.

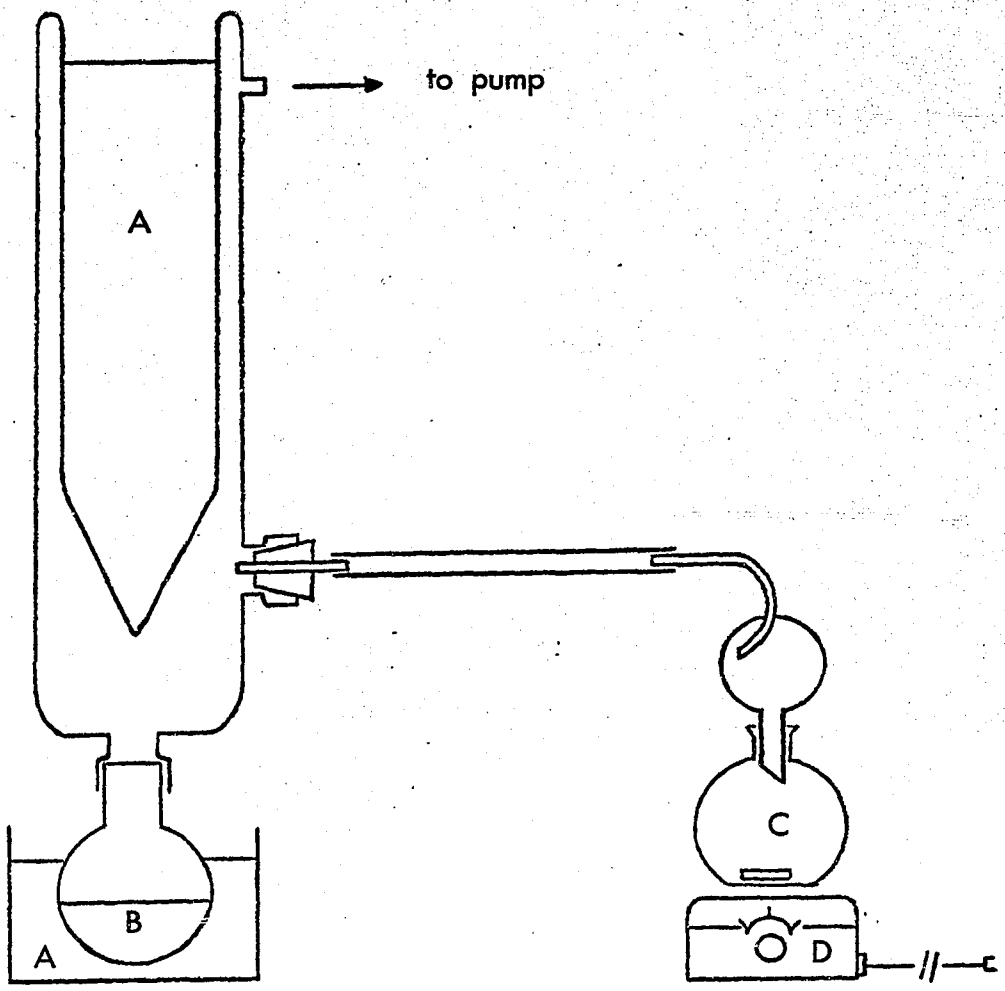
At the conclusion of the synthesis the peptide resin was removed from the synthesis vessel by the following procedure: the resin was suspended in the vessel with 30 ml of ethanol and then poured on to a filter paper in a Buchner funnel. Mild suction was used to remove the ethanol. This procedure was continued until the bulk of the resin was removed from the vessel. Resin adhering to the side of the vessel was removed by repeating the procedure with dimethylformamide. The peptide resin on the filter was washed extensively with methanol to remove dimethylformamide, dried on the bench vacuum for several hours and then in the vacuum desiccator overnight under high vacuum. A weight gain of 472 mg at this stage indicated a 65% incorporation of the protected peptide based on the initial BOC-glycine content (0.591 mmoles) on the resin.

N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-glutaminyl-glycinamide.

The protected peptide resin (2.0 g) was allowed to swell in 80 ml of dry methanol while being stirred magnetically in a stoppered round bottom flask for 3 hours and then cooled to -10°C in an ice salt bath. Ammonia, collected over a dry ice acetone bath and then dried by adding a piece of

freshly cut sodium, was bubbled through this cooled stirred solution under anhydrous conditions for 2.5 hours. The flask was stoppered and stirring allowed to continue for 18 hours, overnight at 4°C in the cold room. The flask was removed from the cold room and left to stir for 2 hours at room temperature before being recooled to -10°C, connected via a splash head adapter and two soda lime U tubes to a water pump and evacuated for three hours to remove the ammonia. During this time the outside of the flask was frequently washed with acetone to prevent excessive cooling caused by evaporation of the ammonia. Complete removal of the methanolic ammonia was accomplished by continuing the evacuation on a high vacuum pump for an additional three hours. An all-glass lyophilizer with the centre flask containing a 100 ml solution of a 1 : 1 mixture of 12 N HCl and acetic acid immersed in a dry ice acetone bath was interposed between the flask and pump to trap the evacuated ammonia vapours as shown in Figure 8
The cleaved peptide was extracted from the resin with dimethylformamide (3 x 30 ml) and methanol (2 x 30 ml). The resin was removed by filtration and the dimethylformamide and methanol in vacuo on the rotary evaporator. Dimethyl formamide was removed by washing with methanol (3 x 30 ml) which was removed after each washing on the rotary evaporator. The residue was dried overnight under high vacuum. The resulting product which gave one major and one minor spot on thin-layer chromatography and a wide melting range was purified by trituration with 95% ethanol. The protected peptide

Figure 8 All Glass Lyophilizer used in Ammonolysis Procedure



A - dry-ice acetone
B - conc.HCl:glacial acetic acid trap
C - methanolic ammonia peptide resin suspension
D - magnetic stirrer

was ground-up using a mortar and pestle and then suspended in 30 ml of 95% ethanol. The suspension was transferred to a filter paper on a Buchner funnel and ethanol and soluble material was removed by suction. The precipitate on the filter paper was washed with ethanol (30 ml) followed by diethyl ether (30 ml) before being dried overnight in vacuo over P_2O_5 to give a white amorphous powder: weight 250 mg, mp 256-258°C, $[\alpha]_D^{21}$ -33.5° (C, 1, dimethylformamide) anal. calcd. for $C_{71}\ O_{15}\ N_{13}\ H_{89}\ S_2$: C, 59.70; H, 6.24; N, 12.75. Found: C, 59.53; H, 6.43; N, 12.65. The yield of the purified protected nonapeptide amide based on the amount of glycine originally esterified to the resin was 50%. Amino acid analysis gave Asp, 1.00; Glu, 2.00; Pro, 1.00; Gly, 1.00; Ile, 0.94; Tyr, 0.81; Bzl-Cys, 2.1; NH_3, 4.0.

The protected nonapeptide amide was adjudged pure by thin-layer chromatography on three different solvent systems giving only one spot in each case with the platinic reagent (Toennies and Kolb, 1951) and none with ninhydrin. The R_f values obtained, compared with those for the protected nonapeptide of oxytocin in the same system are given below:

	Solvent Systems		
Protected nonapeptide amide of	A	B	C
8-glutamine-oxytocin	.61	.71	.67
Oxytocin	.70	.82	.73

A - Butanol (4) : acetic acid (1) : water (5) 2 phases

B - Propanol (2) : water (1)

C - Isoamyl alcohol (7) : pyridine (7) : water (6)

8-Glutamine Oxytocin: Debenzylation of the protected nonapeptide amide was accomplished by sodium in liquid ammonia (Sifferd and du Vigneaud, 1935) following the general procedure used in the synthesis of deamino oxytocin (Hope et al., 1962). 250 ml of sodium dried liquid ammonia was redistilled into a 500 ml round bottom flask immersed in a dry ice acetone bath, under anhydrous conditions. The flask was removed from the bath and the protected peptide (100 mg) was added before the ammonia was brought to its boiling point. Sodium was added by intermittent dipping of a small bore glass tube containing fresh sodium into the stirred refluxing solution until a faint blue colour enveloped the solution for a 15 second period. The blue colour, representing excess sodium metal was discharged by the addition of a few drops of dry acetic acid from a Pasteur pipette. The flask was connected to a water pump via a splash head adapter and two soda lime drying tubes and the ammonia was removed under vacuum. The resulting residue was allowed to dissolve for 30 minutes in 400 ml of 0.2% acetic acid with stirring and then adjusted to pH 6.5 with 2 N ammonium hydroxide. An excess of freshly prepared 0.011 M aqueous potassium ferricyanide solution (Hope et al., 1962) (5 ml) was added by burette to the stirred solution. End point of the reaction was indicated by the persistance of the yellow colour of the ferricyanide ions in the solution. Stirring was continued for 5 minutes before 5 g of AG3X4 resin (chloride form) was added to effect the removal of the ferricyanide and excess ferricyanide ions. The

suspension was filtered through a bed of washed AG3X4 resin (chloride form, weight 20 g) and washed through with 200 ml of 0.2% acetic acid. The filtrate and washings were combined and the pH adjusted to 3 with glacial acetic acid. The solution was lyophilized to give the crude product containing in addition to the active peptide, some peptide dimer and inorganic salt.

