Vasopressin Analog with Extraordinarily High Antidiuretic Potency: A Study of Conformation and Activity

Abstract. *Application of information derived from a three-dimensional model of vasopressin bound to its antidiuretic receptor has resulted in the design and synthesis of a potent analog, [1-deamino,2-phenylalanine,7-(3,4-dehydroproline)]-arginine vasopressin; this analog has a specific antidiuretic activity of 13,000 ± 1,250 units per milligram; noteworthy at these doses is the absence of any detectable pressor activity. Three modifications based on conformational considerations were introduced into the vasopressin molecule in preparing the analog: (i) to enhance binding, a double bond was introduced into the side chain of an amino acid residue occupying a corner position of a β turn in the vasopressin conformation, (ii) the hydroxyl moiety was deleted from Tyr², and (iii) to tighten the backbone structure and to enhance the enzymatic resistance of the analog, the NH_2-terminal amino group was deleted.*

The mammalian antidiuretic hormones, arginine vasopressin (AVP) or lysine vasopressin (LVP), are the major regulatory factors determining the amount of water excreted by the kidney. In addition to their antidiuretic activity, the naturally occurring peptides (*1*)

H-Cys¹-Tyr²-Phe³-Gln⁴-Asn⁵-Cys⁶-
Pro⁷-Arg⁸-Gly⁹-NH_2
(Lys⁸)

exhibit potent vasoconstrictor activities that can give rise to undesirable side effects during therapeutic applications of the hormones (*2, 3*). With the use of a working model of the "biologically active" conformation of the vasopressins for antidiuretic activity (*4*), we now report the rationale for the design, the synthesis, and some of the biological properties of a vasopressin analog [1-deamino,2-phenylalanine,7-(3,4-dehydroproline)]arginine vasopressin (DPD-AVP). The antidiuretic potency of DPD-AVP is dramatically enhanced as compared to that of AVP (*5*) and, at the same time, its pressor activity is effectively reduced.

The biologically active model of the vasopressins is largely based on the preferred conformations of LVP (*6*) (Fig. 1) and of AVP (*7*) both determined in dimethylsulfoxide, but the model includes the stacking interaction of the aromatic side chains of the Tyr-Phe sequence observed for vasopressin in aqueous medium (*8*) (Fig. 1). One surface of the cross β structure of the 20-membered covalent ring of the hormone model is featureless and hydrophobic. Amino acid side chains of residues along the rim of this surface of vasopressin extending from residue 3 by the hydrocarbon portion of Gln⁴ and Pro⁷ to the hydrocarbon portion of the side chain of residue 8 are proposed for initial recognition by the antidiuretic receptor. The other surface of the molecule is overlayed by the glutamine and asparagine side chains as well as the COOH-terminal tripeptide; the latter bends in the direction of the primary amino group of Cys¹. The lysine (or arginine) side chain of LVP (or AVP) is shown in Fig. 1 in an extended conformation. However, in the biologically active model, it is approximately parallel to the peptide backbone in the region occupied by the asparagine residue, but above the β-pleated sheet of the 20-membered ring of vasopressin. It has been proposed that the carboxamide group of Asn⁵ and the basic moiety of residue 8 ["active elements" (*9*)] in this hydrophilic cluster act in concert on the receptor to initiate the sequence of events that lead to the antidiuretic response (*10*). One important feature that differentiates this model of vasopressin from that of oxytocin when bound to the uterotonic receptor (*9*), is the absence of the tyrosine side chain with its hydroxyl group from the hydrophilic cluster.

With the development of a working model of the "antidiuretically" active conformation of vasopressin, the systematic design of vasopressin analogs now rests on a rational foundation. As stated earlier in more general terms (*11*), analogs can be endowed with enhancement or diminished antidiuretic activity, as compared to the natural principle; or they may even be designed to be competitive inhibitors.

