GENERAL AND COMPARATIVE ENDOCRINOLOGY 25, 189–198 (1975)

Pineal Factors Other Than Melatonin

I. EBELS

Department of Organic Chemistry, University of Utrecht, Utrecht, The Netherlands

Received September 1, 1974

Some sheep pineal factors other than melatonin are described.

A "nonmelatonin" antigonadotropic activity has been detected by application of the inhibition of compensatory ovarian hypertrophy (COH) in unilaterally ovariectomized adult Charles River CD-1 mice. The factor has been extracted from sheep pineals under rather simple and mild experimental conditions. This antigonadotropic activity has been partially purified by gel filtration and ultrafiltration and was localized on paper chromatograms and paper electropherograms.

Inhibitory and stimulatory activities on the hypothalamic–hypophyseal system *in vitro* have been detected in sheep pineal fractions obtained after gel filtration and ultrafiltration.

A substance with distinct fluorescence characteristics, which could not be detected in sheep cerebral cortex extracts and which differs from melatonin, has been isolated from a low molecular Sephadex G-25 fraction of an extract of sheep pineals.

Reiter and Fraschini (1969) concluded in their review that relatively little is known about the influence of the pineal on the modulation of reproductive functions. The majority of evidence suggests that the pineal exerts its influence by altering hypophyseal gonadotropin secretion. An inhibitory effect has been ascribed to melatonin (Wurtman *et al.*, 1963), 5-methoxytryptophol (McIsaac *et al.*, 1964), and 5-hydroxytryptophol (Fraschini *et al.*, 1968). Ebels *et al.* (1965) and Moszkowska and Ebels (1971) have detected in two low molecular-weight Sephadex G-25 fractions F2 and F3, which were isolated from sheep pineals, an inhibiting and a stimulating effect on the follicle-stimulating activity of the anterior hypophysis of the male rat *in vitro*. The effects differ from those of melatonin, 5-methoxytryptophol, serotonin creatinine sulfate, and arginine vasotocin. In an *in vivo* bioassay (Sorrentino and Benson (1970)) using the compensatory growth of the remaining ovary after unilateral ovariectomy as an index, Benson *et al.* (1971, 1972) have shown that melatonin-free extracts of bovine pineals possess an antigonadotropic activity.

In this paper the detection and partial purification of some pineal factors other than melatonin are presented. The purification was effected by gel filtration.

MATERIALS AND METHODS

Sheep pineals were collected by ERSCO, San Mateo, CA, in December 1971, March 1972, December 1972; sheep cerebral cortex in Paris in November 1972. These tissues were frozen directly after death, shipped on dry ice, and preserved at −20°C.

Extraction and Chromatography

Hundred-gram quantities of sheep pineals were homogenized with 100 ml of distilled water. The extraction is carried out as described before (Ebels *et al.*, 1972, 1973). The residue is reextracted twice with 75 ml of distilled water. The combined supernatant portions (230 ml) were applied to the Sephadex G-25 column.

Gel filtration has been carried out on Sephadex G-25 (Pharmacia, Uppsala, Sweden) columns (56 × 4.2 cm) with distilled water as the eluant (10 ml/10 min/tube).

The details for the localization of excitation and fluorescence maxima in the eluate have been described before (Balemans *et al.*, 1970, Ebels *et al.*,

