The Composition of Crystalline Complexes of Neurophysin-M with [8-Arginine]-Vasopressin and Oxytocin

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Neurophysin-M, a methionine-containing protein that is the major constituent of neurophysin, has been crystallized as complexes with [8-arginine]-vasopressin. Three moles of vasopressin alone or 2 moles of vasopressin together with 1 mole of oxytocin are bound/mole of protein. An amorphous complex of the protein with oxytocin alone contains 2 moles of the hormone/mole of protein. Deamino-[8-arginine]-vasopressin, a highly active basic analogue of vasopressin, is not bound by neurophysin. The primary amino group of both vasopressin and oxytocin is necessary for binding with neurophysin.

Neurophysin, a protein from the pituitary posterior lobe (Acher & Fromageot, 1957) was recently shown to contain several constituents, all of which form complexes with both oxytocin and vasopressin (Hope & Hollenberg, 1966; Hollenberg & Hope, 1967). The major fraction, neurophysin-M, was characterized by the presence of methionine, an amino acid not previously detected (Block & van Dyke, 1950, 1952). Neurophysin-M consists of two constituents which were separable by electrophoresis at pH 8.1 (Ferguson & Wallace, 1961) but which migrate together at pH 8.6 (Poulik, 1957). Attempts to separate these proteins by fractional precipitation in the presence of vasopressin led to the isolation of crystalline material.

The behaviour of oxytocin and of some analogues of the hormones has also been investigated.

The crystalline complexes afford material of defined chemical composition. The binding capacity of neurophysin-M for the polypeptide hormones in the crystals has been measured and the values will be reported and compared with the findings of other workers who have estimated the binding capacity of neurophysin preparations by gel-filtration and dialysis procedures (Ginsburg & Ireland, 1965; Breslow & Abrash, 1966).

A preliminary report of the crystallization of neurophysin-M has already been published (Hollenberg & Hope, 1966).

MATERIALS AND METHODS

Biological materials. The protein-hormone complex was prepared from acetone-dried posterior lobes of bovine pituitary glands (Chauvet, Lenci & Acher, 1960a). Before fractionation, the protein was freed from the polypeptide hormones by gel filtration in 0.1 N-formic acid on Sephadex

G-25 (Frankland, Hollenberg, Hope & Schacter, 1966). Crude neurophysin was separated from other proteins by gel filtration in 0.1 N-formic acid on Sephadex G-75; neurophysin-M was isolated from the crude neurophysin by ion-exchange chromatography on CM-Sephadex C-50 (Hollenberg & Hope, 1967).

[Arginine]-vasopressin was a gift from Dr R. O. Studer (F. Hoffmann-La Roche and Co.). Dr B. Berde (Sandoz Products Ltd.) kindly provided deamino-[8-arginine]-vasopressin. Bradykinin was obtained from Dr H. O. Collier (Parke, Davis and Co. Ltd.). Natural oxytocin and vasopressin were prepared from the protein-hormone complex as described by Frankland *et al.* (1966).

Bioassay procedures. Oxytocic activity was assayed by the method of Holton (1948) with a Mg^{2+} -free solution suggested by Munsick (1960). The rats were injected subcutaneously 17 hr. before bioassay with 40 μ g. of diethylstilboestrol. Pressor activity was assayed by the method of Dekanski (1952), with male albino rats, anaesthetized with urethane and treated with phenoxybenzamine. Biological activities were assayed against the International Standard (Bangham & Mussett, 1958). With the three-point-assay procedure devised by Gaddum (1959), each solution of a crystalline complex was assayed 12 times; other solutions were assayed six times. The results given are the average of all assays $\pm$ S.D. Bradykinin and histamine were assayed on an isolated strip of guinea-pig intestine suspended in Krebs solution.

Equilibrium dialysis. The ability of crude neurophysin to bind bradykinin, histamine and deamino-[8-arginine]-vasopressin, as well as vasopressin and oxytocin, was assessed by thin-film dialysis. The 'alternate cell' described by Craig & Konigsberg (1961) was used. A solution (0.5 ml.) containing approx. 10 mg. of protein was placed on the inside of the 18/32 Visking membrane, and dialysed against sodium acetate buffer, pH 5.6 and 10.1. The ultraviolet absorption of the diffusate was measured at 280 and 260 $m\mu$, to ensure that no protein had escaped. A solution (7.5 ml.) containing one of the test compounds mentioned above in the acetate buffer, was then placed in the outer compartment of the dialysis cell. The system was allowed to

equilibrate at 4° for 17 hr.; the fluid volumes and the concentrations of hormone in each compartment were then measured by bioassay.

