Influence of the Peptide-Chain Length on Disulphide-Bond Formation in Neurohypophysial Hormones and Analogues

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[8-Arginine]vasopressin, [8-arginine]vasotocin, oxytocin and oxypressin, the 'ring' derivatives pressinamide and tocinamide, and the extended-chain analogues Pro-Arg-Val-[8-arginine]vasopressin and [8-arginine]vasopressinoyl-Ala-Met-Ala-NH₂, were synthesized by the solid-phase method and purified by sequential gel filtration on Sephadex G-15 in 50% acetic acid and 0.2M-acetic acid. Controlled oxidation of the thiol groups of the reduced peptides obtained after deprotection with sodium in liquid ammonia gave rise to products that depended on the length of the peptide chain: (i) nonapeptides gave monomer and dimer species, (ii) hexapeptides produced mixtures containing higher polymers, and (iii) dodecapeptides gave predominantly monomer with some dimerized material. The evidence suggests that the presence of the acyclic tail tripeptide in the nonapeptide hormones induces a conformation in the preceding hexapeptide that favours the formation of an intramolecular disulphide bond. For [8-arginine]vasopressin, intramolecular disulphide-bond formation is enhanced by extension of the peptide chain from either the *N*- or the *C*-terminus. The possible significance of these studies to neurohypophysial hormone-prohormone relationships is discussed.

The formation of an intramolecular disulphide bond occurs at some stage during the biosynthesis of the neurohypophysial hormones, and presumably takes place in an environment that ensures intramolecular alignment of the thiol groups so that intermolecular disulphide bonds are not formed. During the chemical synthesis of neurohypophysial hormones, both intra- and inter-molecular disulphide-bond formation are observed after oxidation of the reduced peptides, suggesting that the hormones

themselves do not represent an 'efficient' environment for intramolecular disulphide-bond formation. The possibility exists that these hormones are biosynthesized in the form of prohormones, which, when released from the ribosomes, are conformationally predisposed to spontaneous and efficient intramolecular disulphide-bond formation.

An index of the precyclic conformation of a given neurohypophysial-hormone analogue may be obtained by determining the degree of difficulty with which its intramolecular disulphide bond is formed, as reflected in the ratio of monomer to polymer(s) obtained after controlled oxidation of the reduced peptide. By using the appropriate analogues, this approach can give information on the relative

Abbreviations used: abbreviations for amino acids and protecting groups are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (1972); Boc, *t*-butoxycarbonyl. Synthetic analogues of neurohypophysial hormones:

pressinamide, Cys-Tyr-Phe-Gln-Asn-Cys-NH₂;

tocinamide, Cys-Tyr-Ile-Gln-Asn-Cys-NH₂;

oxypressin, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Leu-Gly-NH₂;

Pro-Arg-Val-[Arg⁸]vasopressin, Pro-Arg-Val-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂;

[Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-Ala-Met-Ala-NH₂.

roles of different loci or regions of the molecule in stabilizing a precyclic conformational state that favours the formation of an intramolecular disulphide bond.

Experimental

Materials

Boc-amino acid derivatives were purchased from Bachem, Torrance, CA, U.S.A., and examined for purity by t.l.c. before use. Chloromethylated 1%-cross-linked divinylbenzene/polystyrene co-polymer was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. T.l.c. plates (silica gel GF-254 on glass) were obtained from Brinkman Instruments, Rexdale, Ontario, Canada. All other reagents were of the highest grade available. Chloroform, methylene chloride and dimethylformamide were stored over a molecular sieve, type 4A (Davison Chemicals, Baltimore, MD, U.S.A.). In addition, dimethylformamide was stored over Bio-Rex 70 (H^+ form) (Bio-Rad). Triethylamine was re-distilled before use. [Leu⁵]enkephalin and physalaemin were purchased from Bachem. Bacitracin, glucagon and myoglobin were from Sigma Chemical Co., St. Louis, MO, U.S.A. [Sar¹,Sar⁷]angiotensin II was synthesized in this laboratory (Moore *et al.*, 1978).

Methods

All the required hormones and derivatives were synthesized by the Merrifield (1963, 1964) method and were deblocked and purified by described procedures (Manning, 1968; Manning *et al.*, 1968). Pharmacological potencies were determined by rat uterus (Heller & Lederis, 1959) and rat pressor (Dekanski, 1952) assay by comparison with International Posterior Pituitary Standard (USP Reference Standards, Rockville, MD, U.S.A.).