Specifically, in vasopressin the side chains of residues located in the corner positions in the two β turns of neurohypophyseal hormone conformations (positions 3, 4, 7, and 8) are most exposed. These side chains, which are most readily available for intermolecular interactions, contain the "binding elements" (*9*) for hormone-receptor recognition and binding. It has proved possible to selectively enhance a particular biological activity by introducing modifications in the side chains of these very residues and, at the same time, to diminish or even abolish other activities. The important contributions to biological activity of the side chains in the corner positions 3 and 8 are well documented and the role of position 4 is emerging as a result of work from several laboratories (*4, 9*). In an effort to test the vasopressin model, we have subjected the remaining corner position, Pro⁷, which has been least investigated, to structural change designed to enhance binding to the antidiuretic receptor. We believe that one way to enhance antidiuretic activity is to increase, by synthetic modification, the capacity of binding elements (*9*) already present in the hormone. Therefore, we have chosen to substitute the proline residue in position 7 of AVP by 3,4-dehydroproline, which has a double bond, so that such deformable electron clouds may enhance receptor binding of the resultant analog, provided that the steric fit at the receptor is correct (*12*).

Another way to enhance "productive interactions" between the analog and the antidiuretic receptor appears to arise from the flexible nature of peptides. Modifications of the hormone designed to increase the time in which the resultant analog is in the "biologically active" conformation should enhance its biological activity. In our study the primary amino group of AVP has been replaced by a hydrogen atom; the expected conformational effects (*4, 13*) as well as consequences for the enzymatic breakdown of the resultant vasopressin analog have been discussed (*2, 14*). It also follows from the model that modifications that would remove the hydroxy moiety of the Tyr² side chain away from the hydrophilic cluster, either by replacement or sterically induced reorientation of the entire side chain, should also enhance antidiuretic activity (*4*). In order to test this aspect of the model we substituted phenylalanine for tyrosine in position 2 in combination with the other modifications enumerated above.

With these considerations in mind, we synthesized DPD-AVP by a Merrifield method of solid phase synthesis (*15*) using a scheme of deprotection, neutralization, and coupling (*16*). Cyclization of the deprotected dithiol intermediate was accomplished by oxidative disulfide bond formation with ICH_2CH_2I (*17*). The analog was purified by gel filtration on Sephadex G-15 (fine) and G-25 (block polymerizate, 200 to 270 mesh), and partition chromatography (*18*). The purity and identity of the final product was verified by thin-layer chromatography in a mixture of 1-butanol, acetic acid, and

0036-8075/78/0120-0297$00.50/0

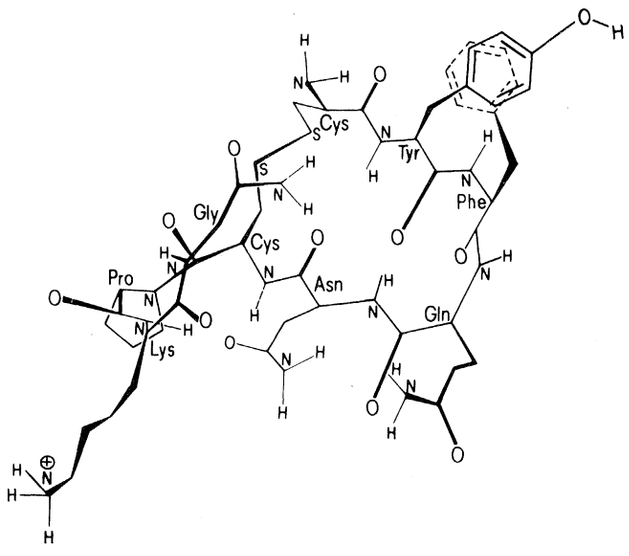

Fig. 1. The preferred conformation of lysine vasopressin determined in solution (*6*).

water (4 : 1 : 1) and 1-butanol, pyridine, acetic acid, and water (15 : 10 : 3 : 6), amino acid analysis, and elemental analysis. The antidiuretic potencies were determined with anesthetized male Sprague-Dawley rats according to the method of Jeffers *et al.* (*19*) as modified by Sawyer (*20*). The pressor assay was performed with anesthetized male rats as described in the U.S. Pharmacopeia (*21*). Either the four-point assay design of Schild (*22*) or matches were used as compared to U.S.P. posterior pituitary reference standard.

