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STUDIES ON A NON-MELATONIN PINEAL ANTIGONADOTROPHIN

By

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ABSTRACT

Continuing investigation of pineal gland function indicates that the antigonadotrophic activity of this organ cannot be attributed solely to the postulated hormone melatonin, the concentration of which is negligible in the pineal body compared to quantities required to produce unequivocal physiological effects. A non-melatonin antigonadotrophic substance recently isolated from bovine pineal glands was further purified by organic solvent extraction, ultrafiltration and gel filtration. Studies of partial blockage of compensatory ovarian hypertrophy in unilaterally ovariectomized Charles River CD-1 mice indicated that this substance is significantly more potent than melatonin in this test system.

Albeit recondite, a functional relationship between the epiphysis cerebri and the gonads cannot be denied in the face of presently increasing evidence. The endocrine nature of this neural structure, for many years a controversial classification, seems to be justified in classical terms through extirpation (Kitay & Altschule 1954; Wurtman et al. 1959; Czyba et al. 1964; Hoffman & Reiter 1965, 1966; Reiter et al. 1966, 1968; Motta et al. 1967) and replacement experiments in which the effects of pinealectomy were reversed by the administration of crude pineal gland extracts (Wurtman et al. 1959; Thiébrot & Blaise 1963) and by transplantation of pineal glands (Gittes & Chu 1965).

Various biologically active principles have been suggested as possible pineal antigonadotrophic hormones (Pavel 1965; Thiébrot et al. 1966; Moszkowska & Ebels 1968). Unfortunately Wurtman's (1967) proposal that the endolic derivative melatonin might be the pineal gland hormone quite overshadowed the search for other active principles. The bases for Wurtman's (1967) assertion

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lie in the facts that melatonin synthesis is unique to the pineal gland (Axelrod et al. 1961) and that melatonin treatment produces well documented, although not unequivocal, antigonadotrophic effects (Ariens-Kappers 1962; Wurtman et al. 1963; Adams et al. 1965; Moszkowska 1965; Motta et al. 1967; Sorrentino 1968). Perusal of such studies reveals, however, that the daily doses of melatonin utilized were large and usually were given over an extended period of time resulting in total dosages commonly in mg quantities. These amounts are grossly out of proportion to those quantities present in the pineal gland itself which are reported to be in the range of 2–7 \times 10^{-4} \mu g/mg of tissue (Lerner et al. 1960; Prop & Ariens-Kappers 1961; Barchas & Lerner 1964; Wurtman et al. 1964; Quay & Baker 1965; Maickel & Miller 1968).

Recently Benson et al. (1971) demonstrated that melatonin-free, aqueous extracts of bovine pineals possess potent antigonadotrophic activity. In the studies reported here the active pineal antigonadotrophin (PAG), believed to be a polypeptide, was further partially purified, and when compared with melatonin was observed to be a substantially more effective antigonadotrophic agent.

**MATERIALS AND METHODS**

*Animals*

Female, Charles River CD-1 mice, 6–10 weeks of age were employed. They were housed under conditions of controlled temperature (25–26°C) and photoperiod (light/dark ratio = 14/10 hours). All received Purina Breeder Mice Chow and water ad libitum.

*Test for antigonadotrophic activity*

Unilateral ovariectomies were performed under ether anaesthesia and the left ovaries were weighed to the nearest 0.1 mg on a torsion balance. The right ovaries were removed and weighed nine days later at sacrifice except in Experiment No. 4 in which the remaining ovaries were taken at five days following unilateral ovariectomy (UO). For each animal the amount of compensatory ovarian hypertrophy (COH) was calculated as the percentage increase in weight of the right over that of the left ovary. The various pineal principles were given by single intraperitoneal injection on the day of UO and the percentage of COH for each test solution was observed. Antigonadotrophic activity was determined by the ability of these extracts to inhibit COH significantly when the mean percentage COH for various groups were compared statistically utilizing the Student's t-test.

*Crude pineal extracts*

An aqueous extract of 5 g of defatted pineal powder (Nutritional Biochem. Co.) was prepared by blending in triply distilled water with subsequent stirring at room temperature for 12 hours. The mixture was centrifuged at 12400 \times g for 30 minutes and the supernatant portion tested by intraperitoneal injection into two groups of
10 mice in Experiment No. 1. On the day of UO each mouse in one test group received an extract of 2.0 mg of the pineal powder in 0.5 ml of tripoly distilled water while each in the second group received 4.0 mg in the same quantity of water. Another group of 10 mice received 0.5 ml of the water only and served as controls.