Boc-glycyl-resin, Boc-alanyl-resin and Boc-cysteinyl(S-benzyl)-resin. These derivatives were prepared from chloromethylated 1% divinylbenzene/polystyrene co-polymer resin (0.89 mequiv. of Cl/g) via the Boc-amino acid caesium salts (Gisin, 1973) and were subsequently acetylated (Stewart & Young, 1969). The Boc-amino acid-resin derivatives thus obtained contained 0.58 mmol of Boc-glycine/g of resin, 0.39 mmol of Boc-cysteine(S-benzyl)/g of resin and 0.40 mmol of Boc-alanine/g of resin respectively [amino acid analysis after hydrolysis in propionic acid/12M-HCl (1:1, v/v) at 160°C for 90 min *in vacuo*].

Solid-phase peptide synthesis. Peptides were synthesized with a Beckman model 990 peptide synthesizer by (a) one 3 h dicyclohexylcarbodi-imide-mediated coupling reaction for each Boc-amino acid [2.5 equiv. in CH_2Cl_2 or $HCONMe_2/CH_2Cl_2$], except for Boc-asparagine and Boc-glutamine, which were coupled via their *o*-nitrophenyl esters [4 equiv. in $HCONMe_2/CH_2Cl_2$ (1:1, v/v)] in a single 12 h

reaction, (b) 25% (v/v) trifluoroacetic acid in $CHCl_3$ for deprotection (2 min and 25 min), (c) 10% (v/v) triethylamine in $CHCl_3$ for neutralization (2 min and 10 min). Solvent volumes were 20 ml (deprotection neutralization and washing steps) and 15 ml (coupling). To minimize dioxopiperazine formation, neutralization of dipeptide-resins was carried out in a single step (2 min) and the subsequent coupling reaction was carried out by adding dicyclohexylcarbodi-imide to the reaction vessel before the addition of the Boc-amino acid. Side-chain protecting groups were: for cysteine and tyrosine, benzyl; for arginine, tosyl; for the α -amino group of the completed peptide, benzyloxycarbonyl.

Deprotection and purification of peptides. After completion of the required cycle procedure, the dry peptide-resin was ammonolysed in 150 ml of methanol saturated with NH_3 (at 0°C) for 2 days in a sealed flask at room temperature (23°C). The mixture was evaporated *in vacuo* through soda-lime and the residue dissolved in dimethylformamide (100 ml). The resin was removed by filtration and dimethylformamide removed *in vacuo*. The residue was triturated with methanol (150 ml), dried and recrystallized from acetic acid/methanol. The fully protected peptide amide (50–150 mg) was dissolved in anhydrous liquid NH_3 (100–200 ml) and the boiling solution titrated with sodium (by dipping a sodium-filled 1 ml pipette into the solution) until a light-blue colour persisted. After 15 s the colour was discharged with 2 drops of dry acetic acid and the ammonia allowed to evaporate through a soda-lime guard tube. The product was dissolved in 0.2% acetic acid (3 ml/mg of protected peptide), the pH adjusted to 7 with 2M- NH_3 soln. and the solution titrated with 0.01M-potassium ferricyanide until a yellow colour persisted. After stirring for 20 min, AG-3X4A resin (Bio-Rad) was added and the solution stirred for a further 20 min. The resin was removed by filtration, and the filtrate adjusted to pH 4 with acetic acid and freeze-dried. The residue was dissolved in 50% (v/v) acetic acid (2 ml) and chromatographed on a column (90 cm $\times$ 2.5 cm) of Sephadex G-15 eluted with 50% acetic acid at a flow rate of 8–10 ml/h. Fractions containing monomer material (fractions were selected for purity rather than yield) were pooled and freeze-dried. The residue was dissolved in 0.2M-acetic acid (2 ml) and re-run through the Sephadex G-15 column eluted with 0.2M-acetic acid at a flow rate of 12–15 ml/h. Fractions containing the required peptide were pooled and freeze-dried.

The ratio of monomer to polymer(s) was determined for each peptide after the first Sephadex G-15 chromatography step from the areas under the peaks. The amount of reduced peptide that formed the monomer was determined as the area under the peak corresponding to the monomer divided by the total area under all the peaks (monomer +

dimer etc.), expressed as a percentage. For each peptide the reduction-reoxidation-Sephadex G-15 chromatography was carried out two to five times and an average yield of monomer was determined.