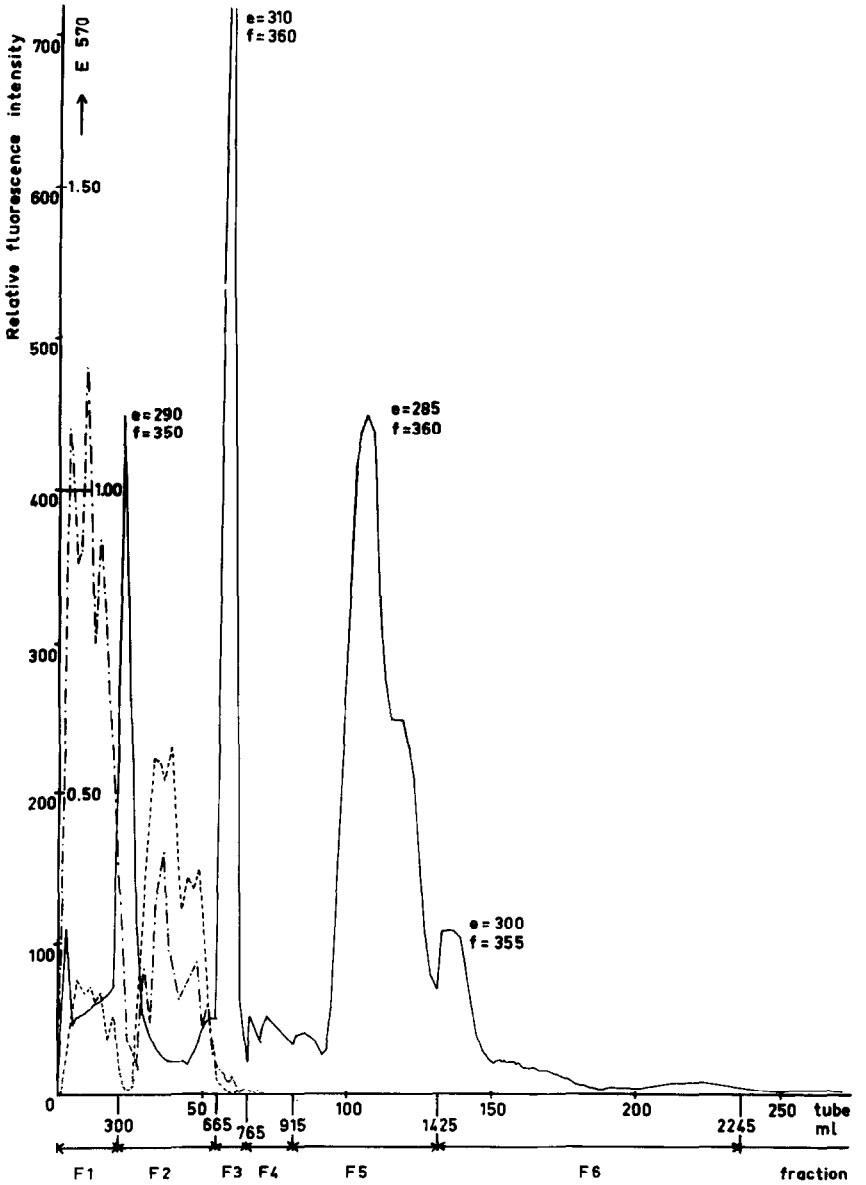

FIG. 1. Separation of an aqueous sheep pineal extract on a Sephadex G-25 column (56 × 4.2 cm) equilibrated, and eluted with distilled water. Relative fluorescence intensity (—); ninhydrin color without hydrolysis (---); ninhydrin color after alkaline hydrolysis (·-·-) ; e = excitation; F = fluorescence.

1972). An Aminco–Bowman spectrofluorometer (4-8203) was used.

Column chromatography on Sephadex G-10 was carried out as described before in detail (Ebels *et al.*, 1973; Zurburg and Ebels, 1974).

Ultrafiltration. Ultrafiltration of the low molecular-weight Sephadex G-25 fractions was performed in an Amicon heavy-duty cell, model 401-S (Amicon, Lexington, MA). Each Sephadex G-25 fraction was divided into a UM-2 residue, a UM-05 residue, and a UM-05 filtrate.[1] For details see Ebels *et al.* (1973).