Purification of the peptide was effected by the method of Manning et al., (1968b) using gel filtration on Sephadex G-15. The lyophilizate (650 mg) was dissolved in 3.0 ml of 50% acetic acid (v/v) and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40-120 μ, column size 110 x 1.2 cm) which had been pre-equilibrated with 50% acetic acid. The sample was washed into the column with a further 2 ml of 50% acetic acid and then eluted with 50% acetic acid at a rate of 10 ml per hour, 2 ml fractions were collected using an automatic fraction collector. The eluate was assayed for peptide by the method of Lowry et al., (1951) and by UV absorption at 280 mμ and the salts were located by flame photometry for potassium and sodium and by precipitation with silver nitrate for chloride. The peptide material emerged into two peaks (Figure 9) with maxima at fractions 25 and 32 clearly separated from the salt by 20 tubes. The fractions corresponding to the two peaks were pooled separately, diluted with two volumes of distilled water and lyophilized to give 70 mg of peptide, almost 100% of that expected from 100 mg of protected

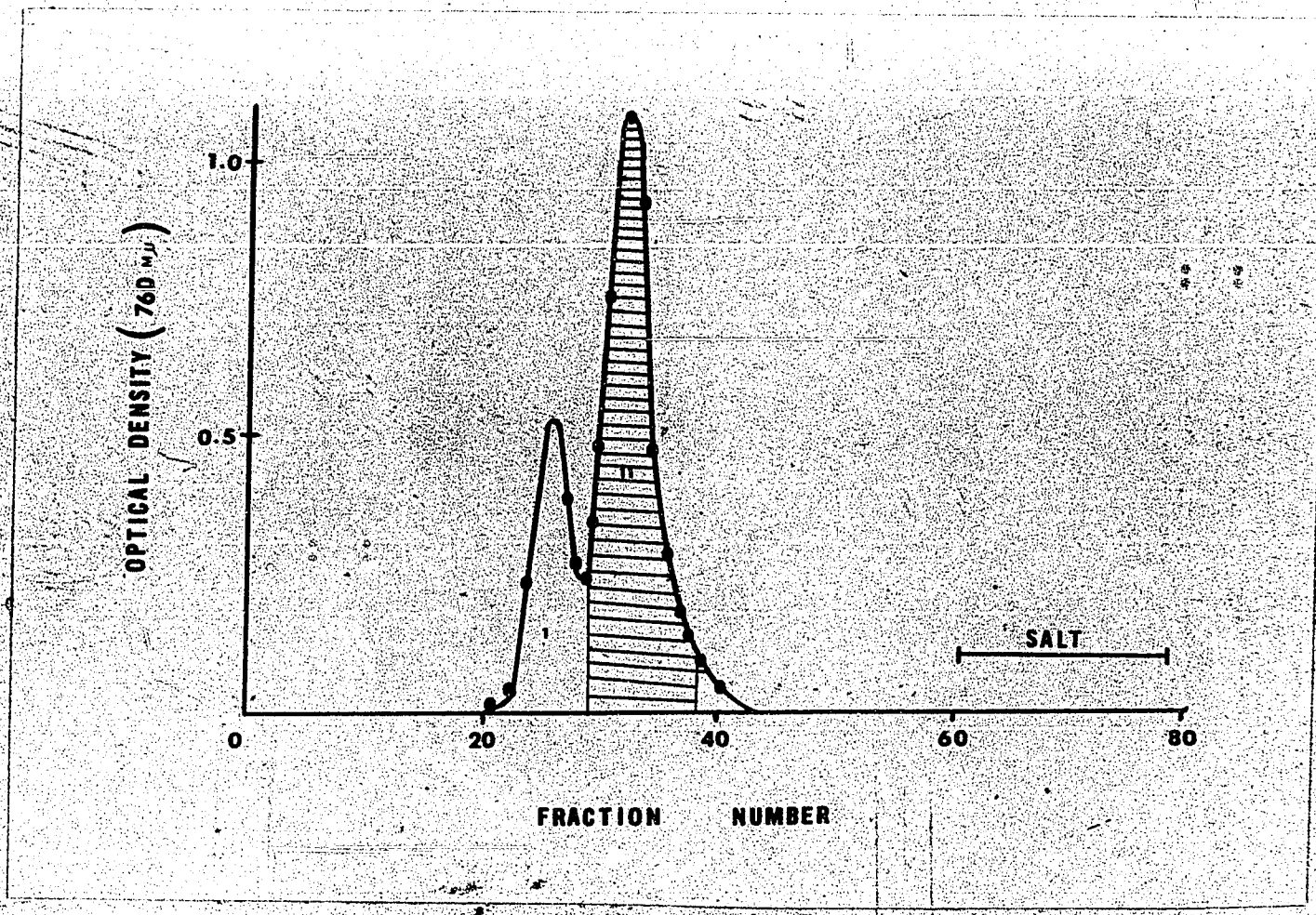


Figure 9. Elution profile for first step in purification of 8-glutamine-oxytocin. 650 mg crude lyophilized material (dimeric peptide, active peptide and salt) dissolved in 3 ml of 50% acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 50% acetic acid, flow rate 10 ml per hour; fraction size 2 ml. Peptide measured by Lowry method (1, dimer; 11, oxytocin). Salt located by standard determinations as mentioned in text. Material from shaded area under peak 11 lyophilized and used in next step.

peptide. The lyophilized material from the second peak (45 mg) including most of the active monomer now free of salt was dissolved in 2.0 ml of 0.2 N acetic acid and applied to the top of the same Sephadex G-15 column, which had in the interim been re-equilibrated with 500 ml of 0.2 N acetic acid over a period of 20 hours. The sample was washed into the column with a further 1 ml of 0.2 N acetic acid and the column eluted with 0.2 N acetic acid at a rate of 10 ml per hour, 2 ml fractions being collected as before. A plot of Folin-Lowry colour values and (absorption at 280 mμ) of the fractions gave a single nearly symmetrical peak (Figure 10) with a maximum at fraction 43. The fractions corresponding to this peak were pooled, diluted with water and lyophilized to give a fluffy white powder: weight 38 mg $[\alpha]_D^{22}$ - 16° (C, 0.5, 1N acetic acid), anal. calcd. for C_{42} O_{13} N_{13} H_{63} S_2 : C, 49.36; H, 6.17; N, 17.83. Found C, 49.24; H, 6.21; N, 17.68. Amino acid analysis gave: Asp, 1.00; Glu, 2.00; Pro, 1.05; Gly, 0.94; Cys, 1.89; Ile, 0.97; Tyr, 0.92; NH_3, 4.18. The peptide was found to give only single spots on thin-layer chromatography on three solvent systems when developed with both platinic reagent (Toennies and Kolb, 1951) and ninhydrin. The R_f values obtained, compared with those of oxytocin, are given below:

	Solvent Systems		
	A	B	C
8-glutamine-oxytocin	.25	.40	.44
Oxytocin	.36	.58	.59

Likewise only one component in the direction of the cathode was observed

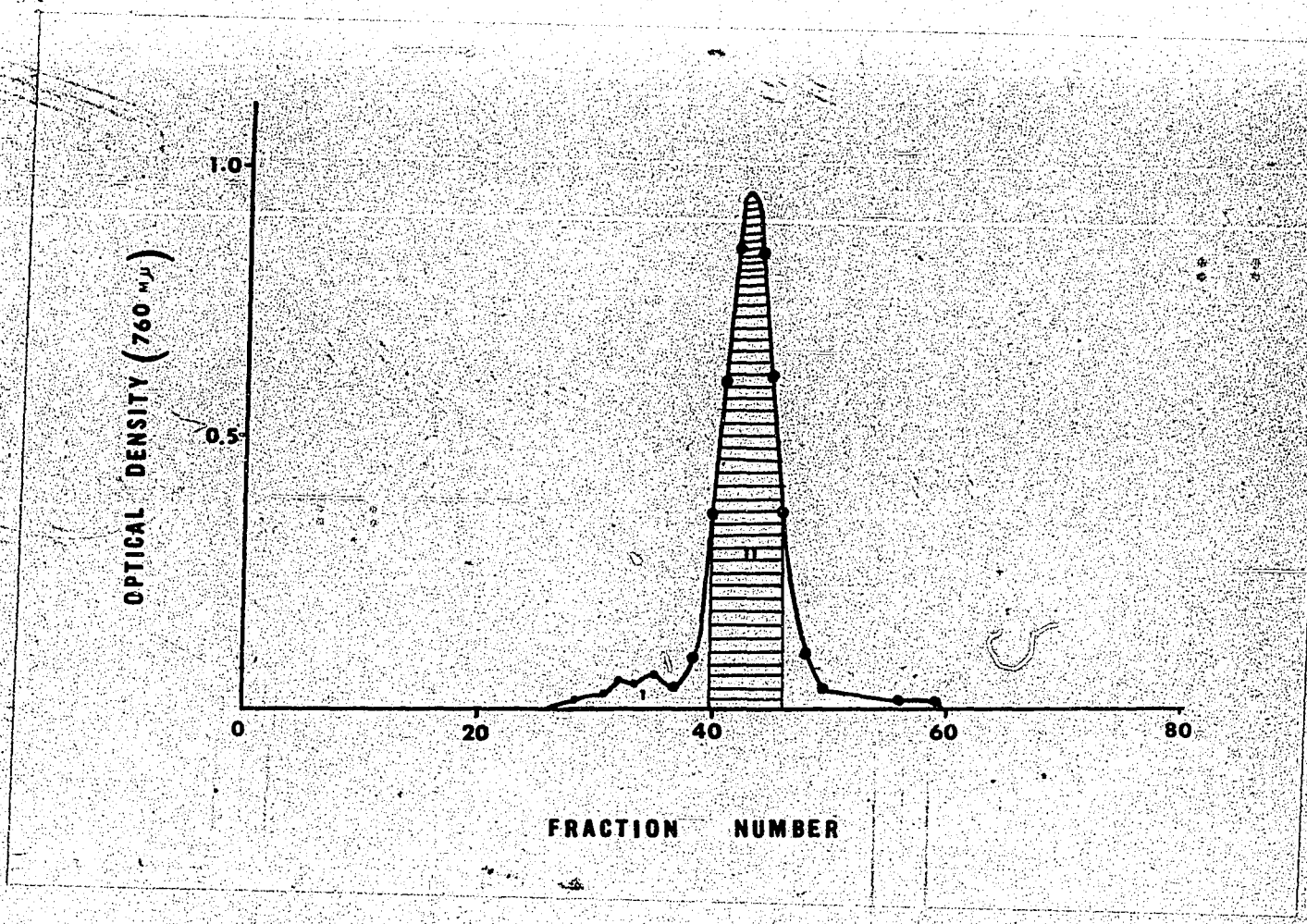


Figure 10 Elution profile for second step in purification of 8-glutamine-oxytocin. 45 mg of active peptide plus small amount of dimer dissolved in 2 ml of 0.2 N acetic acid and applied to a column of Sephadex G-15. Eluting solvent: 0.2 N acetic acid; flow rate 10 ml per hour; fraction size 2 ml. Peptide measured as in Fig. 8 (I, dimer; II, oxytocin). Material from shaded area under peak II lyophilized.

with paper electrophoresis in two pyridine acetate buffers of pH 3.5 and 6.4 using the same detecting reagents.

The overall yield of pure product was 26.8% based on the initial glycine incorporation on the resin.

II. 8-Phenylalanine-Oxytocin (Manning et al., 1968c & d, Baxter et al., 1969a). Synthesis of N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanyl-glycyl-resin.

Three grams of BOC glycine resin containing 0.681 mmoles glycine (0.227 mmole/g) was introduced into the reaction vessel, washed with methanol and methylene chloride and left to swell in methylene chloride overnight.

The following general procedure was used for all washing steps in the synthesis: The resin was washed three times with 30 ml aliquots of each solvent over a 15 minute period. When changing from one solvent to another an intermediate washing with a 1 : 1 mixture (30 ml) of each of the two solvents was employed. The following cycle which incorporates minor variations of the original procedure outlined by Merrifield (1964) was used to introduce each new residue into the growing peptide chain. (1) The resin was first washed successively with methylene chloride, ethanol and glacial

acetic acid. (2) The BOC group was removed by treatment with 1 N HCl in glacial acetic acid using the following procedure: 30 ml of 1N HCl in glacial acetic acid was added and allowed to equilibrate with resin for 5 minutes. This was filtered and a second 30 ml aliquot of the 1N HCl in glacial acetic acid added and allowed to react for a further 25 minutes. During this time the synthesis vessel was occasionally removed from the rocker and shaken manually to prevent caking of the resin on the side of the vessel. For the deprotection of the BOC-glutaminyl residue the procedure outlined previously (Manning, 1968) utilizing trifluoroacetic acid (Takashima et al., 1968) was followed. (3) The resin was washed with acetic acid, ethanol and chloroform. (4) The free amino group was liberated from the hydrochloride or trifluoroacetate by treatment with triethylamine as follows: to a suspension of the resin in chloroform (10 ml) a solution of triethylamine (2 ml) in 20 ml of chloroform was added and allowed to react for 5 minutes with intermittent manual shaking. The resin was filtered, washed with chloroform and the step repeated. (5) The resin was washed successively with chloroform and methylene chloride. (6) The resin was suspended in methylene chloride (10 ml) and a 3-fold excess (2.04 mmole) of the appropriate BOC amino acid, dissolved in 3 ml of methylene chloride, was added and allowed to equilibrate with the resin for 20 minutes. (7) An equivalent amount of dicyclohexylcarbodiimide (2.04 mmole) in a 50% (w/v) solution of methylene chloride (0.8 ml) was added and allowed to react for 4 hours. The synthesis

vessel was removed from the rocker and agitated manually at intermittent intervals during the synthesis to allow complete mixing. Eight cycles of deprotection, neutralization and coupling were carried out in this manner on successive days with the following amino acid derivatives: BOC-L-phenylalanine, BOC-L-proline, BOC-S-benzyl-L-cysteine, BOC-L-asparagine-p-nitrophenylester, BOC-L-glutamine-p-nitrophenylester, BOC-L-isoleucine, BOC-O-benzyl-L-tyrosine, N-benzyloxycarbonyl-S-benzyl-L-cysteine. The incorporation of the BOC-glutamine and BOC asparagine as their nitrophenyl esters led to the elimination of step (7) and the substitution of dimethylformamide for methylene chloride in steps (5) and (6).

After incorporation of the final residue, steps 1-5 were repeated to bring about the removal of all by N-CBZ-S-Bzl-L-cysteine which had been bound by ion exchange during the final coupling step to the quaternary ammonium groups on the resin. Estimation of the chloride content of the filtrate and washings following the neutralization step at this stage gave a chloride value of 0.08 mmole; this value represents the quantity of ion exchange groups formed during the initial esterification of the BOC glycine to the resin and in subsequent neutralization steps.

At the conclusion of the synthesis, the protected peptide resin was washed out of the synthesis vessel with ethanol, dimethyl formamide and methanol; collected on a filter and dried in vacuo, weight 3.728 g. The weight gain of 728 mg (0.574 mmoles) at this stage indicated a 84.3%

incorporation of protected peptide based on the initial BOC glycine content (.681 mmoles) of the resin.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanyl-glycynamide.

The protected peptide resin (2.0 g) was weighed into a round bottom flask and allowed to swell in 80 ml of dry methanol for 3 hours at room temperature before being cooled to-10° in an ice salt bath. Dry ammonia, from a boiling solution saturated with sodium was bubbled through this cooled, stirred resin suspension for 2.5 hours under anhydrous conditions. At this time the methanolic suspension was shown to be 16.4 N with respect to ammonia and the volume had increased to 130 ml. The flask was stoppered and stirring was continued for 18 hours at 4°C in the coldroom. The flask was further stirred for 2 hours at room temperature, recooled to -10°C in an ice salt bath and connected to a water pump via a splash head adaptor and two fresh soda lime drying U tubes and evacuated for 3 hours to remove the ammonia. During this time removal of ammonia was aided by washing down the outside of the flask with acetone thus preventing the formation of ice. At the end of 3 hours the volume had decreased to about 80 ml. The complete removal of methanolic ammonia was accomplished by continuing the evacuation

on a high vacuum pump for an additional 3 hours. An all glass lyophilizer, with the centre flask containing a 100 ml solution of a 1:1 mixture of 12N HCl and glacial acetic acid immersed in a dry ice acetone bath, was interposed between the flask and the pump to trap the evacuated ammonia vapours.