Criteria of purity of peptides. Protected peptides were examined for purity by t.l.c. (on silica gel GF-254) in two solvent systems: butan-1-ol/acetic acid/water (4:1:1, by vol.) and acetonitrile/water (9:1, v/v). Identification was sequentially by fluorescence quenching, ninhydrin spray reagent, and chlorination followed by starch/KI spray reagent.

The purity of the required deprotected peptides was also assessed by high-voltage electrophoresis, amino acid analysis and biological potency.

Results

Table 1 summarizes the properties of the fully protected peptides obtained after their removal from the resin by ammonolysis. After deprotection, oxidation and two sequential Sephadex G-15 chromatography steps the peptides were obtained in the following yields (based on fully protected peptide): [Arg⁸]vasopressin, 51%; [Arg⁸]vasotocin, 29%; oxytocin, 31%; oxypressin, 37%; pressinamide, 8%; tocinamide, 10%; Pro-Arg-Val-[Arg⁸]vasopressin, 22%; [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂, 59%. [It is generally believed that sodium in liquid NH₃ cannot be used to deprotect peptides containing proline. However, the neurohypophysial hormones represent a special case, since a large number of analogues have been successfully synthesized by this procedure (Manning, 1968).] For each peptide, high-voltage electrophoresis on cellulose thin-layer plates at pH2.75 showed the presence of a single spot migrating in the direction of the cathode. Chemical

Table 1. *Chemical data on fully protected peptides*
Yields are based on starting amount of Boc-amino acid-resin. Yield on the resin was determined from the weight gain on the resin. The yield of peptide was determined by drying to constant weight after crystallization (see the text).

Compound	M.p. (°C)	Yield on resin (%)	Yield of peptide (%)
[Arg ⁸]vasopressin	227-228	96	45
[Arg ⁸]vasotocin	230-231	81	28
Oxytocin	230-231	90	25
Oxypressin	236-237	78	21
Tocinamide	234-235	63	20
Pressinamide	224-225	74	16
Pro-Arg-Val-	214-215	78	42
[Arg ⁸]vasopressin			
[Arg ⁸]vasopressinoyl-	226-227	79	27
Ala-Met-Ala-NH ₂			

Table 2. *Chemical and pharmacological properties of neurohypophysial hormones and analogues*
R_F values refer to t.l.c. on silica gel in the following solvents: BPAW, butanol-1-ol/pyridine/acetic acid/water (15:10:3:6, by vol.); CMAW, chloroform/methanol/acetic acid/water (15:10:2:3, by vol.). Biological activity is expressed as units/mg of peptide ± s.e.m.

Peptide	R_F			Amino acid composition (residues/molecule)										Biological activity			
				Asp	Glu	Pro	Cys	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Arg	Rat uterus*
[Arg ⁸]vasopressin	0.33	0.15	1.03	1.07	0.98	1.81	1.00	—	—	—	—	—	0.87	0.98	0.94	—	360 ± 36
[Arg ⁸]vasotocin	0.35	0.14	1.05	1.10	0.90	1.88	1.00	—	—	—	1.04	—	0.87	—	0.96	219 ± 22	181 ± 17
Oxytocin	0.71	0.69	1.06	1.02	0.93	1.79	1.00	—	—	—	1.01	1.05	0.91	—	—	313 ± 34	2.0 ± 0.2
Oxypressin	0.69	0.68	1.07	1.06	0.92	1.92	1.00	—	—	—	—	1.02	0.88	0.95	—	59 ± 3	2.8 ± 0.2
Tocinamide	0.64	0.57	1.07	1.08	—	1.89	—	—	—	—	1.00	—	0.87	—	—	—	—
Pressinamide	0.67	0.60	1.11	1.13	—	1.91	—	—	—	—	—	—	0.84	1.00	—	—	—
Pro-Arg-Val-	0.51	0.63	0.98	1.05	1.90	1.70	1.00	—	1.04	—	—	—	1.00	1.00	1.92	—	0.3 ± 0.01†
[Arg ⁸]vasopressin																	
[Arg ⁸]vasopressinoyl-	0.59	0.70	1.05	1.09	1.09	1.86	1.00	2.19	—	0.98	—	—	1.04	1.02	0.99	—	0.2 ± 0.01‡
Ala-Met-Ala-NH ₂																	

* In the presence of 0.5 mm-Mg²⁺.
† The log (dose)-time response curve (Pliska, 1969) was parallel to that obtained for [Arg⁸]vasopressin, indicating that the observed pressor activity originated from an intrinsic activity of the peptide rather than conversion *in vivo* into [Arg⁸]vasopressin (Rudinger *et al.*, 1972).
‡ Partial agonist.