Paper electrophoresis was carried out on Whatman 3MM paper at pH 3.5 and 6.5; paper chromatography on Whatman 3MM paper in different solvents. For

[1] Diaflo membrane UM-2 generally partition mixtures of solutes above and below the 1000-MW range, and UM-05 will do the same above and below the 500-MW range.

TABLE 1
COH-INHIBITING ACTIVITY IN CD-1 MICE OF THE DIFFERENT FRACTIONS OBTAINED AFTER SEPARATION OF AN AQUEOUS SHEEP PINEAL EXTRACT ON SEPHADEX G-25

Fraction	Corresponding to wet weight of sheep pineals (g)	Weight of the fraction after lyophilization (mg)	% COH[a] for the control animals	% COH for the test animals	% Inhibition	*P* value *t* test
F1	20	1034.27	65.1 ± 9.7	65.7 ± 7.3	*P*–	n.s.
F2	20	495.07	43.7 ± 4.7	17.6 ± 7.1	59.7	$P < 0.01$
F3	20	12.01	43.7 ± 4.7	10.8 ± 5.4	75.3	$P < 0.001$
F4	100	23.41	43.7 ± 4.7	40.0 ± 5.4	8.5	n.s.
F5	100	16.85	43.7 ± 4.7	51.3 ± 8.8	—	n.s.
F6	100	5.08	60.6 ± 2.9	66.1 ± 7.0	—	n.s.

[a] % COH = mean value ± standard error of the mean value for seven or eight mice.

details of these two techniques see Ebels *et al.* (1973).

Thin layer chromatography was carried out on plates of silica gel (DC-Fertigplatten, Kieselgel F254, 20 × 5 cm and 20 × 20 cm from E. Merck A. G., Darmstadt, Germany). For details see Zurburg and Ebels (1974).

Bioassays

Compensatory ovarian hypertrophy. The use of inhibition of compensatory ovarian hypertrophy (COH) for the detection of antigonadotropic activity has been discussed in several studies. Animals of the same inbred strain of CD-1 mice were utilized (Benson *et al.*, 1971, 1972). For details of the method used in these studies see Ebels *et al.* (1973).

Incubation experiments with mouse hypothalami. For the study of each pineal fraction six mouse hypothalami were incubated during 1.5 hr in a Krebs-Ringer solution at 37°C, aerated with 95% O_2 and 5% CO_2, with a pineal fraction. Six mouse hypothalami without a pineal fraction served as a control. The incubation liquid was subsequently used for the incubation of three mouse anterior hypophysis during 3 hr under the same experimental conditions to determine the gonadotropin-releasing activity. Using this test it is also possible to evaluate the action of various pineal fractions on the hypothalamic–hypophysiotropic activity by comparing the gonadotropin-releasing activity of hypothalami incubated alone and in the presence of the pineal fractions. For details of the method see Moszkowska *et al.* (1973); Citharel *et al.* (1973).

TABLE 2
COH-INHIBITING ACTIVITY IN CD-1 MICE OF THE ACTIVE SEPHADEX G-25 FRACTIONS AFTER ULTRAFILTRATION THROUGH DIAFLO MEMBRANES

Experiment no.	Fraction	Corresponding to wet weight of sheep pineals (g)	Weight of the fraction after lyophilization (mg)	% COH[a] for the control animals	% COH for the test animals	% Inhibition	*P* value *t* test
I	F3						
	UM-2 residue	40	5.42	39.0 ± 6.6	38.1 ± 3.7	2.3	n.s.
	UM-05 residue	40	6.66	39.0 ± 6.6	0.9 ± 5.5	97.7	$P < 0.001$
	UM-05 filtrate	40	104.31	39.0 ± 6.6	32.8 ± 6.0	15.9	n.s.
II	F2						
	UM-05 residue	40	12.20	43.7 ± 4.7	20.6 ± 7.2	52.9	$P < 0.02$
	UM-05 filtrate	40	768.72	43.7 ± 4.7	51.2 ± 9.1	—	n.s.
	F3						
	UM-05 residue	40	1.95	43.7 ± 4.7	0.8 ± 8.5	98.1	$P < 0.001$
III	F2						
	UM-05 residue	40	11.74	69.9 ± 4.3	51.9 ± 8.1	25.7	n.s.
	F3						
	UM-05 residue	40	2.90	69.9 ± 4.3	10.7 ± 5.0	84.7	$P < 0.001$

