of the sand and this is a very thin channel—it did not cut deeply enough into lower channels to rework many teeth. The surface of the teeth shows no abrasion; the very tiny and sharp denticles of the theropod teeth show only occlusal attritional wear. Finally, were they to be reworked, one would also expect to find Cretaceous species of *Baiconodon* and *Oxyprimus* that are far more numerous than dinosaur teeth in the only channels from which the dinosaur teeth could possibly be reworked. The most reasonable conclusion is that these are indeed early Paleocene dinosaurs.

It thus appears on present data that KS and BCA are definitely Cretaceous, BCW-SMP may be Cretaceous or Paleocene, and FR is definitely Paleocene. Of the 30 dinosaur genera present in the area 8 million years before the end of the Cretaceous, a maximum of 12 were present just before the K/T boundary event, and between 7 and 11 genera survived into the Paleocene. Depending on the precise level of the K/T boundary with respect to these faunas, all that can be ascribed to the asteroid impact is the extinction of from one to three genera. The remaining genera either became extinct significantly earlier or later.

If dinosaur extinction is not solely due to an asteroid impact (we are convinced it did not help them), what are the other factors? We continue (*3*) to suggest a concurrence of several factors: global temperature lowering over the last 15 million years of the Cretaceous, lowering of sea level during the late Maastrichtian and consequent increase in seasonality, major deterioration of the flora as a result of these two causes, and diffuse competition from new mammalian herbivores most likely introduced to this continent from Asia.

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Crystal Structure Analysis of Deamino-Oxytocin: Conformational Flexibility and Receptor Binding

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Two crystal structures of deamino-oxytocin have been determined at better than 1.1 Å resolution from isomorphous replacement and anomalous scattering x-ray measurements. In each of two crystal forms there are two closely related conformers with disulfide bridges of different chirality, which may be important in receptor recognition and activation.

THE NEUROHYPOPHYSEAL HORMONE oxytocin (Fig. 1) elicits smooth muscle contraction, causing milk ejection and uterine contractions in mammals. The synthesis of oxytocin (*1*) led to a systematic study of the relation of primary structure to biological activity, and more recent studies have highlighted the additive nature of modifications that favor certain pharmacological effects. This has resulted in the design of highly selective, long-acting superagonists and antagonists of therapeutic potential (*2, 3*). One synthetic analogue of particular interest is deamino-oxytocin (1-mercaptopropionateoxytocin), which was the first to be found more active in most tests than the natural hormone (*4*).

Spectroscopic studies such as nuclear magnetic resonance (NMR), laser Raman, and circular dichroism have shown that oxytocin can exist in several conformations although certain well-defined intramolecular hydrogen bonds characterize most conformers in solution (*5, 6*). In view of this inherent flexibility, it is necessary to examine the conformation and dynamics of the hormone and its analogues not only in aqueous conditions, but also in other environments that are models for the hormone in its complex with the receptor. Although crystals of oxytocin were first reported in 1952 (*7*), the crystal structure has proved elusive. In 1965, crystal data for deamino-oxytocin were reported by Low and Chen (*8*) and an active

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6-selenodeamino-oxytocin analogue was later purified and crystallized (*9*). Little further progress has been reported although other crystals of oxytocin have been described and a structure analysis has been completed for two COOH-terminal peptides (*10, 11*).

Here we present the three-dimensional structures of two crystal forms of deamino-oxytocin and a related crystal form of 6-selenodeamino-oxytocin defined by x-ray analyses at resolutions between 1.09 and 2.1 Å. We compare the crystal structures to conformations proposed from NMR and other spectroscopic studies in dimethylsulfoxide (DMSO) and water. We show that even in the crystals the deamino-oxytocin is flexible and that there are at least two well-defined conformers. We suggest that the partially hydrophobic environment in the crystal may have features in common with the receptor, and that the flexibility seen in the deamino analogues may be required for full agonist rather than antagonist activity.