Fig. 1. Chromatography of peptides on Sephadex G-15 after oxidation of the thiol groups

Column dimensions were 90cm $\times$ 2.5cm and the eluent was 50% acetic acid at a flow rate of 8–10ml/h. The fraction size was 3.2ml. —, Pressinamide; ----, [Arg⁸]vasopressin; ····, Pro-Arg-Val-[Arg⁸]vasopressin; — — —, [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂. Calibration standards were: 1, myoglobin; 2, glucagon; 3, physalaemin; 4, bacitracin; 5, [Sar¹,Sar⁷]angiotensin II; 6, [Leu⁵]enkephalin.

data and pharmacological potencies of the peptides are given in Table 2.

The results of a systematic study of the products obtained after oxidation of the reduced peptides are exemplified in Fig. 1. The concentration of peptide was kept constant in the oxidation step because the amount of monomer formed varied with concentration. Thus for the oxidation of reduced [Arg⁸]vasotocin (obtained by sodium/liquid NH₃ reduction of 150mg of the fully protected peptide) the percentage of monomer formed was 35, 57 and 66 when the volume of buffer used was 250, 450 and 650ml respectively.

Elution profiles (Fig. 1) are shown for [Arg⁸]vasopressin, pressinamide, Pro-Arg-Val-[Arg⁸]vasopressin and [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂. The results for oxytocin, [Arg⁸]vasotocin and oxytocin were essentially the same as that for [Arg⁸]vasopressin, and the elution profile obtained for tocinamide was similar to that shown for pressinamide. For the four nonapeptide hormones, oxidation of the thiol groups with ferricyanide to form the disulphide bond gave only monomer and dimer material, the monomer being obtained in 53 $\pm$ 6% (11) [mean $\pm$ s.d. (no. of experiments)] yield (Fig. 1). However, for the hexapeptide-ring derivatives

Fig. 2. Relationship between elution-volume/void-volume ratio and log(molecular weight) for neurohypophyseal peptides and other peptides by Sephadex G-15 chromatography

Taken from the data in Fig. 1. Neurohypophyseal peptides: 1, pressinamide; 2, [Arg⁸]vasopressin; 3, [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂; 4, Pro-Arg-Val-[Arg⁸]vasopressin; 5, pressinamide dimer; 6, [Arg⁸]vasopressin dimer; 7, pressinamide trimer; 8, [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂ dimer; 9, Pro-Arg-Val-[Arg⁸]vasopressin dimer. Calibration standards: 10, [Leu⁵]enkephalin; 11, [Sar¹,Sar⁷]angiotensin II; 12, bacitracin; 13, physalaemin.

tocinamide and pressinamide, oxidation of the thiol groups under the same conditions produced considerable amounts of higher polymers, such that the yield of the required monomer represented 29 $\pm$ 5% (4) of the total peptide material present. For the dodecapeptides Pro-Arg-Val-[Arg⁸]vasopressin and [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂, only monomer and dimer species were formed, the respective monomers being obtained in 80 $\pm$ 5% (2) and 83 $\pm$ 3% (2) yield (Fig. 1).

There is an approximately linear relationship between elution volume and log(molecular weight) for the neurohypophyseal peptides and their polymers on the Sephadex G-15 column (Fig. 2). This correspondence occurs largely because these peptides belong to a homologous family of cyclic peptides. Open-chain peptides ([Leu⁵]enkephalin, [Sar¹,Sar⁷]angiotensin II and physalaemin) lie on a separate calibration curve, whereas bacitracin, a peptide containing a cyclic-hexapeptide moiety (Bodansky & Perlman, 1964), conforms to the neurohypophyseal-

peptide calibration curve (Fig. 2). These findings suggest that, when dealing with small peptides, it would be more accurate to relate elution volume to $\log(\text{molecular diameter})$ (i.e. the length of the longest axis of the peptide) rather than molecular weight.