The antidiuretic activity of DPD-AVP was measured by the intensity of the antidiuretic response, on the basis of the maximal depression of urine flow after injections; this method yields values least subject to errors introduced by differing durations of antidiuretic responses (*23*). The response pattern to equipotent doses of DPD-AVP and AVP were nearly identical. In addition, the specific antidiuretic activity of DPD-AVP determined in rats of the Brattleboro strain, homozygous for diabetes insipidus, were the same within experimental variability as those obtained with Sprague-Dawley rats. This result indicates that the magnitude of the antidiuretic response was due to a direct action of the analog on the kidney and was not augmented by the release of endogenous vasopressin. No autopotentiation or autoattenuation was observed when a series of five equal doses of $1.5 \times 10^{-12} M$ DPD-AVP were administered, preceded and followed by 21 μunit doses of standard (equivalent to $4 \times 10^{-11} M$ AVP at 500 unit/mg). The specific antidiuretic activity found for DPD-AVP was 13,000 ± 1,250 unit/mg (mean ± standard error of the mean) as compared to the 503 ± 53 units per milligram of AVP (Fig. 2).

The antidiuretic and pressor potencies of DPD-AVP are highly dissociated with the pressor activity being, for all practical purposes, insignificant. In order to obtain even a minimal response in the pressor assay with DPD-AVP it had to be administered at a dose 30,000 times larger than that used to obtain an antidiuretic response. At this high dose the analog produced tachyphylaxis which lasted the duration of the assay (about 4 hours) and caused the attenuation of further pressor responses to either standard or the analog.

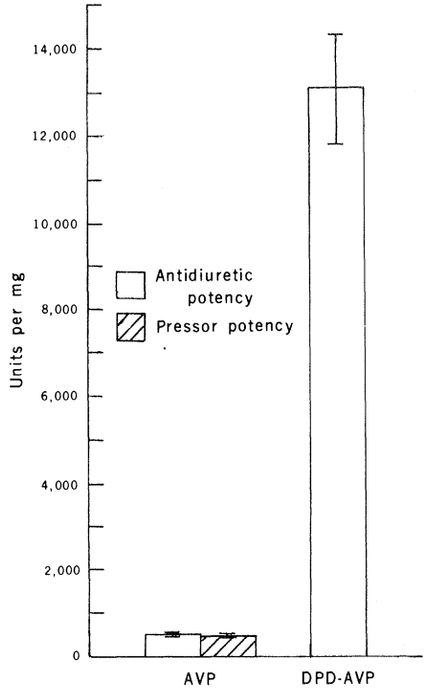

Fig. 2. Comparison of the "vasopressin-like" activities of the antidiuretic hormone, arginine vasopressin (AVP), and its [1-deamino,2-phenylalanine,7-(3,4-dehydroproline)]arginine vasopressin analog (DPD-AVP). Values are given as mean ± standard error of the mean (corrected for the anhydrous peptides). The AVP potencies are those of Meienhofer *et al.* (*5*).

The development of relationships between conformation and activity and their application to the design of analogs on a predictable basis goes beyond the preparation of vasopressin analogs with exceptionally high potencies or specificity for a given activity (or both). This approach is also relevant to the synthesis of analogs of other peptide hormones and should dramatically reduce the enormous expenditure of effort required for the synthesis of a large number of analogs required for the more empirical structure-activity approach to analog design.

CLARK W. SMITH
RODERICH WALTER
Department of Physiology and
Biophysics, University of Illinois
Medical Center, Chicago 60612