In view of the lack of success encountered earlier (*8, 9*), we synthesized fresh materials, grew new crystals, and re-collected x-ray data for deamino-oxytocin (wet form, space group $P2_1$), air-dried deamino-oxytocin (space group $C2$) and 6-selenodeamino-oxytocin (space group $C2$). The similarity in cell parameters of the air-dried $C2$ crystals contrasts with a large angle change reported for dried deamino-oxytocin crystals previously. The sulfur positions in the $C2$ crystal form were located with the use of selenium isomorphous difference, selenium anomalous difference, and sulfur anomalous difference measurements. The selenium single isomorphous replacement with anomalous differences (SIRAS) technique alone did not provide an interpretable map; but, when supplemented with higher quality anomalous differences due to sulfur in the native structure, a best-phased Fourier map at 2.1 Å resolution provided a rough model of the structure. Restrained refinement (*12*) with anisotropic thermal ellipsoids for nonhydrogens and isotropic ellipsoids for hydrogens gave R = 10.0 percent at 1.2 Å resolution for the $C2$ crystal form. The resulting parameters were then used to initiate the refinement of the crystal form with space group $P2_1$ and data to 1 Å resolution giving a final residual of 7 percent after several cycles of modeling from difference maps and refinement. The coordinates have been deposited with the Cambridge Data File (Cambridge, England).

The conformations of deamino-oxytocin are shown in Figs. 2 and 3. Each has a type II β turn between Tyr^2 and Asn^5 stabilized by two hydrogen bonds between the amide and carbonyl of both residues and a type III turn between Cys^6 and Gly^9 stabilized by a

Cys^1 Tyr^2 Ile^3 Gln^4 Asn^5 Cys^6 Pro^7 Leu^8 Gly^9 NH

Fig. 1. The amino acid sequence of oxytocin.

hydrogen bond between the Cys^6 carbonyl (CO) and Gly^9 peptide imino (NH). Other small cyclic peptides with bulky corner residues have been described with type II turns (*13*). The main chain torsion angles lead to a ring curvature (see Fig. 3) with the acyclic tail oriented away from the ring with a *trans* Cys^6–Pro^7 peptide. The top surface of the curve is also made up of Tyr^2, Ile^3, Asn^5, the α and β carbon of Gln^4, the ring of Pro^7, and Leu^8. The underside of the curve is devoid of side chains, the terminal amide of Gly^9 and side chain amide of Gln^4 being at the extremities of the underside cleft. In the crystal there are at least two possible conformations for the disulfide bridge region which differ in S1 position. In the $C2$ cell one conformation (Fig. 2) results in a disulfide torsion angle of +76° with right-handed chirality and the other with a torsion angle of −101° and left-handed chirality. The two S1 positions are about 2 Å apart and equally populated. The alternate positions for S1 can be reached with only small differences of other nonhydrogen atoms due to the parallel nature of the Cβ(6)–S6 and $C\alpha_1$–$C\beta_1$ bonds. In the $P2_1$ cell both molecules (Fig. 3) exhibit the disorder at S1 but different chiral forms, similar to those seen in the $C2$ cell, are predominantly populated (70/30) in each molecule. The torsion angles (Cβ(1)–Sγ(1)–Sγ(6)–Cβ(6)) are *+77°*, −87° and *−94°*, +83°, the dominant isomer being in italics. Furthermore, the dominant conformers are associated with distinct differences in the main chain torsion angles, in the Cα–Cβ rotamers of the Ile^3 side chain, and in the side chains of Tyr^2 and Asn^5. Together these differences in structure between the two molecules of the $P2_1$ cell correlate well with those atoms showing high thermal parameters in the $C2$ cell (compare Fig. 3, A and B).

All the hydrogen bonding potential of the molecule is satisfied either by intramolecular interactions or bonding with water and other oxytocin molecules. There are seven water positions in the $C2$ cell, two of which lie close to crystallographic twofold axes and are disordered. Four water molecules are found in a complex cage network around another water close to the twofold axis and form a hydrophilic zone in the crystal near the underside "cleft" with potential hydrogen bonds to Gln^4 CO, Gln^4 NH, Gln^4 Oε1, Gln^4 Nε2, Asn^5 Oδ1, Gly^9 CO, and the COOH-terminal amide NH_2 of several oxytocin molecules. The other two water molecules, one of which is also disordered close to the twofold axis, are in the region of Tyr^2 (with hydrogen bonds to Tyr^2 OH, Asn^5 CO, and Cys^1 CO) and interrupt an otherwise hydrophobic zone in the crystal formed by Tyr^2, Leu^8, Pro^7 and S1 from symmetry related molecules. In the $P2_1$ cell the water distribution is generally the same, but there are differences in location, numbers, and disorder. The water molecules close to the pseudosymmetry axes are no longer disordered. There is one water molecule more in the $P2_1$ cell and this water is in close contact with Asn^5 CO. Its removal may