TABLE 3
COH-INHIBITING ACTIVITY IN CD-1 MICE OF DIFFERENT FRACTIONS OBTAINED AFTER SEPARATION OF ACTIVE ULTRAFILTRATE FRACTIONS ON SEPHADEX G-10

Experiment	Fraction applied to the Sephadex G-10 column	Fraction from the Sephadex G-10 column	Eluation volume (ml)	Weight of the fraction after lyophilization (mg)	% COH[a] for the control animals	% COH for the test animals	% Inhibition of COH	P value t test
I	F3	F1	0–35	0.49	59.9 ± 5.3	50.9 ± 4.8	15.0	n.s
	UM-05 residue	F2	36–56	0.46	59.9 ± 5.3	31.6 ± 7.1	47.2	$P < 0.001$
		F3	57–161	6.26	59.9 ± 5.3	30.1 ± 4.0	49.7	$P < 0.001$
		F4	162–236	0.47	59.9 ± 5.3	60.4 ± 4.6	—	n.s.
		F5	237–387	2.72	59.9 ± 5.3	73.4 ± 9.3	—	n.s.
II	F2 + F3	F1	0–21	0.40	60.4 ± 8.2	59.6 ± 7.4	1.3	n.s.
	UM-05 residue	F2	22–51	0.40	60.4 ± 8.2	56.0 ± 6.3	7.3	n.s.
		F3	52–68	0.15	60.4 ± 8.2	20.7 ± 4.1	65.7	$P < 0.001$
		F4	69–110	20.90	60.4 ± 8.2	43.0 ± 9.1	28.8	n.s.
		F5	111–256	1.58	60.4 ± 8.2	58.8 ± 6.7	2.6	n.s.
		F6	257–417	0.77	60.4 ± 8.2	60.9 ± 7.3	—	n.s.
	Blue Dextran 2000		60–118					
	NaCl		119–138					
	Melatonin		1700–2260					

[a] % COH = mean value ± standard error of the mean value for seven or eight mice.
[b] n.s. = not significant.

RESULTS AND DISCUSSION

A Nonmelatonin Antigonadotropic Activity in the COH Bioassay[2]

After rather simple and mild aqueous extraction from sheep pineals a substance (s) with antigonadotropic activity has been partially purified by gel filtration on Sephadex G-25 (Fig. 1 and Table 1).

Two low-molecular-weight Sephadex G-25 fractions F2 and F3 contain the antigonadotropic activity. These active Sephadex G-25 fractions are ultrafiltrated through the Diaflo membranes UM-2 and UM-05. It was found that the UM-05 residue contains the substance(s) which inhibits COH in unilaterally ovariectomized mice (Table 2). When this active UM-05 residue was gel filtered on a Sephadex G-10 column, with distilled water as the eluant, an active fraction appeared long before melatonin. The column was tested with the latter compound (Table 3).

The antigonadotropic activity was further localized by paper electrophoresis and paper chromatography (Tables 4, 5, 6). We can only speculate on the nature of the antigonadotropin and the class of compounds to which it belongs. As this antigonadotropic activity is retained by the UM-05 ultrafiltration membrane and passed by the UM-2 membrane, it may be that the active substance (s) has a molecular weight > 500 and < 1000. Tryptic digestion of a similar active fraction destroyed the antigonadotropic activity (Matthews and Benson, 1973). Therefore, the active substance (s) may be a peptide or may contain a peptide moiety essential for activity.