In this first experiment two additional groups of mice were given a single injection of an aqueous extract of rat pineals prepared in the following manner: sixty, fresh rat pineal glands taken from adult Charles River males were homogenized in water and the homogenate was centrifuged at 12,400 × g for 20 minutes. The clear supernatant was lyophilized, and the white powder was dissolved in physiological saline for injection at two dose levels corresponding to 3.4 or 6.8 mg wet weight of fresh pineal glands per mouse.

**Dose response to melatonin**

A dose response relationship between percentage COH and melatonin was demonstrated by Vaughan & Benson (1970) and Vaughan et al. (1970, 1971). The purpose of Experiment No. 2 was to compare the antgonadotrophic activity of pineal gland extracts with that of melatonin. Thirty-eight mice were divided into four groups of 9–10 animals each. Crystalline melatonin (Sigma Chem. Co.) was dissolved in ethyl alcohol and this was subsequently diluted with physiological saline to a final alcohol concentration of 3.0% (v/v). Each mouse in three of the groups received a single injection of melatonin in either 20, 40 or 80 µg quantities on the day of UO. The fourth group served as controls and received 0.2 ml of the 3.0% (v/v) alcohol-saline diluent.

**Purified bovine pineal extracts**

Isobutanol extraction. – An isobutanol extract of bovine pineal glands was made essentially after the method of Cheesman & Fariss (1970). Twenty-six g of fresh pineal glands were frozen at the abattoir, thawed the next day and homogenized in 50.0 ml of acetone in a Virtis Model # 45 homogenizer. Acetone extraction was continued with stirring for 24 hours at 4°C. After filtering the air-dried residues was blended in 50.0 ml of 33% (v/v) aqueous methanol and the mixture was stirred for eight hours at 50–55°C. After centrifugation at 12,400 × g for 20 minutes, the supernatant portion was saturated at room temperature with ammonium sulphate, then stored over night at 4°C. The precipitate (12.1 mg) was filtered and dissolved in 20 ml of 0.05 M ammonium hydroxide. This solution was extracted thrice with 15 ml portions of isobutanol which was later removed in vacuo at 50°C.

In Experiment No. 3 the residue was taken up in a small quantity of physiological saline for injection into mice on the day of UO. A total of 37 mice were divided into four groups of 8–10 mice each. An estimated 1.0 µg representing 1/60 of the total residue from the isobutanol extract was given to each mouse in one group. A second group of ten mice received an aliquot of the residue from the isobutanol extraction of an equivalent amount of bovine cerebral cortex. A third group of eight mice was given 60 µg of melatonin for the purpose of comparison, and the fourth group of nine control animals received 0.5 ml of physiological saline on the day of UO.

Partial purification of the isobutanol extract of bovine pineal glands. – The isobutanol extractable residue was further purified by ultrafiltration and gel filtration techniques. One-half of the total residue from the isobutanol extract, prepared as described above, was dissolved in 100 ml of 0.05 M sodium acetate and placed in an Amicon Model # 402 ultrafiltration cell equipped with a Diaflo XM-100 filter which retains molecular weight (MW) substances greater than 100,000. Under nitrogen
pressure the filtrate, containing material less than 100 000 MW, was run through a series of Diafio filters: PM-10, UM-2 and UM-05 with respective retentions of MW substances less than 10 000, 1000 and 500. In this manner a crude MW separation was achieved and after freeze-drying each fraction was tested for antigonadotrophic activity.

Inasmuch as only the MW 500-1000 fraction possessed significant biological activity, this portion was lyophilized, taken up into 10 ml of water and placed on a 45 × 2.5 cm Sephadex G-25 (fine) column equilibrated with deionized water. The column was eluted at a flow rate of 2.0 ml/min and 5.0 ml fractions were collected and read at 280 nm in a Beckman Model DU spectrophotometer. The various 5.0 ml fractions were combined according to their absorbance at 280 nm and tested for biological activity as described in Experiment No. 4. Each combined fraction was submitted to a quantitative ninhydrin analysis using the method of Saifer et al. (1960).

**Melatonin determination**

Each fraction to be tested was placed on a 45 × 2.5 cm Sephadex G-25 (fine) column equilibrated with deionized water as recommended by van der Veerdonk (1965). Fractions were collected and the volume in which a 1.0 µg melatonin standard had been previously recovered was collected. The melatonin fraction was lyophilized, dissolved in 3.0 ml of 10 % ethanol, and read in an Aminco-Bowen spectrophotofluorometer with excitation set at 304 and emission at 357 nm. The readings were compared with those of melatonin standards (Mann Research Laboratories) that had previously been placed on the column. A 95.6 % recovery has been observed with this method, the details of which are to be published elsewhere (Matthews & Rodin, in preparation).