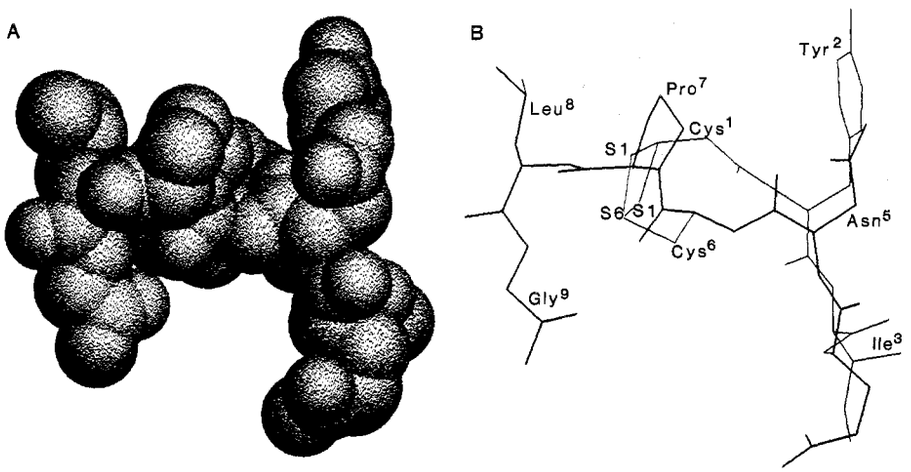

Fig. 2. The space-filling (A) and equivalent stick model (B) for deamino-oxytocin showing the "pleat" of the 20-membered ring and the accentuation of this form by the "tail" peptide and the Gln^4 side chain dispositions. The main chain torsion angles (ϕ, ψ)$^{in\ degrees}$ for one molecule of the $P2_1$ cell are (____, 101)^1, (−126, 164)^2, (−65, 125)^3, (56, 29)^4, (−158, 66)^5, (−128, 98)^6, (−73, −12)^7, (−77, −33)^8, (−176, 168)^9. The three intramolecular hydrogen bonds have O–N distances and N–H–O angles of (i) Tyr^2 CO–Asn^5 NH: 2.93 Å, 156.31°, (ii) Tyr^2 NH–Asn^5 CO: 3.09 Å, 162.62°, and (iii) Cys^6 CO–Gly^9 NH: 3.37 Å, 134.19°.

explain the change in spacegroup on drying.

There are intermolecular hydrogen bonds between Ile[3] NH and Pro[7] CO, Asn[5] Nδ2 and Leu[8] CO, Cys[6] NH and Gln[4] Oε1, Cys[1] CO and Asn[5] Nδ2, Ile[3] CO and the COOH-terminal amide NH. The packing of the molecules around the twofold axis brings residue 1 in each of two molecules close together. If the terminal amino group were in position, then electrostatic repulsion would not favor this packing, and this may explain why oxytocin itself is not crystallized in this form.

The structure of the 6-seleno analogue at 2.1 Å resolution is very similar except in the region of the disulfide bridge. The torsion angles (Cβ(1)–Sγ(1)–Sγ(6)–Cβ(6)) are distorted to +79° and −87° and the relative occupancies are shifted to 62 percent for the left-handed and 38 percent for the right-handed conformer. Again the S1 positions are about 2 Å apart and the disorder cannot be analyzed in detail away from the bridge.

The conformation defined in the crystal structures has features in common with conformations proposed on the basis of spectroscopic studies in DMSO and in water (*14–18*). All have a turn between residues 2 and 5 with a hydrogen bond between Tyr[2] CO and Asn[5] NH. Although the initial definition of the turn in DMSO was not explicit, the bulky corner residues at positions 3 and 4 were considered more consistent with a type I turn which is generally preferred unless Gly or D-Ala are located at residue $i + 2$. However Glickson and co-workers (*5, 19*) suggested that unique conformational constraints due to the 20-membered ring might stabilize a type II turn. An additional hydrogen bond between Asn[5] CO and Tyr[2] NH has been indicated by NMR for deamino-oxytocin in DMSO as observed in the crystal structure.

The chirality of the disulfide bridge in solution has also been the subject of much discussion. A right-handed screw with a dihedral angle close to +90° was proposed on the basis of the sign and rotatory strength of the near ultraviolet circular dichroism of oxytocin in water (*20*) but the validity of this interpretation has been questioned (*5, 7*). Although laser Raman spectroscopy indicates that most molecules in solution have dihedral angles close to ±90°, the preponderance of a particular screw cannot be determined. It is in fact possible that both conformers exist in equilibrium and that the population of each conformer, in solution as in the crystals, depends on the analogue and the environment.