At the conclusion of ammonolysis when the resin was free of methanol and ammonia the resulting cleaved peptide was extracted from the resin with dimethylformamide (3 x 30 ml) followed by methanol (2 x 10 ml). The resin was removed by filtration using Whatman No. 1 in a glass Buchner funnel and the dimethylformamide and methanol were removed in vacuo by a rotary evaporator. The residue was then washed with methanol (3 x 30 ml) to remove the dimethylformamide and was removed each time on the rotary evaporator. Increasing care had to be taken at this stage with the material as continued washings with methanol resulted in a dry flaky product which could easily be lost on the rotary evaporator. The residue was then dried in vacuo overnight. The resulting product which gave a rather large melting range and two spots on thin layer was purified by trituration with 95% ethanol. The protected peptide was ground up using a mortar and pestle and then suspended in 30 ml of 95% ethanol. The suspension was transferred to filter paper on a Buchner funnel and the ethanol removed by gentle suction. The precipitate was washed with ethanol (2 x 10 ml) and diethyl ether (3x 10 ml) and dried in vacuo over P_2O_5 to give the required protected nonapeptide amide as a white amorphous powder: weight 268 mg, mp 248-250°C, $[\alpha]_D^{22}$ - 50°

(C, 0.5, dimethylformamide). Anal calc. for C_{75} O_{14} N_{12} H_{90} S_2: C, 61.98; H, 6.28; N, 11.83. Found C, 62.22; H, 6.27; N, 11.61.

The yield of the purified protected nonapeptide amide from ammonolytic cleavage and trituration was 70% of the amount expected based on the weight gain of the resin. The yield based on the amount of glycine originally esterified to the resin was 59.2%. Amino acid analysis gave: Asp, 1.01; Glu, 1.02; Pro, 0.97; Gly, 1.05; Ile, 1.00; Tyr, 0.90; Phe, 1.00; Bzl-Cys, 1.86; NH_3, 3.1.

The protected nonapeptide amide gave single spots on thin-layer chromatography with three solvent systems when sprayed with platinic reagent (Toennies and Kolb, 1951). No spots were detected with ninhydrin. The R_f values obtained, compared with those of the protected nonapeptide of oxytocin are given below:

	Solvent Systems		
Protected nonapeptide amide of	A	B	C
8-phenylalanine-oxytocin	.74	.82	.70
Oxytocin	.70	.82	.73

A - Butanol (4) : acetic acid (1) : water (5) 2 phases;

B - Propanol (2) : water (1);

C - Isoamyl alcohol (7) : pyridine (7) : water (6).

8-Phenylalanine-oxytocin: The protected nonapeptide amide (75 mg, 0.052 mmole) was dissolved in 350 ml of anhydrous liquid ammonia, which was at

its boiling point. Sodium was added by intermittent dipping of a small-bore glass tube containing sodium into the magnetically stirred refluxing solution until a faint blue colour enveloped the solution for 15 seconds. Dry acetic acid (3 ml) was added by Pasteur pipette to discharge the colour and the ammonia was evaporated *in vacuo* under anhydrous conditions at the water pump. The residue was dissolved up in 400 ml 0.2% acetic acid for 30 minutes and after adjustment of the pH to 6.8 with 2 N ammonium hydroxide, an excess of 0.011 M potassium ferricyanide (5 ml) (Hope *et al.*, 1962) was added to the stirred solution. After stirring for 10 minutes, 5 g of AG3X4 resin (Cl^-form) was added to remove ferrocyanide and excess ferricyanide ions. The suspension was filtered through a bed of AG3X4 resin (Cl^-form) (20 g wet weight) and washed through with 100 ml of 0.2% acetic acid. The filtrate and washings were combined and adjusted to pH 3 with glacial acetic acid. The solution was lyophilized to give 700 mg of crude product consisting of the required peptide and as well some dimer and inorganic salts.

Purification of the peptide was effected by the method of Manning *et al.*, (1968b) using the technique of gel filtration on Sephadex G-15. The lyophilizate was dissolved in 3.0 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40-120 μ, column size 110 x 1.2 cm) which had been preequilibrated with 500 ml of 50% acetic acid. The sample was washed into the column with a further 2 ml of 50% acetic acid and eluted with 50% acetic acid

at a flow rate of 7.6 ml per hour. Fractions (2.3 ml) were collected using an automatic fraction collector. A plot of the Folin-Lowry colour values (Lowry et al., 1951) of the fractions showed the presence of two peaks with maxima at tubes 24 and 31 clearly separated from the salt by 15 tubes. The peaks were pooled separately, diluted with 2 volumes of distilled water and lyophilized to give a total of 50 mg of peptide material. The contents of the second peak (tubes 29-38) contained the majority of active material as detected by oxytocic assay (Munsick, 1960). During this time the Sephadex column was reequilibrated with 500 ml of 0.2N acetic acid over a period of 20 hours. The lyophilized powder (34.5 mg) from the second peak was dissolved in 2.0 ml of 0.2 N acetic acid, applied to the column, washed with a further 1 ml of 0.2 N acetic acid and eluted and collected as before, this time at a rate of 9.2 ml per hour. The required peptide emerged in a single nearly symmetrical peak preceded by a small amount of dimer. The contents of this main peak (tubes 50-65) were pooled, diluted with two volumes of distilled water and lyophilized to give the desired product as a white fluffy powder (30 mg). This represents a yield of 55% in the reduction-oxidation step. $[\alpha]_D^{22.5}$ - 9.0° (C, 0.5; 1N acetic acid). Anal. calcd. C, 53.07; N, 16.15; H, 6.15; Found: C, 52.9; N, 15.95; H, 6.20. Amino acid analysis gave: Asp, 1.07; Glu, 1.14; Pro, 0.89; Gly, 1.00; Cys, 1.98; Ile, 1.05; Phe, 0.95; Tyr, 0.90; NH_3, 3.4.

The peptide gave only a single spot on thin-layer chromatography on

three different solvent systems when developed both with platinic (Toennies and Kolb, 1951) and ninhydrin reagents. The R_f values obtained by direct comparison with oxytocin are given below:

	Solvent Systems		
	A	B	C
8-Phenylalanine-oxytocin	.38	.59	.61
Oxytocin	.36	.58	.59

using the same solvents as for the protected peptides. Likewise only one component in the direction of the cathode was observed with paper electrophoresis in two pyridine acetate buffers of pH 3.5 and 6.4 using the same detecting reagents as above.

The overall yield of pure product was 32.8% based on the initial glycine incorporation on the resin.

III. 4-Proline 8-glutamine-oxytocin: Synthesis of N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-prolyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-phenylalanyl-glycyl resin.

4.57 g of BOC-glycine resin containing 0.197 mmoles BOC-glycine per gram substituted resin (0.90 mmoles) was placed in a synthesis vessel, washed with methanol and methylene chloride and allowed to swell in 50 ml of methylene chloride overnight.

The following procedure was used for all washing steps in the synthesis:

The resin was washed three times with 50 ml aliquots of each solvent over a 15 minute period. When changing from one solvent to another an intermediate wash containing a 1 :1 ml (50 ml) of each of the two solvents was employed.

The following cycle incorporating some variations from the original procedure outlined by Merrifield (1964) was used to introduce each new residue into the growing peptide chain. (1) The resin was first washed sucessively with methylene chloride, ethanol and glacial acetic acid. (2) The BOC group was removed from the terminal amino group by treatment with 1 N HCl in acetic acid using the following procedure: 50 ml of 1 N HCl in glacial acetic acid was added to the reaction vessel and allowed to equilibrate with the resin for 5 minutes with shaking. The resin was filtered and a second 50 ml aliquot of 1 N HCl in glacial acetic acid was added and allowed to react for a further 25 minutes. During this time the reaction vessel was occasionally removed from the rocker and shaken manually to prevent caking of the resin on the sides of the vessel. For the deprotection of the BOC-glutaminyl residue the procedure outlined previously (Manning, 1968) utilizing trifluoroacetic acid (Takashima et al., 1968) was followed. (3) The resin was washed successively with acetic acid, ethanol and chloroform. (4) The free amino group was liberated from the hydrochloride or trifluoroacetate by treatment with triethylamine as follows: 1 ml triethylamine in 20 ml of chloroform was added to the resin and the vessel was rocked for 7 minutes. At

this point a further 1 ml of triethylamine was added and rocking continued for a further 10 minutes. (5) The resin was filtered and washed successively with chloroform and methylene chloride. (7) The resin was suspended in 15 ml of methylene chloride which contained a three times excess (2.7 mmoles) of the appropriate BOC amino acid and the vessel was rocked for 15 minutes to allow the BOC amino acid to equilibrate with the resin. An equivalent amount of dicyclohexylcarbodiimide (2.7 mmoles) in a 50% (w/v) solution of methylene chloride was added and the resulting mixture was allowed to react for 2 hours. The vessel was removed from the rocker and shaken manually at intervals during this period. At the end of the 2 hour coupling the resin was filtered, washed with methylene chloride and the coupling procedure was repeated in exactly the same way for another 2 hour coupling. Eight cycles of deprotection, neutralization and coupling were carried out in this manner with the following protected amino acids: BOC-L-glutaminyl-p-nitrophenyl ester, BOC-L-proline, BOC-S-benzyl-L-cysteine, BOC-L-asparagine-p-nitrophenyl ester, BOC-L-proline, BOC-L-isoleucine, BOC-O-benzyl-L-tyrosine, N-benzyloxycarbonyl-S-benzyl-L-cysteine.

