Isolation and Characterization of a Gonadotropin-Inhibiting Substance from the Bovine Pineal Gland[1] (34664)

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The presence of a gonadotropin-inhibiting substance in the pineal gland has been the subject of considerable controversy for many years. In the last decade several groups of workers have shown that such a compound does exist, and a variety of methods have been employed to estimate its activity. Soffer *et al.* (1) used a concentrated aqueous extract of the bovine pineal gland and showed inhibition of the stimulatory effect of human chorionic gonadotropin (HCG) and human menopausal gonadotropin (HMG) in the mouse. Meyer and Wurtman (2) and Reiss *et al.* (3) obtained a significant lowering of mouse ovarian and uterine weights after administering a protein-free pineal extract. Attempts to isolate and characterize the active principle have been made by Bianchini (4) who purified a compound, which he named anestrine, and he showed that it would prolong anestrus in mice. Thieblot *et al.* (5), in a partial purification of the aqueous extract, showed that the active material was a mixture of 7 Ninhydrin-positive spots.

Milcu *et al.* (6) investigated the relation between the pineal gland and neurosecretory system, and concluded that there were cyclic octapeptides with oxytocic and pressor activities. From chromatographic data and enzymatic studies they inferred that the porcine pineal contained 8-lysine vasotocin and the bovine pineal contained 8-arginine vasotocin.

In this laboratory, the gonadotropin-inhibiting material has been isolated in homogeneous form, and structural elucidation involving amino acid analysis and mass spectrometry has been carried out (7).

Isolation. Fresh glands (7 lb) were homogenized in a Waring blender and extracted with acetone (12 liters) under constant agitation for 18 hr at 4°. After filtering, the air-dried solid (310 g) was suspended in water (800 ml) and methanol (400 ml), and the mixture was stirred at 50° for 6 hr, cooled, and centrifuged at 10,000 rpm for 15 min. The supernatant was concentrated at 50° *in vacuo* to 700 ml, and after cooling to room temperature it was saturated with ammonium sulfate and kept at 4° for 8 hr. The resultant precipitate (2.5 g) was filtered and dissolved in 0.05 *M* ammonium hydroxide (50 ml). The solution was extracted with isobutanol (5 × 15 ml), saturated with 0.05 *M* ammonium hydroxide solution, and after drying the combined extracts with anhydrous magnesium sulfate, the solvent was removed *in vacuo*.

The residue was dissolved in ammonium acetate solution (0.05 *M*, 10 ml), saturated with trichlorbutanol, and applied to a Sephadex G-25 column (4 × 45 cm) prepared in the same buffer. The column was eluted with the buffer at a flow rate of 5 ml/min and the active material was obtained by pooling the fractions between 500 and 580 ml. The pooled fraction was lyophilized and the resultant powder was dissolved in ammonium acetate buffer (pH 7, 0.05 *M*, 1.0 ml) saturated with trichlorbutanol. The solution was chromatographed on a Bio-Rex 70 column (200–400 mesh, 1 × 40 cm) prepared in the same buffer. The flow rate was adjusted to 4 ml/hr and the active material as followed by measuring the fluorescence at 395 mμ of each fraction after treatment with an alkaline solution of 5,6-phenanthrenequinone (8). Fractions between 90 and 110 ml contained the peptide, and these were

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pooled and lyophilized, yielding a white powder (1.4 mg).

Electrophoresis (1200 V, 4 hr) in a pyridine–acetate buffer at pH 3.5 using Whatman 3 MM and detection with hypochlorous acid/*ortho*-tolidine gave 3 bands, and the active compound (at 25 cm) was eluted and lyophilized. Descending paper chromatography on Whatman filter paper No. 1, using butanol:acetic acid:water (4:1:5) for 12 hr, and elution of the band at 14.5 cm gave the homogeneous microcrystalline peptide (380 μg).

Discussion. Isloation of the pure gonadotropin-inhibiting peptide was complicated by a much larger concentration of a second peptide which possessed similar partition and electrophoretic properties to the active compound. This peptide, which exhibited no effect on the action of gonadotropins, had the same amino acid composition as 8-arginine vasotocin with the addition of threonine, serine, and alanine residues.

Fresh pineal glands, after having been defatted with acetone, were extracted with aqueous methanol to minimize the extraction of extraneous protein. The active compound, being soluble in isobutanol, could be further purified from the more polar impurities by a partition between 0.05 *M* ammonium hydroxide and isobutanol, with a negligible amount of the active material lost. Residual protein was removed by chromatography on a Sephadex gel column, care being taken to avoid enzymatic degradation of the peptide by saturating the buffer with trichlorbutanol. After further concentration by chromatography on a cation exchange resin, the compound was followed by a convenient method of intense fluorescence produced by the monosubstituted guanidine condensing with 5, 6-phenanthrenequinone (8). Electrophoresis revealed the presence of 3 components (the second peptide mentioned above), exhibiting similar migration to the active compound. Paper chromatography in butanol:acetic acid:water gave the pure peptide.