Crystallization of protein-hormone complexes. Crystalline complexes were prepared in 0.5M-sodium acetate buffer, pH 3.9, at 4°. The protein concentration was approx. 2mg./ml. and the concentration of peptide material was 1mg./ml. In all experiments, the total volume was 5.0ml. Crystallization was achieved by the addition of small portions (0.1 ml.) of NaCl solution, saturated at 4°. The solution remained clear after the first addition of NaCl, and dust particles were removed at this point by centrifugation. Upon adding the second portion of saline, an amorphous precipitate formed. In the presence of vasopressin, fine crystalline material could be observed under the microscope within 2 days, and after 7 days all of the amorphous material had become crystalline. The final chloride concentration, measured by electrometric titration, was 0.2M.

Separation and analysis of crystals. Crystals in 0.5ml. of a suspension were collected by centrifuging (10000g_{av.} min. at 0°) in a small glass tube (1.5 cm. × 8 cm.). The supernatant was removed and the sediment was resuspended in 0.75ml. of NaCl solution, saturated at 4°. Crystals were sedimented by centrifuging, as above, and the washing procedure was repeated three more times. Microscopic examination showed that washing the crystals did not affect their appearance. The final sediment was dissolved in 5ml. of 0.067M-sodium phosphate buffer, pH 7.4. The protein content and the biological activity of this solution were measured.

Estimation of protein. The concentration of protein was measured by the method of Waddell (1956). A calibration curve was prepared from the absorption at 215 and 225 mμ of pure neurophysin-M of known moisture content. The plot of $(E_{215\text{m}\mu}^{1\text{cm.}} - E_{225\text{m}\mu}^{1\text{cm.}})$ versus protein concentration in μg./ml. was linear in the range 0–100 μg. of protein/ml. and the value of the slope was 122. Within this concentration range $E_{1\text{cm.}}$ at 215 mμ varied from 0 to 1.020 at 70.1 μg. of protein/ml.; that at 225 mμ varied from 0 to 0.446. The concentration of protein was thus given, in μg./ml., by: $(E_{215\text{m}\mu}^{1\text{cm.}} - E_{225\text{m}\mu}^{1\text{cm.}}) \times 122$.

Zone electrophoresis of protein. Protein solutions were submitted to zone electrophoresis in starch gels by using the discontinuous buffer systems suggested by Poulik (1957) and by Ferguson & Wallace (1961). Samples were dissolved in the same buffer as that used to prepare the starch gels. Protein was detected by staining with Ninrosine (0.05%, w/v).

RESULTS

Neurophysin-M, prepared by the method of Hollenberg & Hope (1967), was homogeneous, both in the ultracentrifuge and on electrophoresis in starch gels using a buffer at pH 8.6 (Poulik, 1957). However, electrophoresis at pH 8.1 (Ferguson & Wallace, 1961) revealed the presence of two constituents in approximately equal quantities. Both components had been eluted together from a CM-Sephadex column. This behaviour indicated that they are similar in size and charge. It was not known whether both were hormone-binding pro-

teins, and during attempts to fractionate the mixture by differential salt precipitation in the presence of vasopressin, crystalline material was obtained.

Crystals containing neurophysin-M and vasopressin

The crystals disintegrated on drying, but were stable indefinitely at room temperature when a suspension (20 μl.) was placed on a microscope slide, covered with a cover slip and the edges of the cover slip were sealed with silicone grease to prevent evaporation. Examination under the microscope (Fig. 1) showed that the size of the crystals varied; the maximum length observed was approx. 100 μ.

The composition of the crystals was established by electrophoretic analysis and bioassay. A suspension of crystals (0.5ml.) was centrifuged and the sediment was washed with saturated sodium chloride as described previously; 0.12mg. of protein was found in the sediment.