There is evidence for the maintenance in DMSO solution of the type III β turn between residues 6 and 9 with a hydrogen bond between the Cys[6] CO and the Gly[9] peptide NH, and the existence of a *trans* Cys[6]–Pro[7] peptide, both of which are observed in the crystals. However, the tocin ring and acyclic tail were proposed to fold over in DMSO so that a hydrogen bond is formed between the Asn[5] amide carbonyl and the Leu[8] NH. This appears to be replaced by intermolecular hydrogen bonds in the crystal structure which lead to a more open structure. In fact such an open conformation is consistent with the NMR in water (*6*) where there is no evidence for such intramolecular hydrogen bonds. In any case, it is likely that there is conformational flexibility between the tocin ring and the β turn of the acyclic tail. To understand the flexibility of oxytocin further we have carried out a normal mode analysis for deamino-oxytocin and calculated 1000 picoseconds of molecular dynamics simulation in vacuo (*21*). These studies demonstrate considerable flexibility in the oxytocin molecule and the existence of two major conformations involving differences in sulfur positions. Similar work on vasopressin has been presented by Hagler *et al.* (*22*).

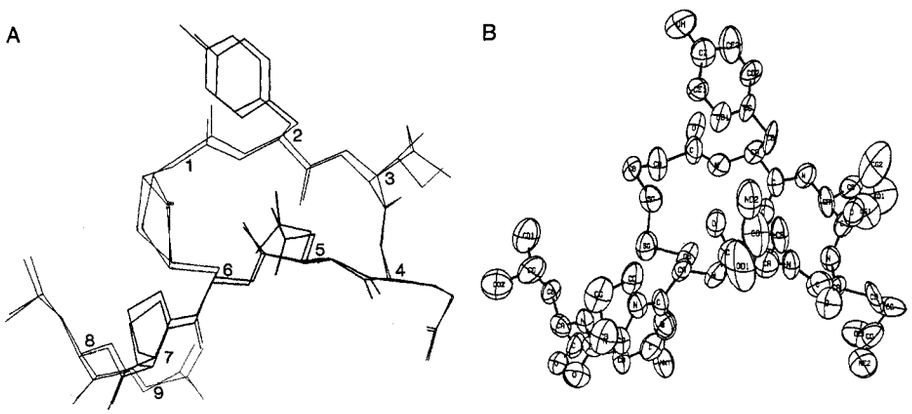

Fig. 3. The two molecules of the $P2_1$ asymmetric unit are shown superimposed (A) to demonstrate the two conformations in the S1 region, the associated Ile[3] rotamers and other conformational differences. These can be compared to the anisotropic thermal ellipsoids for the *C*2 structure of deamino-oxytocin (B). One disulfide chirality only is shown. Locations for the nitrogens (N), carbons (C), and sulfurs (S) are indicated by A, α; B, β; G, γ; D, δ; E, ε; Z, ζ; and so forth.

The conformations in the crystal lattice can provide information about the conformation at the receptor. Although oxytocin in the natural state is usually in an aqueous environment, water will be excluded from much of the surface of the hormone at the receptor. Experimental data from analogues indicate that hydrophobic and hydrogen bonded interactions—possibly through the free main chain CO and NH groups as well as through side chains are likely to dominate the receptor interaction. The molecule probably binds as a neutral species so that the deprotonated amino terminus can form a hydrogen bond to the receptor. This is consistent with the enhanced activity of analogues in which the amino terminus is replaced by an hydroxyl group (*6*). The receptor binding may also require the formation of the two hydrogen bonds between residues 2 and 5 as found in deamino-oxytocin and analogues with glycine at residue 4. All these observations indicate that the conformation of the neutral deamino-oxytocin in an environment which excludes water from much of the hormone—as in the crystal—might reveal important aspects of the conformation at the receptor.

The first and most comprehensive model for receptor binding and biological activation, proposed by Walter and co-workers, was based on the solution conformation in DMSO as defined by NMR together with an assessment of the analogue assay data available for the uterine target (see *23* for a review). Ile[3], Gln[4], Pro[7], and Leu[8] were suggested to constitute the binding message while the proximity of Tyr[2]-OH and Asn[5] carboxamide were important for activation. In this model the 20-membered tocin ring was seen as essentially planar with an almost featureless underside and a topside dominated by Tyr[2] and Asn[5] in a cleft between ring and acyclic tail. Although some features of this model may be correct, the x-ray analysis shows that the ring is likely to be pleated with Tyr[2] and Asn[5] close together on one side and the Gln[4] protruding on the other.