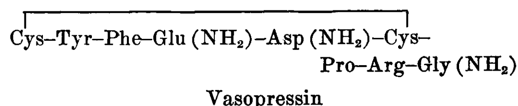
As mentioned above, detailed structural elucidation of the peptide has been reported in a separate paper (8). The composition of the molecule was determined by amino acid

TABLE I. Twenty-Four hr Hydrolysate of Peptide.

Amino acid	No. of moles	No. of residues[a]
Arginine	0.080	1
Aspartic acid	0.082	1
Glutamic acid	0.078	1
Proline	0.075	1
Glycine	0.081	1
Half-cystine	0.063	2
Isoleucine	0.082	1
Tyrosine	0.065	1

[a] Assuming arginine equals 1.

analysis using a Technicon-TSM dual column analyzer (Table I). Assuming there is one arginine residue, the compound contains 8 amino acids and one disulfide bridge. Apart from the presence of isoleucine and the absence of phenylalanine, the compound has the same components as the cyclic nonapeptide, vasopressin, which is found in the pituitary gland:



Vasopressin

Electrophoretic comparison of the two compounds at pH 2 and 6 showed their migration to be identical, implying that the pineal peptide was a nonapeptide containing glutamine, asparagine, and glycinamide, and not the free acids. Obviously, these three primary amides would be hydrolyzed during treatment with 6 *N* hydrochloric acid.

Amino acid sequence of the peptide was determined by mass spectrometry since usual techniques for elucidating peptide structure were not applicable owing to the extremely low concentration of material in the glands. It was necessary to enhance volatility to the molecule and, accordingly, several structural modifications were performed. This involved selective aspartic acid cleavage and then reduction of the disulfide bridge with mercaptoethanol to produce two tetrapeptides. After carboxymethylation of the resulting free thiols, the compounds which were separated by electrophoresis were methylated and the free amines were acylated with trifluoracetic anhydride.

TABLE II. Effect of Pineal Polypeptide and Synthetic Nonapeptides on the Stimulatory Effect of HCG on Mouse Uterine Weight.

Compound	No. of mice	Uterine wt (mg/100 g ± SE)	Probability of chance occurrence
Saline	10	43 ± 6	
HCG, 0.5 IU	9	178 ± 16	
+ pineal peptide, 2 μg	10	87 ± 8	<0.001
+ synthetic arginine vasotocin, 2 μg	10	94 ± 7	<0.001
+ oxytocin, 2 μg	10	123 ± 10	<0.01
+ vasopressin, 2 μg	9	185 ± 15	>0.7

Comparison of the mass spectra of these compounds with the two tetrapeptides obtained by treating vasopressin in a similar manner, enabled an unequivocal structure to be deduced.

Bioassay. During development of the isolation, the most efficient method of following the active compound proved to be the mouse uterine assay, since minimal amounts of material were required and the assay was completed within 3 days. Obviously, any assay using large amounts of material or requiring a longer time would not be acceptable. HCG, HMG, and pregnant mare serum have been used by various workers to stimulate the mouse uterus; however, in this laboratory an intramuscular injection of 0.5 IU of HCG daily for 3 days gave satisfactory stimulation. This dose produced approximately a 4-fold increase in the uterine weight of 27-day-old Swiss albino mice, weighing 15–18 g, and the data were significant in groups of 10 animals (Table II).

Inhibitions produced by the pineal peptide and synthetic 8-arginine vasotocin are almost identical. Vasopressin exhibits no effect on the uterus; however, oxytocin produces a significant inhibition, and from this it might be inferred that the interference with gonadotropin produced by the pineal peptide may be brought about by an oxytocic effect. Attempts to show that the pineal peptide demonstrated specific LH activity, using the ventral prostate assay as outlined by Greep *et al.* (9) were unsuccessful. In fact, with all compounds mentioned above there was no significant inhibition of the stimulated prostate gland. The data suggest that the inhibitory effect of the pineal peptide on a stimulated mouse uterus is actually oxytocic in nature.

Summary. A cyclic nonapeptide has been isolated in homogeneous form from an extract of the bovine pineal gland. Structural elucidation, involving amino acid analysis and mass spectrometry, has shown the compound to be 8-arginine vasotocin. Comparing synthetic 8-arginine vasotocin to the pineal hormone by mouse uterine and ventral prostate assays, shows their activities to be identical. The possibility that the pineal peptide is exhibiting an oxytocic effect is discussed.

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