Fig. 1. Crystalline neurophysin-M-vasopressin complex. A suspension of crystals (20 μl.) was pipetted on to a microscope slide. The crystals were protected from desiccation by sealing the edges of a cover slip with silicone grease. Magnification is indicated by the 50 μ scale.

Electrophoretic analysis. The electrophoretic analysis of the crystalline complex compared with that of neurophysin-M in the buffer system of Ferguson & Wallace (1961) is shown in Fig. 2. It can be seen that the crystals contain the same protein constituents as those present in neurophysin-M.

Protein analysis and bioassay. Measurements of the protein concentration were made on solutions of crystals in 0.067M-phosphate buffer, pH 7.4. These had been diluted to an extinction 0.2–0.6 at 215mμ. The same solutions were analysed for their hormone content by a bioassay procedure. The analysis of the crystalline complex of neurophysin-M showed that each milligram possessed 64.1±8.5 i.u. of pressor activity (Table 1). This figure can be used to calculate the number of vasopressin molecules present in the complex. The work of Acher (1958) on the electro dialysis of the protein-hormone complex shows that the free hormones and the hormones in the complex possess

the same specific activity. In addition, the calculation requires a knowledge of the specific activity (i.u./mg.) of [8-arginine]-vasopressin and of the molecular weight of neurophysin-M. Studer (1963) reported that anhydrous [8-arginine]-vasopressin acetate possessed 430 i.u. of pressor activity/mg. Hollenberg & Hope (1967) found that the molecular weight of neurophysin-M is close to 20000. Thus each milligram of protein-hormone complex contained 64.1/430=0.149 mg. of vasopressin, and there would be 2980 g. of vasopressin in an amount of complex equal to the gram molecular weight of neurophysin-M. This corresponds to 2.75 moles of vasopressin. We conclude that there are three vasopressin molecules associated with each molecule of protein in the complex. The molecular weight of the complex would thus be close to 23000; from this figure a more precise calculation of the number of molecules of vasopressin in a molecule of the complex was possible and was found to be 3.16.

Fig. 2. Starch-gel electrophoresis of neurophysin-M (channel a) and of the crystalline complex of neurophysin-M with [8-arginine]-vasopressin (channel b). Samples (approx. 0.2mg. of protein), dissolved in the gel buffer, pH 8.1 (Ferguson & Wallace, 1961), were applied to filter paper and inserted into the gel. Electrophoresis (gradient 10 v/cm.) was allowed to proceed for 4 hr. The gel was sliced in two and protein was detected with Nigrosine. The origin and the visible buffer boundary are indicated by arrows; protein migrated towards the anode.

Crystals containing neurophysin-M, vasopressin and oxytocin

Electrophoretic analysis. The electrophoretic analysis of crystals containing both hormones in the buffer system at pH 8.1 revealed the presence of the same protein components as those found in the crystals containing vasopressin alone. Judging by the intensity of the bands, the relative proportion of the two components was the same as had been observed previously.

Protein analysis and bioassay. The crystals exhibited both pressor and oxytocic activities (Table 1). Quantitatively, there was more pressor than oxytocic activity. A calculation was made for the number of hormone molecules present in the complex by the procedure described above. The oxytocic activity of pure oxytocin has been found to be 546±14 i.u./mg. (Chan, O'Connell & Pomeroy, 1963). This figure was used in the calculations. The calculation showed that there were,

Table 1. *Crystalline complexes of neurophysin-M*

The preliminary estimate of the number of peptide residues in a complex was based on the molecular weight of neurophysin-M (20000). Depending on the result of this calculation, a mol.wt. 22000 or 23000 for the complex was used to calculate the number of peptide molecules in a molecule of complex. The oxytocin complex was not obtained in crystalline form; *n* represents the number of bioassays.

Complex containing	Biological activity (i.u./mg.)		Molecules of hormone/ molecule of protein in complex
	Pressor	Oxytocic	
Vasopressin	64.0±8.5 (<i>n</i> 12)	—	3.16 (3)
Vasopressin and oxytocin	48.5±3.2 (<i>n</i> 9)	22.4±7.8 (<i>n</i> =9)	2.39 (2)
Oxytocin	—	49.1±7.6 (<i>n</i> =12)	0.94 (1)
			2.07 (2)

to the nearest whole number, two (2.39) molecules of vasopressin and one (0.94) of oxytocin in a complex of molecular weight 23000.