The incorporation of the BOC glutamine and BOC asparagine residues as their nitrophenyl ester derivatives led to the elimination of step (7) and the substitution of dimethyl formamide for methylene chloride in steps (5) and (6). After the incorporation of the final residue, steps 1-5 were repeated to bring about the removal of all the N-CBZ-S-Bzl-L-cysteine which had

been bound by ion exchange during final coupling step to the quaternary ammonium groups on the resin.

At the conclusion of the synthesis, the protected peptide resin was washed out of the synthesis vessel with ethanol, dimethylformamide and methanol, collected on a filter and dried in vacuo, weight 5.356 g. The weight gain of 786 mg represents a 70% incorporation of the protected peptide based on initial glycine content of the resin.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-prolyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-glutaminyl-glycinamide

The protected peptide resin (3.0 g) was allowed to swell in 85 ml of dry methanol for 1 hour before being cooled to -10° in an ice salt bath. Dry ammonia, from a boiling solution saturated with sodium was bubbled through the cooled, magnetically stirred suspension for 2.5 hours. The flask was stoppered and stirring continued in the coldroom at +4°C for 18 hours overnight. After stirring for 2 hours at room temperature the flask was re-cooled to -10°C on an ice salt bath before being connected via a splash head adapter and two soda lime U tubes to the water pump and evacuated at room temperature for 3 hours.

Complete removal of the methanolic ammonia was accomplished by continuing the evacuation on a high vacuum for an additional 3 hours. An

all glass lyophilizer with the centre flask containing a 100 ml solution, a 1 :1 mixture of 12 N HCl and acetic acid immersed in dry ice acetone bath was interposed between the flask and the pump to trap the evacuated ammonia vapours.

The cleaved peptide was extracted from the resin with dimethylformamide (3 x 30 ml) followed by methanol (1 x 30 ml). The resin was removed by filtration and the dimethylformamide and methanol removed after each extraction by flash evaporation on the rotary evaporator. The residue was washed with methanol (3 x 30 ml) to remove dimethylformamide and after each wash methanol was removed on the rotary evaporator. The product was dried overnight under high vacuum followed by purification by trituration with 95% ethanol. The protected peptide was ground up using a mortar and pestle before being suspended in 30 ml of 95% ethanol and transferred to a filter paper in a Buchner funnel where the ethanol was removed by gentle suction. The peptide was washed successively with ethanol and diethyl ether and then dried under high vacuum to give the product as a white amorphous powder: 360 mg, mp 225 - 227°, $[\alpha]_D^{22.5}$ - 46.8° (C, 1, dimethylformamide). Anal. calcd. for C_{65} O_{14} N_{12} H_{88} S_2 : C, 61.03; H, 6.30; N, 12.03; Found: C, 60.96; H, 6.47; N, 12.06.

The yield of the purified protected nonapeptide amide from the ammonolytic cleavage and trituration was 51.5% of the amount expected based on the weight gain on the resin. The yield based on the amount of

glycine originally esterified to the resin was 36%. Amino acid analysis gave: Asp, 1.00; Glu, 1.03; Gly, 1.08; Bzl-Cys, 2.08; Ile, 0.99; Tyr, 0.62; Pro, 1.51; NH_3, 2.74.

The protected nonapeptide amide gave single spots on thin-layer chromatography with three solvent systems when detected using the platinic reagent (Toennies and Kolb, 1951). No spots could be detected with ninhydrin. The R_f values obtained by direct comparison with the protected nonapeptide amide of oxytocin (Manning, 1968) are given below:

	Solvent Systems		
Protected nonapeptide amide of	A	B	C
4-Proline 8-glutamine-oxytocin	.71	.72	.75
Oxytocin	.70	.82	.73

A - Butanol (4) : acetic acid (1) : water (5) 2 phases;

B - Propanol (2) : water (1);

C - Isoamyl alcohol (7) : pyridine (7) : water (6).

4-Proline 8-Glutamine-Oxytocin: The protected nonapeptide amide (125 mg) was added to a solution of 200 ml of anhydrous liquid ammonia at its boiling point. Sodium was added to the stirred solution by intermittent dipping of a small bore glass tube containing sodium into the solution until a medium blue colour lasting 15 seconds enveloped the solution. The colour was discharged with a few drops of dry acetic acid from a Pasteur pipette. The

ammonia was removed by connecting the flask via a splash head adaptor and two soda lime drying tubes to a water pump and evacuating for 3 hours at room temperature. The resulting residue was dissolved up in 125 ml 0.2% acetic acid for 15 minutes before adjusting the pH to 6.5 with 2 N ammonium hydroxide, the dithiol was oxidized by the addition of 7 ml of 0.011 M potassium ferricyanide (Hope et al., 1962). After stirring the solution for 10 minutes 5 g of AG3X4 resin (Cl^-form) was added to the solution to remove ferrocyanide and excess ferricyanide ions. The suspension was filtered through a bed of AG3X4 resin 15 g wet weight and further washed with 375 ml of 0.2% acetic acid and 100 ml of 1% acetic acid. The resulting filtrate and washings were lyophilized. The lyophilizate containing, in addition to the desired peptide, dimer and inorganic salt was purified according to the method of Manning et al., (1968b) by gel filtration on Sephadex G-15. The lyophilizate (400 mg) was dissolved in 3.00 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40-120 μ, column size 110 x 1.2 cm) which had been pre-equilibrated with 500 ml of 50% acetic acid. The sample was washed into the column with a further 2 ml of 50% acetic acid and eluted with 50% acetic acid at a rate of 10 ml per hour, 2 ml fractions collected using an automatic fraction collector. A plot of Folin-Lowry colour values (Lowry et al., 1951) of the fractions showed the presence of two peaks with maxima at tubes 31 and 38 separated from the salt by 15 fractions. The fractions

corresponding to the two peaks were pooled separately, diluted with distilled water and lyophilized to give 72 mg of peptide material. The contents of the second peak (tubes 35-42), 50 mg, which contained most of the monomeric peptide was dissolved in 2.0 ml of 0.2 N acetic and applied to the column which had been reequilibrated with 500 ml of 0.2 N acetic acid. The sample was washed into the column with a further 1 ml of 0.2 N acetic and then eluted with 0.2 N acetic acid and collected as before. The required peptide emerged as a single nearly symmetrical peak preceded by a small amount of dimer. The contents of this peak (tubes 40-49) were pooled, diluted with two volumes of distilled water and lyophilized to give the desired peptide as a fluffy white powder (45 mg). This represents a yield of 50% in the reduction-oxidation step of the protected nonapeptide and an overall yield of 18% based on initial glycine incorporation on the resin. $[\alpha]_D^{21.5}$ - 15.5° (C, 0.5, 1 N acetic acid). Amino acid analysis gave: Asp, 1.00; Glu, 0.99; Gly, 1.10; Pro, 1.61; Cys, 1.89; Ile, 1.14; Tyr, 0.99; NH_3, 3.6. The peptide was found to give single spots on thin-layer chromatography on three solvent systems when developed with platinic (Toennies and Kolb, 1951) and ninhydrin reagents. The R_f values obtained compared with those of oxytocin are given below.

	Solvent Systems		
Peptide	A	B	C
4-Proline 8-glutamine-oxytocin	.25	.37	.48
Oxytocin	.36	.58	.59

using the same solvent systems as for protected peptides. Similarly only one component in the direction of the cathode was observed with paper electrophoresis in two pyridine acetate buffers of pH 3.5 and 6.4 using both platinic and ninhydrin reagents.

IV. 8-Valine-Oxytocin: Synthesis of N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-valinyl-glycyl resin.

5 g of the BOC-glycine resin containing 0.210 mmoles glycine per gram substituted resin (1.05 mmoles total) was introduced into the reaction vessel, washed with ethanol and methylene chloride and then left to swell in 50 ml of methylene chloride over the weekend.