References and Notes

1. Abbreviations follow the IUPAC-IUB Tentative Rules on Biochemical Nomenclature, *J. Biol. Chem.* **247**, 577 (1972). Optically active amino acids are of the L configuration.
2. A. S. Aronson, K.-E. Anderson, C. G. Bergstrand, J. L. Mulder, *Acta Pediat. Scand.* **62**, 133 (1973).
3. J. P. Rado and E. Juhos, *J. Clin. Pharmacol.* **16**, 333 (1976).
4. R. Walter, C. W. Smith, P. K. Metha, S. Boonjarern, J. A. L. Arruda, N. A. Kurtzman, in *Disturbances in Body Fluid Osmolality*, T. E. Andreoli, J. Grantham, F. C. Rector, Jr., Eds. (American Physiological Society, Bethesda, Md., 1977), p. 1.
5. J. Meienhofer, A. Trzeciak, R. T. Havran, R. Walter, *J. Am. Chem. Soc.* **92**, 7199 (1970).
6. R. Walter, J. D. Glickson, I. L. Schwartz, R. T. Havran, J. Meienhofer, D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1920 (1972).
7. R. Walter, A. Ballardin, I. L. Schwartz, W. A. Gibbons, H. R. Wyssbrod, *ibid.* **71**, 4528 (1974).
8. R. Deslauriers and I. C. P. Smith, *Biochem. Biophys. Res. Commun.* **40**, 179 (1970); P. H. Von Dreele, A. I. Brewster, J. Dadok, H. A. Scheraga, F. A. Bovey, M. F. Ferger, V. du Vigneaud, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2169 (1972).
9. R. Walter, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1872 (1977).
10. For several recent reviews see: R. M. Hays and S. D. Levine, *Kidney Int.* **6**, 307 (1974); S. Jard and J. Bockaert, *Physiol. Rev.* **55**, 489 (1975); T. P. Dousa and H. Valtin, *Kidney Int.* **10**, 46 (1976); in *Disturbances in Body Fluid Osmolality*, T. E. Andreoli, J. Grantham, F. C. Rector, Jr., Eds. (American Physiological Society, Bethesda, Md., 1977).
11. R. Walter, I. L. Schwartz, J. H. Darnell, D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1355 (1971).
12. R. Walter, in *Proceeding of Fifth International Congress of Endocrinology, Hamburg 1976*, V. H. T. James, Ed. (Excerpta Medica, Amsterdam, 1976), vol. 2, p. 18; S. Moore, A. M. Felix, J. Meienhofer, C. W. Smith, R. Walter, *J. Med. Chem.* **20**, 495 (1977).
13. J. D. Glickson, D. W. Urry, R. T. Havran, R. W. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2136 (1972).
14. J. Rudinger, V. Pliška, I. Krejčí, *Recent Progr. Hormone Res.* **28**, 131 (1972); I. Vavra, A. Machová, V. Holeček, J. H. Cort, M. Zaoral, F. Šorm, *Lancet* **1968-II**, 948 (1968).
15. R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963).
16. C. W. Smith and M. F. Ferger, *J. Med. Chem.* **19**, 250 (1976).
17. F. Weygand and G. Zumach, *Z. Naturforsch. B* **17**, 807 (1962).
18. D. Yamashiro, *Nature (London)* **201**, 76 (1964).
19. W. A. Jeffers, M. M. Livezey, J. H. Austin, *Proc. Soc. Exp. Biol. Med.* **50**, 184 (1942).

20. W. H. Sawyer, *Endocrinology* **63**, 694 (1958).
21. *Pharmacopeia of the United States of America* (Mack, Easton, Pa., rev. 18, 1970), p. 771.
22. H. O. Schild, *J. Physiol. (London)* **101**, 115 (1942).
23. W. H. Sawyer, M. Acosta, L. Balaspiri, J. Judd, M. Manning, *Endocrinology* **94**, 1106 (1974).
24. We thank C. R. Botos for assistance in the synthesis and for performing the amino acid analysis and G. Skala and S. Chan for performing the bioassays. The *t*-butyloxycarbonyl-3,4-dehydroproline was the gift of J. Meienhofer, Hoffmann–La Roche, Nutley, N.J. Supported in part by PHS grant AM-18399 and a Pharmaceutical Manufacturer's Foundation Research Starter Grant.

19 July 1977; revised 29 August 1977

Perfusion Preservation of Hearts for 6 to 9 Days at Room Temperature

Abstract. *The combination of a defined medium with single-pass perfusion has made possible long-term maintenance of beating rat hearts at 22°C in vitro. The 6- to 9-day survival period appears to be the longest so far reported for hearts. This method provides a stable system which should be useful for investigating the role of single factors in myocardial preservation and evaluating the effects of exposure to pharmacological and toxicological agents.*

Techniques for maintaining single cells or small tissue sections in culture for extensive periods have been known for several decades. However, the maintenance in vitro of perfused parenchymatous organs, such as the heart and kidney, has been severely limited. The development of transplantation medicine has focused renewed attention on perfusion techniques for the maintenance of organs in vitro, and it is essential to preserve cadaver organs for sufficiently long periods to permit accurate cross matching and transportation [for reviews, see (*1*–*5*)]. The diversity of the approaches to preservation has precluded a general consensus on the factors that limit survival (*5*, *6*). Attempts have included a broad temperature range, various physical techniques, and different perfusion fluids. Among these, hypothermic perfusion with plasma-based fluids and cold storage after flushing with an "intracellular" (high potassium and magnesium) electrolyte solution appears clinically useful. Most current preservation techniques utilize deep hypothermia (< 10°C). The experimental preservation of hearts for 4 days after continuous perfusion with a modified Ringer's solution (*7*) and the preservation of kidneys for 7 days after perfusion with a modified plasma preparation (*8*) have been reported, but the results with kidneys were not consistently achieved, possibly because of variations in the plasma-based media. Methods involving recirculating perfusion and simple cold storage are invariably complicated by multiple, unquantifiable variables arising from tissue metabolism and cellular solute exchange even in deep hypothermia. No system has been developed in which single variables can be evaluated.