Complex containing neurophysin-M and oxytocin

Under the conditions used for the crystallization of the previous two complexes, neurophysin-M and oxytocin formed a precipitate, but even after a period of 3 months at 4° no crystals had appeared. Previously, when vasopressin had been present with oxytocin, the transformation of amorphous material into crystals had occurred within a week. The precipitate was analysed by the methods used for the crystalline complexes.

Electrophoretic analysis. Electrophoresis at pH 8.1 showed that both protein components were present in the proportions observed previously in the crystalline complexes. In no instance had fractional precipitation of these components occurred.

Protein analysis and bioassay. The analysis of the amorphous precipitate showed (Table 1) that it possessed 49.1 ± 7.6 i.u. of oxytocic activity/mg. This corresponds to two (2.07) molecules of oxytocin in a complex of molecular weight 22000.

Crystalline complex of crude neurophysin and vasopressin

After the successful crystallization of the neurophysin-M-vasopressin complex, which contained both of the protein constituents, it was of interest to attempt a crystallization of crude neurophysin. This material contained several distinct protein fractions with similar amino acid compositions (Hollenberg & Hope, 1967). A complex containing crude neurophysin and vasopressin was precipitated as described in the Materials and Methods section and seeded with a few crystals of the neuro-

physin-M-vasopressin complex. After a week, all of the amorphous material had crystallized.

Electrophoretic analysis. Electrophoresis at pH 8.1 showed that all the components present initially in crude neurophysin were found in the crystals in approximately the same proportions.

It is not known whether the protein components were present in mixed crystals or in separate crystal forms. However, the similarity of their molecular weights (18000–20000) and amino acid compositions (Hollenberg & Hope, 1967) suggest that solid solutions of the complexes would form.

Protein analysis and bioassay. The analysis of the crystals containing crude neurophysin showed that they possessed a lower biological activity than the analogous crystals containing neurophysin-M. The pressor activity was 32.5 ± 2.4 i.u./mg. of complex. The exact molecular weight of the crude neurophysin was not established, but assuming it to be close to that of the major component, neurophysin-M, it was calculated that 1.66 molecules of vasopressin were bound to a molecule of protein. This suggests that the binding capacity of the minor components present in crude neurophysin may be lower than that of neurophysin-M.

Equilibrium dialysis

The results of equilibrium dialysis are summarized in Table 2. It can be seen that, at equilibrium, the concentrations of deamino-[8-arginine]-vasopressin, histamine and bradykinin were the same on both sides of the 18/32 Visking membrane. In contrast, both oxytocin and vasopressin were concentrated about 100-fold on the inside of the membrane.

DISCUSSION

The biological activities associated with an amorphous protein-hormone complex (van Dyke,

Table 2. *Equilibrium dialysis*

The protein used in these experiments was crude neurophysin. Protein was placed in the inner compartment (0.5 ml.) of a dialysis cell (Craig & Konigsberg, 1961). The low-molecular-weight compound was placed in the outer compartment (7.5 ml.) and dialysed against the protein. At equilibrium (17 hr. at 4°) the inner and outer solutions were withdrawn, their volumes measured and analysed for the concentration of test compound. A ratio of concentration (inner/outer) greater than 1.0 indicates a binding of the compound tested. In each experiment the assay of biological activity in the two compartments was performed three times. Results are expressed as an average $\pm$ s.d. from the mean.