Bensinger *et al.* (1973) have isolated from bovine pineal glands a nonmelatonin lipophylic antigonadotropic factor also using the COH-inhibition model in mice for the detection of active fractions. These authors stated that the active principle is a small, nontryptophan-containing polypep-

[2] The experiments on the nonmelatonin antigonadotropic activity have been carried out in collaboration with Dr. B. Benson and co-worker, during his sabbatical leave in our institute; present address: The University of Arizona, Arizona Medical Center, College of Medicine, Department of Anatomy, Tucson, AZ.

TABLE 4

LOCALIZATION OF PINEAL ANTIGONADOTROPIC ACTIVITY BY PAPER ELECTROPHORESIS

Experiment	Band of the strip No.	cm[a] From	To	% COH[b]	% Inhibition	P
I	E1	−16.50	−8.75	104.9 ± 19.1	—	n.s.[c]
	E2	−8.75	−1.00	97.8 ± 16.4	2.2	n.s.
	E3	−1.00	−0.25	47.0 ± 8.5	53.0	<0.02
	E4	−0.25	+0.75	51.0 ± 14.4	49.0	<0.05
	E5	+0.75	+2.00	100.0 ± 17.4	—	n.s.
	E6	+2.00	+3.50	98.3 ± 26.9	1.7	n.s.
	E7	+3.50	+17.00	100.9 ± 15.2	—	n.s.

[a] Start = 0; − = cathode side; + = anode side.

[b] % COH = mean value ± standard error of the mean value for seven or eight mice expressed as a percentage of the mean of an appropriate group of control mice treated with 0.9% NaCl.

[c] n.s. = not significant at 5% level.

TABLE 5

LOCALIZATION OF PINEAL ANTIGONADOTROPIC ACTIVITY BY PAPER CHROMATOGRAPHY

Solvent	Distance from start to front, cm	Band of the strip No.	Cm[a]	% COH[b]	% Inhibition	P
n-Butanol–acetic acid–water 4:1:5 (v/v/v)	41.0	PC1	−0.5 → 0.5	92.5 ± 14.3	7.5	n.s.[c]
		PC2	0.5 → 2.0	105.0 ± 14.3	—	n.s.
		PC3	2.0 → 5.0	85.6 ± 33.7	14.4	n.s.
		PC4	5.0 → 8.0	90.0 ± 29.4	10.0	n.s.
		PC5	8.0 → 9.0	66.3 ± 17.4	33.7	n.s.
		PC6	9.0 → 11.0	19.8 ± 23.4	80.2	<0.01
		PC7	11.0 → 13.0	116.0 ± 16.8	—	n.s.
		PC8	13.0 → 15.0	100.0 ± 13.2	—	n.s.

[a] −0.5 = 0.5 cm above the start.

[b] % COH = mean value ± standard error of the mean for seven or eight mice expressed as a percentage of the mean of an appropriate control group of mice treated with 0.9% NaCl.

[c] n.s. = not significant at the 5.0% level.

TABLE 6

WEIGHTS AND R_f VALUES FOR ACTIVE PINEAL FRACTIONS LOCALIZED BY PAPER CHROMATOGRAPHY

Solvent	Experiment	Weight of the applied UM-05 residue (mg)	Weight of the eluted active fraction (mg)	Approximate R_f value
n-Butanol–acetic acid–water 4:1:5 (v/v/v) upper phase	I	5.10	1.28	0.24
	II	3.96	1.67	0.23
n-Butanol–acetic acid–pyridine–water 15:3:10:12 (v/v/v/v)	I	11.52	2.14	0.26
	II	3.78	1.72	0.27
n-Butanol–pyridine–water 6:4:3 (v/v/v)	I	11.67	2.52	0.16
Tert. amylalcohol–pyridine–water 7:7:6 (v/v/v)	I	3.23	0.80	0.07
n-Butanol–acetic acid–water 12:5:3 (v/v/v)	I	14.20	3.48	0.39

tide. At the present time the identity of the ovine and the bovine COH-inhibiting activities are unproved.