Discussion

The synthesis of neurohypophysial hormones usually requires the oxidative formation of the disulphide bridge as a final chemical step. This reaction can theoretically produce not only the required monomer (intramolecular disulphide) but also dimers and higher polymers arising from the formation of intermolecular disulphides. To obtain a pure product the unwanted polymers must be removed, and this has traditionally been carried out by countercurrent distribution (Schroder & Lubke, 1966), gel-exclusion chromatography (Manning *et al.*, 1968), partition chromatography (Yamashiro, 1964) or ion-exchange chromatography (Kasafirek *et al.*, 1966). Although chromatographic data have often not been reported in detail, it is clear from the available evidence that the oxidation step in the synthesis of neurohypophysial hormones produces only monomer and dimer species (Manning *et al.*, 1968). Furthermore, analogues of neurohypophysial hormones in which single- or multiple-site amino acid replacements have been made produce essentially the same situation, although small amounts of higher polymers have been observed with some analogues (M. Manning, personal communication).

In the present study, the products obtained after controlled oxidation of the thiol groups of the hexapeptide 'ring' derivatives of the neurohypophysial hormones were found to differ considerably from those of the hormones themselves. Thus both pressinamide and tocinaamide produced substantial quantities of trimers, tetramers, pentamers etc., which were not observed with the nonapeptide hormones (Fig. 1). Since there is no precedent for the existence of polymeric species held together by non-covalent binding forces under the extreme denaturing conditions used in the Sephadex G-15 chromatography step, i.e. 50% acetic acid, these products arise from the formation of intermolecular disulphide bonds. [This conclusion has been substantiated by demonstrating that (i) similar elution profiles are obtained when these peptides are chromatographed on a Sephadex G-15 column equilibrated with 8M-urea, (ii) reduced [Arg⁸]vasopressin is eluted from Sephadex G-15 in 50% acetic acid essentially as a single peak in a position immediately preceding that of the cyclic monomer, and (iii) reduction and reoxidation of [Arg⁸]vasopressin dimer material results in the formation of monomer and dimer.] This indicates that the presence of the acyclic tripeptide in the nona-

peptide hormones induces a conformation in the preceding hexapeptide portion of the molecule that favours the formation of the intramolecular disulphide bond, whereas in the absence of the tripeptide tail oxidation of the thiol groups produces a more random interaction leading to a greater percentage of intermolecular disulphide bonds. It is also possible that the acyclic tripeptide may impose a steric restraint on intermolecular thiol interaction, although this seems unlikely if [Arg⁸]vasopressin assumes a conformation in solution that is similar to that proposed for oxytocin in dimethyl sulphoxide (Urry & Walter, 1971).

A possible factor contributing to a precyclic conformational state that favours the formation of an intramolecular disulphide bond is the presence of the Cys⁶-Pro⁷ bond, which, owing to the rigid structure of the cyclic proline moiety, would create a bend in this region of the molecule. However, for the synthesis of an analogue of [Lys⁸]vasopressin in which Pro⁷ was replaced by Val (Boissonas *et al.*, 1963), and which was obtained from the fully protected peptide precursor by procedures that were essentially the same as those described here, no polymer formation was reported and the required compound was obtained from the fully protected peptide in 48% yield. This seems to indicate that Pro⁷ is not the sole determining factor in orienting the pre-cyclic conformation of neurohypophysial hormones. Other factors arising from the interactions of the acyclic tripeptide with the ring portion of the molecule, e.g. the proposed hydrogen-bonding between the side-chain -C=O of Asn⁵ and the -NH of Leu⁸ in oxytocin (Urry & Walter, 1971), may also contribute to the pre-cyclic conformational state.

These findings provide independent evidence in support of the suggestion made by Walter (1972), based on n.m.r.-spectroscopy studies on oxytocin and [Lys⁸]vasopressin, that the tripeptide seems to exert some influence on the conformation of the ring of neurohypophysial hormones. In retrospect, these studies show that before disulphide formation the acyclic tripeptide may orientate the conformation of the preceding hexapeptide in such a way as to favour the intramolecular disulphide linkage, but do not show that the ring, once formed, remains under the influence of the tripeptide. However, a continued interaction in the completed cyclic structure would be strongly implicated.