The following general procedure was used for all washing steps in the synthesis: the resin was washed three times with 50 ml of each solvent over a 15 minute period. When changing from one solvent to another an intermediate washing with a 1 : 1 mixture (50 ml) of each of the two solvents was employed.

The following cycle which incorporates minor variations of the original procedure as outlined by Merrifield (1964) was used to introduce each new residue into the growing peptide chain (1). The resin was washed successively with methylene chloride, ethanol and glacial acetic acid (2). The BOC

group was removed by treatment with 1 N HCl in acetic acid using the following procedure: 50 ml of 1 N HCl in acetic acid was added to the resin and allowed to equilibrate for 5 minutes. The resin was filtered and a second 50 ml aliquot of 1 N HCl in acetic acid was added and allowed to react for a further 25 minutes. During this time the vessel was occasionally removed from the rocker and shaken by hand to prevent caking of the resin on the sides of the vessel. For the deprotection of the BOC glutaminyl residue the procedure outlined previously (Manning, 1968), utilizing trifluoroacetic acid (Takashima et al., 1968) was followed. (3) The resin was washed successively with acetic acid, ethanol and chloroform. (4) The free amino group was liberated from the hydrochloride or trifluoroacetate by treatment with triethylamine as follows: to a suspension of the resin in chloroform (10 ml) a solution of triethylamine (2 ml) in 20 ml of chloroform was added and allowed to shake with the resin for 5 minutes. The resin was filtered, washed with chloroform and the step repeated. (5) The resin was washed with chloroform and methylene chloride. (6) The resin was suspended in methylene chloride (15 ml) and a three-fold excess (3.15 mmoles) of the appropriate BOC amino acid dissolved in 5 ml of methylene chloride was added and allowed to mix with the resin for 20 minutes. (7) An equivalent amount of dicyclohexylcarbodiimide (3.15 mmoles) in a 50% (w/v) solution of methylene chloride was added and allowed to react for 4 hours. The reaction vessel was agitated manually at intervals during this period. At the end of

4 hours the resin was filtered, washed with methylene chloride and the coupling step repeated. Eight cycles of deprotection, neutralization and coupling were carried out on successive days with the following amino acid derivatives: BOC-L-valine, BOC-L-proline, BOC-S-benzyl-L-cysteine, BOC-L-asparagine-p-nitrophenyl ester, BOC-L-glutamine-p-nitrophenyl ester, BOC-L-isoleucine, BOC-O-benzyl-L-tyrosine, N-benzyloxycarbonyl-S-benzyl-L-cysteine. The incorporation of the BOC-glutamine and BOC-asparagine residues as their nitrophenyl ester derivatives led to the elimination of step (7) and the substitution of dimethylformamide for methylene chloride in steps (5) and (6). After the incorporation of the final residue, steps 1-5 were repeated to bring about the removal of any N-CBZ-S-Bzl-L-cysteine which had been bound by ion exchange during the final coupling step to the quaternary ammonium groups on the resin.

At the conclusion of the synthesis, the protected peptide resin was washed out of the reaction vessel with ethanol, dimethylformamide and methanol, collected on a filter and dried in vacuo, weight 6.298 g. The weight gain of 1.298 g at this stage represents a 98% incorporation of protected peptide based on the initial BOC glycine content (1.05 mmoles) on the resin.

N-benzyloxycarbonyl-S-benzyl-L-cysteinyl-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-valinyl-glycinamide.

The protected peptide resin (3.0 g) was allowed to swell for 1 hour in 70 ml of dry methanol before being cooled to -10°C in an ice salt bath. Dry ammonia, from a boiling solution saturated with sodium was bubbled through the cooled stirred suspension for 2.5 hours. The flask was stoppered and stirring continued at +4° for 18 hours. The flask was stirred at room temperature for 2 hours then cooled to -10°C in an ice salt bath. The flask was connected via a splash head adaptor and two soda lime drying tubes to a water pump and evacuated for 3 hours to remove the ammonia. Complete removal of the methanolic ammonia was accomplished by continuing the evacuation on a high vacuum pump. An all glass lyophilizer with the centre flask containing a 100 ml solution of a 1 : 1 mixture of 12 N HCl and glacial acetic acid immersed in a dry ice acetone bath, was interposed between the flask and the pump to trap the evacuated ammonia vapours. The cleaved peptide was extracted from the resin with dimethylformamide (3 x 30 ml) and methanol (2 x 10 ml). The resin was removed by filtration and the dimethylformamide and methanol were removed in vacuo on a rotary evaporator. The residue was washed with methanol (3 x 30 ml) which was removed each time by evaporation and dried in vacuo overnight. The product was purified by trituration with 95% ethanol (30 ml) and the insoluble precipitate was collected on a filter, washed with ethanol (2 x 10 ml) and diethyl ether (3 x 10 ml) and dried in vacuo over P_2O_5 to give the required protected nonapeptide amide as a white amorphous powder, weight 475 mg,

mp 258 - 261°, $[\alpha]_D^{21}$ - 43° (C, 1 dimethylformamide), anal. calcd. for C_{71} O_{14} N_{12} H_{90} S_2 : C, 60.94; H, 6.44; N, 12.02. Found : C, 60.90; H, 6.64; N, 11.86.

The yield of the purified protected nonapeptide amide from the ammonolytic cleavage and trituration was 72% of the amount expected, based on the weight gain of the resin. The yield based on the amount of glycine originally esterified to the resin was 71.5%. Amino acid analysis gave Asp, 1.00; Glu, 1.09; Pro, 0.94; Gly, 1.05; Val, 1.01; Ile, 1.01; Tyr, 0.64; S-Bzl-Cys, 2.01; NH_3, 3.4.

The protected nonapeptide amide was adjudged pure by thin-layer chromatography on three solvent systems giving only one spot with platinic reagent (Toennies and Kolb, 1951) and none with ninhydrin. The R_f values compared with those for the protected nonapeptide of oxytocin are given below:

	Solvent Systems		
Protected nonapeptide amide of	A	B	C
8-Valine-oxytocin	.67	.75	.70
Oxytocin	.70	.82	.73

A - Butanol (4) : acetic acid (1) : water (5) 2 phases;

B - Propanol (2) : water (1);

C - Isoamyl alcohol (7) : pyridine (7) : water (6).

8-Valine-~~Oxytocin~~: The protected nonapeptide amide (150 mg) was dissolved in 200 ml of anhydrous liquid ammonia, which was at its boiling point. Sodium was added from a small bore glass tube until a faint blue colour enveloped the solution for 10 seconds. Dry acetic acid (3 drops) was added to discharge the colour and the ammonia was evaporated in vacuo under anhydrous conditions at the water pump. The residue was dissolved in 200 ml of 0.2% acetic acid for 20 minutes and after adjustment of the pH to 6.8 with 2 N ammonium hydroxide, an excess of 0.011 M potassium ferricyanide (8 ml) (Hope et al., 1962) was added to the stirred solution. After 10 minutes, 5 g of AG3X4 resin (Cl^- form) was added and stirring was continued for 5 minutes to remove the ferrocyanide and excess ferricyanide ions. The suspension was filtered through a bed of AG3X4 resin (Cl^-form) 20 g wet weight, and washed through with 320 ml of 0.2% acetic acid. The filtrate and washings were combined and the pH adjusted to 3 with glacial acetic acid. The solution was lyophilized to give 730 mg of crude product consisting of the required peptide, dimer and inorganic salts. Purification was effected by the method of Manning et al. (1968b) using gel filtration on Sephadex G-15. The lyophilizate was dissolved in 3.0 ml of 50% acetic acid and applied to the top of a column of Sephadex G-15 (Pharmacia Fine Chemicals, Uppsala, particle size 40-120 μ, column sizw 110 x 1.2 cm) which had been preequilibrated with 500 ml of 50% acetic acid. The sample was washed into the column with a further 2 ml of 50% acetic acid and eluted

with 50% acetic acid at a rate of 10 ml per hour. 2 ml fractions were collected. A plot of the Folin-Lowry colour values (Lowry et al., 1951) of the fractions showed the presence of two peaks with maxima at 27 and 35, clearly separated from the salt. The fractions corresponding to the two peaks were pooled separately, diluted with distilled water and lyophilized to give 95 mg of peptide material. Meanwhile the Sephadex column was reequilibrated with 500 ml of 0.2 N acetic acid. The lyophilized powder (46 mg) from the second peak containing most of the active monomer was dissolved in 2.0 ml of 0.2 N acetic acid and eluted and collected as before at a rate of 10 ml per hour. The required peptide emerged as a single nearly symmetrical peak preceded by a small amount of dimer. The contents of this main peak (tubes 38-44) were pooled, diluted with two volumes of water and lyophilized to give the desired peptide as a fluffy white powder (42 mg). This represents an overall yield of 28.6% based on the initial glycine incorporation on the resin. $[\alpha]_D^{22}$ - 22° (C, 0.5 1N acetic acid). Amino acid analysis gave: Asp, 1.00; Glu, 1.09; Pro, 0.89; Gly, 0.93; Val, 1.06; Ile, 0.93; Tyr, 0.99 ; Cys, 2.14; NH_3, 3.2. The peptide gave single spots on thin-layer chromatography with three different solvent systems when developed with both platinic (Toennies and Kolb, 1951) and ninhydrin reagents. The R_f values obtained by direct comparison with oxytocin are given below:

	Solvent Systems		
Peptides	A	B	C
8-Valine-oxytocin	.29	.42	.59
Oxytocin	.36	.58	.59

The same solvent systems were used as for protected peptides. Similarly, only one component in the direction of the cathode was observed with paper electrophoresis in two pyridine acetate buffers of pH 3.5 and 6.4 using the same detecting reagents as above.