The definition of the two conformers in the crystals allows us to comment on and extend the model of Hruby and co-workers (*6*). This model highlights the importance of flexibility in agonist activity and stresses the use of conformationally restricted antagonists such as 1-L-penicillamine oxytocin to probe binding, activation events, and energy barriers between such states (*6, 24*). Circular dichroism and Raman and NMR spectroscopy (*6, 24*) show that they have much reduced flexibility in the ring moiety, a preferred disulfide torsion angle of between 110° and 115°, and a conformation in which

the Tyr^2 aromatic group cannot be over the tocin ring. We assume that the receptor is also flexible and can adopt two conformations. In the absence of hormone, it adopts a conformation that binds the oxytocin conformers with right-handed disulfide chirality. This inactive state is then stabilized by rigid right-handed disulfide containing analogues such as 1-penicillamine derivatives that act as antagonists (*24*). In contrast, strong agonists stabilize the receptor in a conformation complementary to the left-handed disulfide oxytocin conformer. An efficient transduction of the biological response would then depend on both a low energy barrier between conformers as in deamino-oxytocin and a strong interaction with the receptor in the active form.

Our model predicts that agonist activity will be enhanced either by kinetic effects involving flexibility and interconvertibility of the two conformers or thermodynamic effects that stabilize the left-handed conformer or increase its interactions with the receptor. Modifications at side chains (for example $Phe^2 \rightarrow Tyr^2$ and $Gln^4 \rightarrow Thr^4$) that increase potency (*6, 25*), may often be due to optimization of direct interactions with the receptor—in this case formation of hydrogen bonds. However, substitutions at the same positions may also affect flexibility and have kinetic effects. Thus, replacement of Gln^4 by glycine may result in a decrease of activity due to loss of a direct interaction with the side chain but a compensatory increase of potency due to increased flexibility; there is evidence that the existence of Gly^4 leads to rearrangements in the tocin ring similar to those in deamino-oxytocin.

In summary, x-ray analysis of crystal structures of deamino-oxytocin emphasizes the importance of intramolecular hydrogen bonds which appear to be retained in solution and probably at the receptor. They also highlight the conformational flexibility of this peptide hormone and define conformers that may play a role in receptor binding and biological activation.

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Circulating Atrial Natriuretic Peptides in Conscious Rats: Regulation of Release by Multiple Factors

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Cardiocytes in the atria contain a prohormone that gives rise to atrial natriuretic peptides (ANP's), which have intrinsic hemodynamic regulatory activity. The distribution of ANP's in the brain suggests the involvement of these peptides in central cardiovascular regulation. In conscious rats with chronic indwelling catheters, volume loading with isotonic saline or glucose increased the amount of circulating immunoreactive ANP's by a factor of 4 to 5, as determined by radioimmunoassay. Hyperosmotic challenge with a hypertonic NaCl solution or anesthesia with halothane caused similar increases in plasma ANP's. Results obtained with the denervated-heart preparation indicate that neuronal influences are important in the release of ANP's induced by volume loading. As judged from reversed-phase high-performance liquid chromatography of extracted plasma and radioimmunoassay of collected fractions, the circulating physiologically important ANP's in the conscious rodent appear to be α-rANP(5–28) (atriopeptin III) and either α-rANP(3–28) [ANF(8–33)] or α-rANP(1–28) (ANF).

FOR SEVERAL DECADES, THE MAMMALIAN cardiac atria have been known to have a role in blood volume homeostasis (*1*), and more recently atrial cardiocytes have been shown to contain secretory storage granules that are characteristic of endocrine or peptide secretory cells (*2*). Intravenous infusion of atrial extracts revealed potent natriuretic, diuretic, and vasorelaxant substances (*3*) termed atrial natriuretic factors. More recent investigations have shown that atrial tissue contains a number of biologically active peptides (*4*) collectively termed atrial natriuretic peptides (ANP's). Cardiac ANP's have been isolated, synthesized, and shown to have intrinsic hemodynamic regulatory activity (*5*). The amino acid sequence of the preprohormone in the atria has been deduced from sequence analysis of the DNA in several mammalian species; the rat preprohormone consists of 152 amino acids (*6*). Multiple ANP's (*6*), which constitute the carboxyl terminus of the precursor, have been isolated from rat atria. They vary in length from 21 to 126 amino acids. Each peptide contains a core sequence with a disulfide bridge, which is required for biological activity. Some of the substances isolated from the atria and thought to be ANP's, could be purification artifacts. Therefore, it is not yet certain which of the circulating ANP's obtained from conscious animals are physiologically important.

Although the primary sources of ANP's are the cardiac atria, immunoreactive ANP's

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