**RESULTS**

**Antigonadotrophic activity of crude pineal extracts**

In Experiment No. 1 the whole aqueous extracts of rat or of defatted bovine pineal glands were observed to possess significant antigonadotrophic activity when administered by single injection at two dose levels. In both cases inhibition of COH increased relative to the increase in the amount of extract (refer to Fig. 1).

For purposes of comparison a linear dose response between inhibition of COH and melatonin was obtained in Experiment No. 2 (see Fig. 2) since the antigonadotrophic effects observed with the pineal gland extracts were detectable even though negligible amounts of melatonin were present. The effect of the 4.0 mg of pineal gland powder in inhibiting COH in Experiment No. 1 was chosen for comparison. The antigonadotrophic activity of 4.0 mg of bovine pineal powder, which reduced COH from 69 % in the controls to 36 % (Fig. 1), was equivalent to the activity of approximately 32 µg of melatonin or 8 µg/mg. This amount is in great excess of the 0.001 µg/mg found in the pineal powder which had been passed through the Diafio filters or the 0.003 µg/mg detected in the untreated powder.
Dose response of partial blockage of COH is obtained with both fresh rat pineals and bovine pineal powder. The partial blockage of COH observed with the larger dosages was in both cases highly significant \( (P < 0.01) \) when compared with the controls and that observed with both the lower dosages was significant \( (P < 0.05) \).

**Antigonadotrophic activity of partially purified pineal gland extracts**

When tested for biological activity the isobutanol extractable material, although small in quantity, demonstrated potent antigonadotrophic activity. The effect of an approximate 1.0 \( \mu \)g was comparable to that of 60 \( \mu \)g of melatonin tested in the same manner. On the other hand the residual material from an isobutanol extract of cerebral cortex was without significant antigonadotrophic activity (refer to Fig. 3).

After the ultrafiltration of the isobutanol extractable material, an aliquot of each MW fraction was tested and only the MW 500–1000 fraction was found to contain the pineal antigonadotrophin (PAG). The remainder of this MW fraction was placed on a Sephadex G-25 column and the elution profile shown in Fig. 4 was obtained. The various samples were combined into five fractions (F1, F2, etc.) as designated. After lyophilization 25% of each of these fractions was tested for biological activity. In Fig. 5 it is seen that only fraction F3 contained PAG. All peaks were essentially ninhydrin negative except for F3 which contained an equivalence of 0.187 \( \mu \)mole of ninhydrin positive material when compared with a tryptophan standard.
Partial blockage of COH is illustrated in response to increasing doses of melatonin. Each point represents the mean for 10 mice and the range is the ± se. The partial inhibition of COH by 4 mg of bovine pineal gland powder (36%+) corresponded to the activity of 32 µg of melatonin.

**DISCUSSION**

The test for antigonadotrophic activity of pineal extracts utilized in this study was inhibition of COH, the mechanism of which, uncomplicated by inhibiting factors, is thought to be dependent upon the secretion of gonadotrophin (Benson et al. 1969). The increase in ovarian weight appears to be finely regulated allowing accurate predictability with respect to time. The inhibition of COH is considered to be due either to the suppression of gonadotrophin secretion or diminution of gonadotrophin action. The relationship of this process to pineal gland function was established by Sorrentino (1969, 1970) who showed that optic enucleation partially inhibited COH in the rat, an effect that was reversed by pinealectomy (see also Sorrentino & Benson 1970). These results were confirmed by Dickson et al. (1971). Little & Benson (1971) have also obtained similar results in the hamster.

Inhibition of COH has been utilized in a variety of studies related to pineal function. Moszkowska (1963) first showed that aqueous pineal gland extracts inhibited COH and the formation of cyclic corpora lutea in the guinea pig.
Partial blockage of COH by a pineal antigonadotrophin (PAG) present in an isobutanol extract of bovine pineal glands. Approximately 1 µg was equivalent to 60–70 µg of melatonin. Cerebral cortex had no effect on COH. Each bar represents the mean ± se for 9 or 10 mice. The difference between the control and PAG is highly significant ($P < 0.01$).