V. Pharmacological Evaluation of Synthesized Peptides

The assays were carried out by Dr. Wilbur H. Sawyer, College of Physicians and Surgeons, Columbia University.

Samples of the synthetic lyophilized peptides were each dissolved in 50 mM acetic acid containing 5 g/litre of chlorobutanol/USP. The solutions were stored under refrigeration and samples were removed as needed for biological assays. Activities on the rat uterus in vitro, suspended in solutions without Mg^{++} and with 0.5 mM Mg^{++}, were performed as described by Munsick (1960) using the assay originally outlined by Holton (1948). Milk ejection assays were performed on lactating rabbits by the method of Cross and Harris (1952) using the modification of van Dyke et al. (1955). Isolated frog bladder (hydro-osmotic) assays were done according to the method of Sawyer (1960) and fowl vasodepressor as described by Munsick et al. (1960). Antidiuretic assays were carried out on ethanol-anesthetized rats using the method described by Sawyer (1958) and vasopressor assays on pithed rats according to Sawyer (1966). All activities are reported in terms of the USP

Posterior Pituitary Reference Standard. The results of the assays compared with those of oxytocin are given in Table II.

TABLE II Some Pharmacological Activities[a] of Oxytocin and the Synthesized Analogues

	Rat Uterus	Fowl Vaso-depressor	Rabbit Milk-ejecting	Rat Vaso-pressor	Rat Anti-diuretic	Frog Bladder
Oxytocin[b]	450	450	450	5	5	450
8-Gln-Oxytocin[c]	58	95	256	34	6	1270
8-Phe-Oxytocin[d]	108	191	341	1	1	1700
8-Val-Oxytocin[d]	265	-	-	17	5	1676
4-Pro 8-Gln-Oxytocin[e]	<0.006	-	-	ca. 0.001	<0.00006	<0.25

[a] Expressed in units/mg; [b] Values reported from Berde and Boissonnas (1968); [c] Values reported from Baxter et al. (1969b); [d] Values reported from Baxter et al. (1969a); [e] Values reported from Sawyer et al. (1969a).

DISCUSSION

I. Synthesis and Purification

The Merrifield method of Solid Phase peptide synthesis has been successfully applied to the synthesis of four analogues of oxytocin: 8-glutamine-oxytocin, 8-phenylalanine-oxytocin, 8-valine-oxytocin and 4-proline 8-glutamine-oxytocin, the structures of which are shown in TableIII. The analogues, adjudged pure by the standard physical and chemical criteria were all readily obtained in good yield. Following after the solid phase syntheses in this laboratory of oxytocin (Manning, 1968) and glumitocin (4-serine 8-glutamine-oxytocin) (Manning et al., 1968a) their syntheses further serves to demonstrate the efficacy of the Merrifield method to the synthesis of peptides of this type.

In the syntheses, the incorporation of the peptide on the resin as estimated by the weight gain of the resin was generally less than that theoretically estimated. The reason for this incomplete incorporation is not entirely clear. The possibility that some of the glycine esterified to the resin initially might prove inaccessible to coupling with other residues was felt to be minimized by using a low incorporation of around 0.2 mmoles glycine per gram of resin. Ives (1968), in comparison, has reported using 0.71 mmoles glycine per gram resin. The coupling at each step was carried

TABLE III Amino Acid Sequences of Oxytocin and the Synthesized Analogues

	1 2 3 4 5 6 7 8 9
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

	Amino Acids in Position	
	4	8
8-Glutamine-Oxytocin	Gln	Gln
8-Phenylalanine-Oxytocin	Gln	Phe
8-Valine-Oxytocin	Gln	Val
4-Proline 8-Glutamine-Oxytocin	Pro	Gln

out using three equivalents of the appropriate amino acid, in excess to that considered adequate (Stewart and Young, 1969). Furthermore in later syntheses quantitative coupling was assured using a double addition of the amino acid at each coupling step. It is known that the glutamine residue in the terminal position of the peptide chain during synthesis can undergo a chain terminating rearrangement possibility to pyroglutamate when 1 N HCl in acetic acid is used for its deprotection (Stewart and Young, 1969). Despite the fact that the modification of Takashima et al. (1968) using trifluoroacetic acid in place of 1 N HCl in acetic acid for this deprotection was employed it is possible that some rearrangement was still occurring. Thin-layer chromatography of the protected peptides after ammonolysis consistently showed one extra minor spot with platinic spray. This minor contaminant was subsequently removed by trituration with 95% ethanol. Moreover with the synthesis of 4-proline 8-isoleucine-oxytocin, an analogue containing no glutamine, a 100% incorporation based on weight gain of the resin was achieved (M.Manning, personal communication). The product obtained directly from ammonolytic cleavage of this peptide from the resin gave, interestingly enough, only a single spot on thin-layer chromatography with the platinic reagent. It would therefore appear that further studies are called for on this question of incorporation and glutamine cyclization in the solid phase synthesis.

The sodium liquid ammonia procedure for deblocking protected peptides originally introduced by Sifferd and du Vigneaud (1935) and subsequently

used in the original synthesis of oxytocin (du Vigneaud et al., 1954) has been the cause of some concern, especially with respect to cleavage of proline bonds (Merrifield and Marglin, 1967). Our experience with this procedure has been generally favourable. The recovery of the peptide material after the reduction, followed by oxidation with ferricyanide, ion exchange removal of the oxidizing ions and desalting with Sephadex, was in the range of 90-95% of the weight of peptide calculated. In the case of the analogue containing two prolines, 4-proline 8-glutamine-oxytocin, the yield was slightly lower, although the only material obtained after oxidation was the expected monomer and dimer.

Complete removal of the benzyl groups from the sulfur of cysteine as could be detected by amino acid analysis could be achieved by a treatment with sodium which resulted in the final light to medium blue colour lasting 15 seconds or less. These conditions are considerably less than those cited by Benisek and Cole (1967) as being necessary for cleavage of the most labile of proline bonds. It would therefore appear that the method, if carefully controlled, can be successfully used in this type of situation, even when two of the nine residues in the peptide are proline.

The method of purification using gel filtration on Sephadex G-15 developed in our laboratory during the course of studies proved a rapid and effective way of purifying these and other synthetic analogues of the neurohypophysial hormones. In contrast to other methods commonly employed for

this purpose, namely counter current distribution and partition chromatography which require development of suitable solvent systems for each type of analogue this method requires the use of only two standard solvents, water and acetic acid in all cases. Whereas solvents commonly used in counter current distribution and partition chromatography such as pyridine and benzene are difficult to remove from the final product by lyophilization both water and acetic acid are easily removed.