Protein (mg.)	Substance tested	Relative concn. at equilibrium: inside/outside
9.8	Deamino-[8-arginine]-vasopressin (60 i.u. of pressor activity)	1.02 $\pm$ 0.18
9.7	Histamine (46 μ g.)	0.70 $\pm$ 0.08
9.7	Bradykinin (500 μ g.)	0.97 $\pm$ 0.07
9.8	Oxytocin (90 i.u. of oxytocic activity)	99.0 $\pm$ 12.9
9.8	Vasopressin (119 i.u. of pressor activity)	87.9 $\pm$ 5.2

Chow, Greep & Rothen, 1942) were used by Landgrebe, Ketterer & Waring (1955) to calculate that one molecule of vasopressin and one of oxytocin were associated with a molecule of protein. In contrast, the crystalline complex formed from [8-arginine]-vasopressin and neurophysin-M contained 3 moles of peptide/mole of protein, whereas the amorphous complex with oxytocin contained 2 moles of hormone/mole of neurophysin. These values differ from the results of Ginsburg & Ireland (1965), who reported that as many as 7 moles of oxytocin and 4 of [8-arginine]-vasopressin could be bound by a molecule of bovine neurophysin. However, the binding capacity of neurophysin-M for oxytocin estimated by our method agrees with the data of Breslow & Abrash (1966) for their bovine neurophysin fraction E. They found that two molecules of oxytocin were bound/molecule of neurophysin, but in a gel-filtration experiment only 2 moles of [8-lysine]-vasopressin were bound/mole of protein. Our results show that 3 moles of [8-arginine]-vasopressin are bound/mole of protein in the crystals. This capacity for an additional molecule of vasopressin may only become apparent when crystals are formed in the presence of a large molar excess of hormone.

The crystalline complex formed in the presence of both hormones contained two molecules of vasopressin and one of oxytocin/molecule of protein. The work of Breslow & Abrash (1966) presented evidence of the ability of oxytocin and [8-lysine]-vasopressin to compete for the same binding sites. In addition, Acher and his co-workers had shown earlier that [8-arginine]-vasotocin was bound to mammalian neurophysins which would normally bind only oxytocin and [8-arginine]-vasopressin (Acher, Chauvet & Lenci, 1960; Chauvet, Lenci & Acher, 1960b; Acher, Chauvet, Lenci, Morel & Maetz, 1960).

The crude neurophysin fraction could also be crystallized as a complex in the presence of vasopressin. The binding capacity of the crude fraction for [8-arginine]-vasopressin was lower than that of neurophysin-M, but approximately 2 (1.66) moles of vasopressin were still bound per 20000g. of neurophysin.

It was established some time ago that the primary amino group of oxytocin was essential for the binding of this hormone to neurophysin (Stouffer, Hope & du Vigneaud, 1963). This indicated that the binding was of an electrostatic nature. Deamino-oxytocin, which possesses no other charged group that could lead to an electrostatic interaction, was not bound. On the other hand, deamino-[8-arginine]-vasopressin possesses the strongly basic guanidino group. The equilibrium-dialysis experiments showed that the presence of this group alone was not sufficient to lead to the formation of a

complex. Thus for both hormones the primary amino group is a prerequisite for binding. This led us to study the binding of histamine and of bradykinin, which like vasopressin contain both primary amino and guanidino groups. Neurophysin did not appear to possess any affinity for the compounds tested. These experiments indicate that although a primary amino group is a prerequisite for the binding of the hormones, it is not sufficient for the binding of compounds with markedly differing structures. Breslow & Abrash (1966) have shown that the binding also depends on the conformation at positions 2 and 3 of the oxytocin and vasopressin molecules. The results of the crystallization experiments suggest that groups other than those proposed by Breslow & Abrash (1966) may be involved in the binding process. The unique ability of [8-arginine]-vasopressin to induce crystallization (even when oxytocin is present as well) may reflect an influence of this peptide on the conformation of neurophysin. Both hormones render the protein insoluble, but only vasopressin facilitated the formation of crystals. The side chain of [8-arginine]-vasopressin that could lead to this effect would most probably be the guanidino group. Vasopressin would contribute two positive charges per molecule in a complex, whereas oxytocin could only contribute one. Furthermore, in a region of a binding site having a low dielectric constant, the guanidinium ion could interact strongly with the carboxylate ions of neurophysin. Such an interaction has been demonstrated in the X-ray analysis of amidinium salts of carboxylic acids (Kennard & Walker, 1963). The dialysis experiment with deamino-[8-arginine]-vasopressin indicated that such an interaction could only come about after the vasopressin has been attached to neurophysin through the primary amino group. It is thus possible that a co-operative effect of both the primary amino group and the guanidino group of vasopressin plays a role in the formation and crystallization of the complexes we have described.

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