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Inhibition of Induced Ovulation by a Highly Purified Extract of the Bovine Pineal Gland (38180)

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In the course of our studies with 8-arginine vasotocin isolated from the bovine pineal gland (1), it became apparent that a partially purified extract of the gland would inhibit ovulation induced in mice with exogenous gonadotropin.

This material was separated from the vasotocin and numerous indole compounds present in the pineal by subjecting the crude extract to ultrafiltration and then fractionation on a polyacrylamide gel. Consequently, the inhibition of ovulation reported could not be attributed to the presence of either vasotocin or any indolic compound.

Materials and Methods. Isolation of the material. Fresh pineal glands (1.5 kg) were homogenised in a Waring blender and extracted with acetone (8 liter) under constant agitation for 18 hr at 4°. After filtration, the solid (190 g) was suspended in 0.01 *M* Tris buffer (1200 ml) at pH 8 and the mixture stirred for 12 hr at 4°. Following centrifugation (15,000 rpm for 30 min), ammonium sulfate (45 g/100 ml) was added to the supernatant and stored at 4° for 24 hr. The resulting precipitate was collected and extracted twice with 0.05 *M* Tris buffer (200 ml) at pH 8 with constant stirring at 4°. The combined extracts were centrifuged at 25,000 rpm for 45 min and subjected to ultrafiltration using a Pellicon PSED membrane. The portion retained by the membrane was collected and chromatographed on a Biogel P200 column (2 × 50 cm) prepared in 0.01 *M* Tris buffer at pH 8. Five milliliter fractions were collected and the active material was eluted in 30 ml of the buffer after the initial protein peak. This solution was adjusted to pH 7 with hydrochloric acid and used in the bioassay as outlined below.

Bioassay. Thirty-day-old female mice of the C57Br strain and 40-day-old Swiss Webster female mice (from Simenson vendors) weighing from 21 to 24 g were used in the assays. As the control, 1.0 IU of pregnant mare serum (PMS) was administered by intraperitoneal injection, followed by 1.0 IU of human chorionic gonadotropin (HCG) 48 hr later. Ovulation occurred within the next 10 hr, each mouse yielding between 10 and 16 ova. To test the pineal compound, 0.1 ml of the extract described above was administered simultaneously with the HCG. Inhibition of ovulation was evidenced by the absence or reduced number of ova in the animals.

The technique used for determining the number of ova involved removal of the oviducts and flushing the ova with phosphate buffered saline solution by inserting a hypodermic needle in the tubal ostium (2, 3). To facilitate counting, the ova were freed from the mass of cumulus cells by the addition of a solution of hyaluronidase and then collected with a micropipette.

Results. The first experiments using Swiss Webster mice showed that 0.1 ml of the active fraction would completely inhibit ovulation (Table I). The C57Br strain was used in later experiments, since the number of ova per mouse in the control animals of this strain showed less variation than with the control animals of the Swiss Webster strain. A dose-response relationship was also established with the C57Br strain: 0.01 ml of the extract produced a 70% inhibition with a significant reduction in the

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TABLE I. Inhibitory Effect of Pineal Extract on the Ovulation Induced in Swiss Webster and C57Br Mice with PMS and HCG.

Number of animals[a]	Strain	Compound	% Ovulation	Ova/mouse (mean ± SE)
Control	Swiss Webster			
15		0.1 ml Tris buffer (0.01 *M*, pH 7)	100	14.2 ± 1.1
Experimental				
15		0.1 ml pineal extract[b]	0	0
Control	C57Br			
9		0.1 ml Tris buffer (0.01 *M*, pH 7)	100	12.4 ± 0.9
Experimental				
9		0.1 ml pineal extract	0	0
10		0.01 ml pineal extract	30	7.2 ± 0.4
10		0.005 ml pineal extract	70	10.4 ± 1.2

[a] Each mouse received 1 IU PMS followed by 1 IU HCG.

[b] 0.1 ml pineal extract represents the material obtained from approximately 0.6 g defatted tissue.

number of ova in those animals that did ovulate; and, at a dose of 0.005 ml of extract, only 30% of the treated animals failed to ovulate (Table I).

Table II summarizes the effects of 8-arginine vasotocin on induced ovulation. At levels of 2, 10, and 50 μg/mouse, no inhibitory action was observed.

Discussion. In the last decade, numerous authors have suggested that the gonadotropin-inhibiting properties of the pineal gland may be explained by the presence of indole compounds. Melatonin and 5-methoxytryphtophol will cause some inhibitory effect on ovulation with prolonged administration (4); however, it has been reported that melatonin does not suppress ovulation induced with PMS and HCG (5). This confirms the conclusions of Soffer *et al.* (6) that melatonin is not a gonadotropin-inhibiting substance. Motta *et al.* (7) have suggested that the pineal inhibits the secretion of luteinizing hormone (LH) and of follicle-stimulating hormone (FSH), and at high levels exogenous melatonin suppresses the secretion of LH but does not interfere with FSH release.

In this laboratory (1) it was shown that inhibition produced by pineal extracts on the stimulatory effect of HCG on uterine

TABLE II. Effect of 8-Arginine Vasotocin on the Ovulation Induced in Swiss Webster Mice with PMS and HCG.

Number of animals[a]	Compound	% Ovulation	Ova/mouse (mean ± SE)
Control			
10	0.1 phosphate buffered saline (PBS)	100	13.5 ± 0.8
Experimental			
5	2 μg 8-arginine vasotocin in 0.1 ml PBS	100	12.8 ± 1.2
5	10 μg 8-arginine vasotocin in 0.1 ml PBS	100	14.6 ± 1.4
5	50 μg 8-arginine vasotocin in 0.1 ml PBS	100	13.9 ± 0.8

[a] Each mouse received 1 IU PMS followed by 1 IU HCG.

enlargement is due to the presence of 8-arginine vasotocin, but this compound does not inhibit ovulation (Table II). A small polypeptide present in the pineal extract is capable of inhibiting compensatory ovarian hypertrophy in mice (8), and it has been shown that the pineal extracts inhibit the effects of both exogenous and endogenous gonadotropins and diminish pituitary storage of gonadotropin (9).

In a recent paper, a significant inhibition of the LH serum rise in rats after castration is shown, with a pituitary LH reduction after 4 days of treatment with bovine pineal extract (10). The authors conclude that the compound is probably a small polypeptide in the 500–1,000 range.

Assuming that the compound purified in our laboratory is a globular protein, on the basis of the gel fractionation data we estimate the mol wt to be of the order of 100,000. Although this is a crude approximation, this compound is obviously different from that described above.

Ota *et al.* (11) have demonstrated the presence of a gonadotropin-inhibiting substance in the urine of intact rats that is not present in the urine of pinealectomized animals. They suggest that the pineal may be a secreting organ of this substance or is at least involved in its secretion.

At present we are designing experiments to investigate both the structure and physical properties of this compound and the nature of its inhibitory action. In the light of these data it is hoped that we can establish any possible similarity of this compound with the substance described in the work of Ota *et al.* (11).

Summary. A highly purified extract of the bovine pineal gland was shown to prevent ovulation induced by exogenous gonadotropin in mice. The potency of this extract may be expressed thusly: The active material obtained from approximately one gram of defatted tissue would inhibit ovulation in 2 mice.

Immature female mice were made to ovulate by administration of PMS and HCG. When the extract was administered simultaneously with the HCG, ovulation was completely inhibited.

The isolation procedure used affects complete separation of this fraction from indolic substances and vasotocin, which are known to possess some antigonadotropic properties.

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