II. Phylogenetic and Structure-Activity Studies

The pharmacological activity of the synthesized analogue 8-glutamine-oxytocin clearly differs in several assays from the activity obtained from EOP I (Table IV). This difference is particularly evident in the frog bladder assay; the ratio of activity to rat uterus without magnesium (R_{FB}) being 22 ± 3 for the synthetic analogue while only 2.4 - 4.2 for EOP I. It must therefore be concluded on the basis of this study that EOP I is not 8-glutamine-oxytocin. This however does not rule out the possibility that 8-glutamine-oxytocin does exist in nature. The fact that an 8-glutamine analogue, glumitocin has been found in cartilaginous fish (Acher et al., 1965a) plus the comparatively large number of principles which have been found in this class makes it entirely possible the analogue does exist in some hitherto unexamined species. Should this turn out to be the case the knowledge of its pharmacological

TABLE IV Pharmacological Activities[a] and Activity Ratios[b] with Standard Errors of 8-Gln-Oxytocin[c], 8-Phe-Oxytocin[d], 8-Val-Oxytocin[d] and EOP I[e]

	8-Gln-Oxytocin	8-Phe-Oxytocin	8-Val-Oxytocin	EOP I
Rat uterus (RUsMg) (no Mg in bath)	58	108	265	-
Rat uterus (RUcMg) (0.5 mM Mg in bath)	122	366	-	-
RUcMg/RUsMg = R_{Mg}	2.1 ± 0.05	3.4 ± 0.2	-	1.8 - 3
Rat vasopressor (RVP)	34	0.97	17	-
RVP/RUsMg = R_{VP}	0.58 ± 0.02	0.009 ± 0.0005	0.064	0.02
Rat antidiuretic (RAD)	5.9	1.16	5	-
RAD/RUsMg) = R_{AD}	0.10 ± 0.02	0.011 ± 0.0013	0.019	0.007
Isolated bullfrog bladder water permeability (FB)	1270	1700	1676	-
FB/RUsMg = R_{FB}	22 ± 3	16 ± 3	6.3	2.4 - 4.2
Rabbit milk-ejecting (ME)	256	341	-	-
ME/RUsMg = R_{ME}	4.4 ± 0.2	3.16 ± 0.17	-	1.5 ± 0.05
Fowl vasodepressor (FV)	-	191	-	-
FV/RUsMg = R_{FV}	-	1.77 ± 0.11	-	1.55 ± 0.11

[a] Expressed in units/mg; [b] Biological assays were carried out and activity ratios were calculated as described in Sawyer (1966); [c] Baxter et al. (1969b); [d] Baxter et al. (1969a); [e] See Sawyer (1965), Sawyer et al. (1969a), Sawyer (1967).

properties given here should lead to its rapid detection if and when the appropriate tissue extracts become available.

The chromatographic properties of 8-phenylalanine-oxytocin were, as expected, very similar to those quoted for EOP I. As was the case for EOP I the analogue moved slightly faster than oxytocin on a hydrophobic solvent system. Comparison of the pharmacological properties of 8-phenylalanine-oxytocin with those of EOP I (Table IV) indicate that the two are indeed quite similar. However, 8-phenylalanine-oxytocin does differ sufficiently from EOP I on two assays, rabbit milk-ejection and bullfrog bladder to warrant the conclusion that the two are not identical.

In the same way the spectrum of activities of 8-valine-oxytocin while as yet not complete would appear to differ from those of EOP I sufficiently to rule out the possibility that they are the same principle (Sawyer, personal communication).

While the idea that EOP I is an substituted analogue of oxytocin is certainly an attractive one it is difficult at the moment to see an analogue with a substitution in the 8 position which would be sufficiently hydrophobic to give chromatographic properties similar to that found for EOP I. Serine, threonine and methionine however can all be obtained by single point mutations from both isoleucine and arginine (Figure 6) and so the possibility that one of these in the 8 position might yield the sought after analogue cannot be ruled out. The alternative, that the analogue

could contain substitutions in other positions as well must be considered. The glutamine-serine transition in position 4 of course offers some intriguing possibilities. It would seem highly likely that an intermediate or intermediates are involved and it is possible that one of them might be EOP I.

In contrast to the other analogues synthesized in this study, the analogue 4-proline 8-glutamine-oxytocin seems to possess only very limited biological activity (Table III). This has also been found to be the case with other 4-proline analogues synthesized in the laboratory (Sawyer et al., 1969a) as well as with one recently synthesized by Rudinger et al., (1969). While they do seem to possess some intrinsic activities on the rat uterus, in the presence of 0.5 mM Mg^{++} and on rat milk ejection, the activities are extremely weak being at the most 1/10,000 of the respective activities of their corresponding 4-glutamine analogues (Sawyer et al., 1969a). It is obvious that these analogues because of their extremely low activity would have escaped detection had they been present in any study of pituitary extracts. It should be noted that the assay systems on which the 4-proline analogues were tested and found essentially inactive are all based on responses from tissues of tetrapod vertebrates. Therefore it cannot be considered outside the realm of possibility that one or more of these analogues might have important physiological activity of some more primitive species of vertebrate, the target tissue of which respond differently from those of

higher vertebrates. One of the problems faced here is that as of yet satisfactory assay systems have yet been developed on any of these lower vertebrates. As to the more immediate question of the identity of EOP I however, it would appear that this 4 position change at least has been eliminated from contention. Rudinger et al. (1969) have suggested the possibility of a 4-leucine analogue as a possible evolutionary intermediate in the glutamine-serine transition and the synthesis of 4-leucine-oxytocin has recently been reported. It would be interesting to contemplate such a 4 position change with EOP I.

The structure-activity relationship of the 8 substituted analogues of oxytocin are also of interest. As can be seen from Table V the 8 position can accommodate large variations in its side chain and still maintain appreciable biological activity. Of particular interest here is the 8-phenylalanine analogue, where the aliphatic side chain in position 8 of oxytocin has been replaced with an aromatic ring. While the oxytocic activity of this analogue has decreased to 24% of the activity of oxytocin it still possesses 76% of the milk-ejecting potency, the highest reported for the 8-substituted analogues of oxytocin. The frog bladder activities of the 8-substituted analogues synthesized are all greater than that reported for oxytocin (Table V).

TABLE V Pharmacological Activities (in USP units/mg of 8 Substituted Analogues of Oxytocin

Oxytocin and Analogues	Rat uterus	Fowl vaso-depressor	Rabbit milk-ejection	Rat vaso-pressor	Rat anti-diuretic	Frog bladder[a]
Oxytocin (8-Leu)[b]	450	450	450	5	5	450
8-Ile-oxytocin[b]	289	498	328	6	1	1070
8-Ile-oxytocin[c]	388	724	264	-	0.53	-
8-Val-oxytocin[b]	200	280	310	9	0.8	-
8-Val-oxytocin[d]	265	-	-	17	5	1676
8-Cit-oxytocin[e,f]	202	ca. 250	238	17	4	800
8-Ala-oxytocin[b]	141	135	208	12	0.08	357
8-Ala-oxytocin[g]	166	240	-	13	-	-
8-Arg-oxytocin[b]	114	285	210	245	250	100,000
8-Phe-oxytocin[d]	108	191	341	1	1	1700
8-Lys-oxytocin[b]	78	210	180	130	24	15,000
8-Gln-oxytocin[h]	58	95	256	34	6	1270
8-Orn-oxytocin[b]	42	90	95	103	2.5	ca. 80
8-Gly-oxytocin[b]	16	7	47	0.6	0.16	530

[a] All frog bladder assays have been reported in Sawyer (1965), Sawyer et al. (1969a) and Baxter et al. (1969a); [b] Values reported by Berte and Boissonnas (1968); [c] Values reported by Rudinger et al. (1969); [d] Values reported by Baxter et al. (1969a); [e] Values reported by Bodanszky and Birkhimer (1962); [f] Values reported by Van Dyke et al. (1963); [g] Values reported by Walter and du Vigneaud (1966); [h] Values reported by Baxter et al. (1969b).

Conclusion

The Merrifield method of peptide synthesis has been used here in an attempt to elucidate the structure of an unknown neurohypophysial principle as well as for the synthesis of other analogues which were considered as possible intermediates in the evolution of these principles. The speed and efficiency with which the analogues can be synthesized has made the method particularly attractive to this type of study as it allows rapid testing of a proposed structure of an unknown intermediate with the synthetic product. Work is continuing at the present time in the laboratory on the synthesis and pharmacological evaluation of other analogues considered interesting both from the point of view of evolution and structure-activity and it is expected that this approach will achieve even more wide-spread use in this type of study in the future.

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APPENDIX: List of Abbreviations

Amino Acids

Ala	Alanine	Ile	Isoleucine
Arg	Arginine	Leu	Leucine
Asp-NH_2 or Asn	Asparagine	Lys	Lysine
Cit	Citrulline	Orn	Ornithine
Cys	Cysteine	Phe	Phenylalanine
Glu-NH_2 or Gln	Glutamine	Pro	Proline
Gly	Glycine	Ser	Serine
		Tyr	Tyrosine
		Val	Valine

Protecting Groups

BOC	t-Butyloxycarbonyl
BZL	Benzyl
CBZ or Z	Benzyloxycarbonyl
pGlu	Pyroglutamic acid
OBZL	Benzyl ester
OEt	Ethyl ester
Tos	Tosyl

Coupling Methods

CHL	Acid chloride
DCC	Dicyclohexylcarbodiimide
MCA	Mixed carboxylic anhydride
ONP	p-Nitrophenyl ester
TEP	Tetraethylpyrophosphate