Perfusion methods are also used to maintain organs in vitro for basic physiological, biochemical, and pharmacological studies at normothermia or moderate hypothermia. Studies at normothermia or moderate hypothermia have been limited because signs of deterioration appear within 3 to 5 hours, depending whether a working or Langendorff preparation is used (*9*). Furthermore, no wholly satisfactory perfusate has been found for preserving the myocardium during open-heart surgery. There is no evidence that it is possible to maintain functioning organs in vitro for prolonged periods under normothermic or moderately hypothermic conditions.

We report here the maintenance of beating rat hearts at 22°C for 6 to 9 days (Table 1), using a defined medium (Table 2) in a single-pass perfusion system. To our knowledge, survival of functioning hearts for this length of time has not been previously reported. By using the single-pass method, the composition of the medium can be nearly completely controlled throughout the preservation period. The medium is patterned for the most part after normal plasma so that it would potentially be able to meet metabolic needs over a broad temperature range. It is not a minimum essential medium, as some components were included simply because they are present in plasma, as long as no harmful effect appeared when they were added to a modified Ringer's solution. Other components (such as putrescine) were used because they are essential for tissue culture fluids (*10*) or (such as adenosine) for physiological regulatory functions (*11*).

Table 1. Survival times of hearts perfused with synthetic plasma-simulating solution at 22°C. The criteria for survival are described in the text. All gas mixtures included 5 percent CO_2 to maintain the *p*H at 7.4. The numbers of hearts are given in parentheses.

Gas mixture (% O_2, % N_2)	Survival times (days)
0.0, 95.0	5 (2), 6 (1)
47.5, 47.5	6 (6), 7 (1)
63.3, 31.7	6 (3), 7 (2), 8 (2), 9 (1)

The medium was prepared in 9-liter batches by combining the stock solution and dry chemicals (Table 2) with ultrapure water while bubbling rapidly with a mixture of 95 percent N_2 and 5 percent CO_2. Water was purified in a Millipore Super Q system and, in order to eliminate any organic impurities, repurified in an Ultrascience still (model 102S). Perfusate was sterilized by pressure filtration through Millipore filters (0.22-μm pore size) and stored under the N_2-CO_2 gas mixture for a maximum of 3 days before use. Serum albumin was added to minimize the loss of insulin and glucagon by adsorption during filtration. Care was taken to avoid surface foaming after the protein components were added. The filtration through 0.22-μm filters was repeated before the medium was passed into the perfusion apparatus. The perfusate came in contact only with borosilicate glass, silicone rubber, polypropylene, and cellulose acetate surfaces.

At the start of each experiment hearts were quickly removed from decapitated Wistar rats, cannulated for coronary perfusion through the aorta (Langendorff technique), and mounted in controlled-temperature chambers. The ischemic time of hearts before the start of perfusion was limited to 2 to 3 minutes. A perfusion flow of 2 to 4 ml g^{-1} min^{-1} was maintained by having a 60- to 70-cm column of the medium in narrow-bore tubing open to the atmosphere and fed by bilateral cam action of a peristaltic pump (Harvard Apparatus, model 1203). Temperature was held constant by using refrigerated circulating temperature controllers. All procedures were carried out in a sterile area in a laminar-flow hood.

Perfusion with the synthetic medium was continued until the first signs of deterioration, such as arrhythmia and discoloration, appeared. Survival time (Table 1) was counted as the time from the start of perfusion to the time at which readily visible achromatic areas appeared on the surface of the myocardium, usually on the right ventricle near the insertion point in the pulmonary artery. Most experiments were terminated at this time. Discoloration was usually accompanied by arrhythmia. If perfusion was continued, the achromatic area spread gradually over the total myocardium. Total discoloration accompanied the eventual cessation of all signs

0036-8075/78/0120-0299$00.50/0