Elution pattern of the isobutanol extract on Sephadex G-25. The five fractions (F1, F2, etc.) were combined as indicated.
Figure 5. Partial blockage of COH by the F3 fraction of the isobutanol extract from fresh bovine pineal glands is shown. Each bar represents the mean ± se for 9 or 10 mice. The difference between the control and the F3 fraction is highly significant ($P < 0.01$).

In this same year Reiss et al. (1963) reported blockage of COH by a suspension of dried bovine pineal glands injected daily for 14 days into UO rats. Sorrentino (1968) first showed that melatonin partially blocked COH and reduced serum FSH levels in the rat. These observations were confirmed and extended by Vaughan & Benson (1970) and Vaughan et al. (1970) who showed that a single injection of melatonin into mice on the day of UO inhibited COH when the remaining ovaries were observed 10 days later. Further studies revealed that the amount of inhibition was dose related and that mice were more sensitive to melatonin during the phase of vaginal dioestrus (Vaughan et al. 1970, 1971). The inhibition of COH is advantageous for studying pineal function since smaller dosages of pineal gland extracts or melatonin are required to produce noticeable effects than those quantities needed to reduce ovarian, seminal vesicle or ventral prostate weight in intact animals.

The evidence for non-melatonin pineal antgonadotrophic factors has been mounting since Ebels et al. (1965) prepared extracts of acetone powdered pineal glands which were subjected to gel filtration on Sephadex G-25. Two of the peaks from the column eluent possessed biological activity: F2, capable of augmenting the hypophyscal secretion of FSH in vitro, and F3, which diminished hypophyscal FSH secretion. The inhibitory fraction F3 was further partially purified by gel filtration on Sephadex G-10 and by paper chromatography (Ebels 1967). The MW of the substance in the F3 fraction has recently been reported to be about 700 after purification (Moszkowska et al. 1971). The data of Ebels et al. (1965) suggest that the antgonadotrophic activity which they observed was due to small polypeptides present in the pineal gland.
Recently cyclic nonapeptides have been isolated from the pineal gland (Milcu et al. 1963; Pavel 1965; Moszkowska & Ebels 1968). Cheesman & Fariss (1970) developed an isolation procedure for the purification of arginine vasotocin which was subsequently identified by mass spectrography. The initial steps utilized by these investigations were used also for the isolation of PAG in our study; however, Cheesman & Fariss (1970) accredited all of the »antigonadotrophic activity« to an oxytocic effect on the HCG stimulated uterus of the immature mouse. Their highly purified material was not effective in an anti-LH assay which involved reduction in ventral prostate weight in the male mouse. More recent experiments have shown that PAG causes a reduction in ventral prostate and seminal vesicle weight in the male mouse and the involution of corpora lutea in the ovaries of intact females (Benson et al., in press). This would indicate an anti-LH effect for PAG as opposed to arginine vasotocin. Since we did not follow the final extraction procedures and our extract proved to be impure on Sephadex G-25, it is impossible to determine whether PAG and arginine vasotocin might be identical or not. Moszkowska & Ebels (1970) also indicate that their inhibitory F3 fraction differs from synthetic arginine vasotocin in vitro.

The most recent evidence for non-melatonin pineal antigonadotrophic principles is the study by Benson et al. (1971) which showed that melatonin-free aqueous extracts of bovine pineals were capable of inhibiting COH in a dose response fashion. The active material was confined to the molecular weight fraction of 500–1000 as defined by ultrafiltration techniques.

In the present study antigonadotrophic activity was demonstrable in whole aqueous extracts of rat and bovine pineals in which negligible quantities of melatonin were present. The non-melatonin pineal antigonadotrophin was partially purified by aqueous methanol and isobutanol extraction. After ultrafiltration only the MW 500–1000, melatonin-free fraction contained PAG. Gel filtration of this MW fraction on Sephadex G-25 yielded multiple peaks, one of which was ninhydrin positive and contained PAG. These studies suggest the presence of an antigonadotrophic pineal gland factor in the MW range of a small polypeptide which possesses strong antgonadotrophic activity. PAG was estimated to be 60–70 times as active as melatonin when compared in the biological system utilized even though it was only in partially purified form.

REFERENCES

Reiss M., Mauer I., Sideman M. B., Davis R. H. & Plichta E. S.: J. Neurochem. 10
(1963) 851.
University of Tennessee Medical Units, Memphis, Tennessee, USA (1969).
Wurtman R. J. In: Martini L. and Ganong W. F., Eds. Neuroendocrinology 2,

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