It is perhaps not surprising that the presence of the complete nonapeptide structure of the neurohypophysial hormones is required to give rise to a conformation in solution that brings the thiol groups of the reduced peptide into close proximity with one another. A similar situation has been proposed for the hypophysiotropic principle somatostatin, which contains a disulphide bond maintaining a cyclic dodecapeptide structure. On the basis of the

observation that [Cys(Me)³] somatostatin, an analogue unable to form the disulphide bond, has a potency of 4% of that of the native hormone, and full intrinsic activity, it has been suggested that intramolecular associations other than the disulphide bond maintain the molecule in a conformation that can be recognized by somatostatin receptors (Vale *et al.*, 1976).

Extension of the peptide chain of [Arg⁸]vasopressin from the *N*-terminus, as in Pro-Arg-Val-[Arg⁸]vasopressin, results in a molecule that, in the reduced state, forms dimerized material less readily than [Arg⁸]vasopressin (Fig. 1). Similarly, elongation of the peptide chain from the *C*-terminus, as in [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂, confers favourable intramolecular-disulphide-bond-forming properties to the open-chain molecule (Fig. 1). These observations result from the imposition of steric and/or conformational restraints on the molecule due to the increased length of the peptide backbone. This phenomenon could be influenced by the nature of the 'extra' amino acids, although several analogues of the neurohypophysial hormones in which the peptide chain was extended from the *N*-terminus apparently gave low amounts of dimers under similar conditions of synthesis (this deduction is inferred from Fig. 1 of the paper of Kasafirek *et al.*, 1966).

It is not possible to predict how the attachment of three extra amino acids, at either the *N*- or the *C*-terminus of [Arg⁸]vasopressin, might influence the conformation of the reduced peptide. Possibly, the conformation may be altered in such a way that the intramolecular thiol groups are brought into an environment that favours their combination, e.g. by being in closer proximity. Alternatively, the extended chain may fold in such a way that an unfavourable interaction of intermolecular thiol groups results because of steric-hindrance effects. Either or both of these possibilities could account for the observed decrease in dimer formation of the synthetic prohormones compared with the native hormone. Radioimmunoassay evidence has been obtained that militates against steric-hindrance effects in that both Pro-Arg-Val-[Arg⁸]vasopressin and [Arg⁸]vasopressinoyl-Ala-Met-Ala-NH₂ exhibit a considerable ability to bind (10 and 5% respectively compared with [Arg⁸]vasopressin) to an antiserum that has previously been shown to be highly specific towards all features of the [Arg⁸]vasopressin molecule (Moore *et al.*, 1977). This would indicate that, for both synthetic prohormones, the extra amino acids fold away from the cyclic-hexapeptide 'face' of the molecule and do not effectively shield any primary antigenic site in the [Arg⁸]vasopressin portion of the molecule from interaction with the antibodies of the antiserum. In particular, the side chain of Arg⁸ would

have to occupy a three-dimensional position similar to that in [Arg⁸]vasopressin in order to account for the observed binding to the antiserum. Thus, on the basis of the proposed conformation of neurohypophysial hormones (Urry & Walter, 1971) and assuming some similarity in conformation between reduced and oxidized forms of the peptides, steric-hindrance effects should not have a major influence in thiol-group interactions.

The choice of amino acids added to the *N*- and *C*-termini respectively of [Arg⁸]vasopressin was dictated by the amino acid sequence of bovine neurophysin II (Schlesinger *et al.*, 1975; Wu & Crumm, 1976). In ox, neurophysin II is the specific carrier protein of [Arg⁸]vasopressin (Dean *et al.*, 1968) and may at some stage in the biosynthesis of [Arg⁸]vasopressin form part of a putative prohormone (Hope & Pickup, 1974). These experiments were carried out in an attempt to obtain evidence that might differentiate between two possibilities concerning the covalent attachment of [Arg⁸]vasopressin in its putative prohormone: via either (i) the *N*-terminus or (ii) the *C*-terminus of [Arg⁸]vasopressin. In retrospect, the evidence favours the alternative possibility that the neurohypophysial hormone may exist in a more central location in the sequence of a precursor molecule, i.e. covalently attached via both the *N*- and the *C*-termini. However, the formation of the disulphide bond was carried out under non-physiological conditions, and interpretations should be treated with due caution.

Further experimentation on disulphide stability with extended-chain analogues of neurohypophysial hormones may give further insight into the resolution of the prohormone-hormone relationship. Indeed, until better techniques are available for the isolation of hormone precursors, comparisons of the structural and conformational stabilities of synthetic prohormones with their respective hormones may, in general, provide an alternative approach to this problem.

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