An Inhibiting and a Stimulating Activity of UM-2 and UM-05 Residues on Mouse Hypothalami in Incubation Experiments[3]

Hypothalami incubated in the presence of UM-2 residues (UM-2R) showed a highly significant decrease in the secretion of hypophysiotropic hormones compared with the controls. In addition these hypothalami showed a certain decrease in the hypophysiotropic factors content (Fig. 2).[3] We can only define the molecular weight of the substances in UM-2R as being more than 1000. Hypothalami incubated with UM-05 residues (UM-05R) showed an increased hypophysiotropic activity compared with control experiments (Fig. 3).[3] It seems, therefore, that this factor, which stimulates hypothalamic activity, has a molecular weight >500 and <1000. Thus,

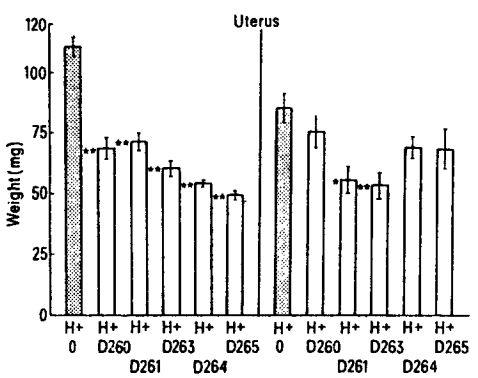

FIG. 2. Weights of the uterus of mice after injection of the incubation liquid (left) and extract (right) of mice hypothalamus incubated with and without a sheep pineal fraction UM-2R. D260, D261, D263, D264, D265 are the codes of five different Sephadex G-25 columns from which UM-2R fractions are prepared. H = hypothalamus; $*P < 5\%$; $**P < 1\%$.

[3] These *in vitro* experiments have been carried out in collaboration with Madame A. Kagan-Moszkowska and co-workers, Laboratoire d'Histophysiologie du Collège de France, Paris, France.

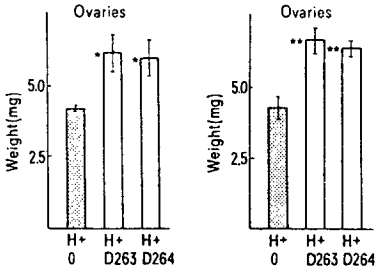

FIG. 3. Weights of the ovaries of mice after injection of the incubation liquid (left) and extract (right) of mouse hypothalamus incubated with and without a sheep pineal fraction UM-05R. D263, D264 are the codes of two different Sephadex G-25 columns from which UM-05R are prepared. $*P < 5\%$; $**P < 1\%$.

we may conclude that sheep pineals contain active principles which differ from melatonin (MW 232) and which are capable of acting via the hypothalamus *in vitro*. For details on the activity of melatonin and other indoles on the hypothalamus see Moszkowska *et al.* (1973).

A Substance with Special Fluorescence Characteristics Isolated from Sheep Pineal Fractions

One of the low-molecular Sephadex G-25 fractions from an aqueous extract of sheep pineals and a comparable fraction of sheep cerebral cortex have been separated in parallel runs on Sephadex G-10. From both materials several distinct peaks are detectable, which show excitation and fluorescence maxima resembling those of indoles. However, in sheep pineal extracts one peak has been observed with a different excitation and fluorescence maximum which has not been observed in sheep cerebral cortex extract (Figs. 4 and 5). Using two-dimensional thin-layer chromatography, a substance designated A has been isolated and purified, showing excitation maxima at 298 nm and 358 nm and a fluorescence maximum at 440 nm (Fig. 6). R_f values of this substance A in seven different solvent systems in thin layer chromatography are presented (Table 6). In paper electrophoresis studies substance A moves slowly to the cathode at pH 3.5 and

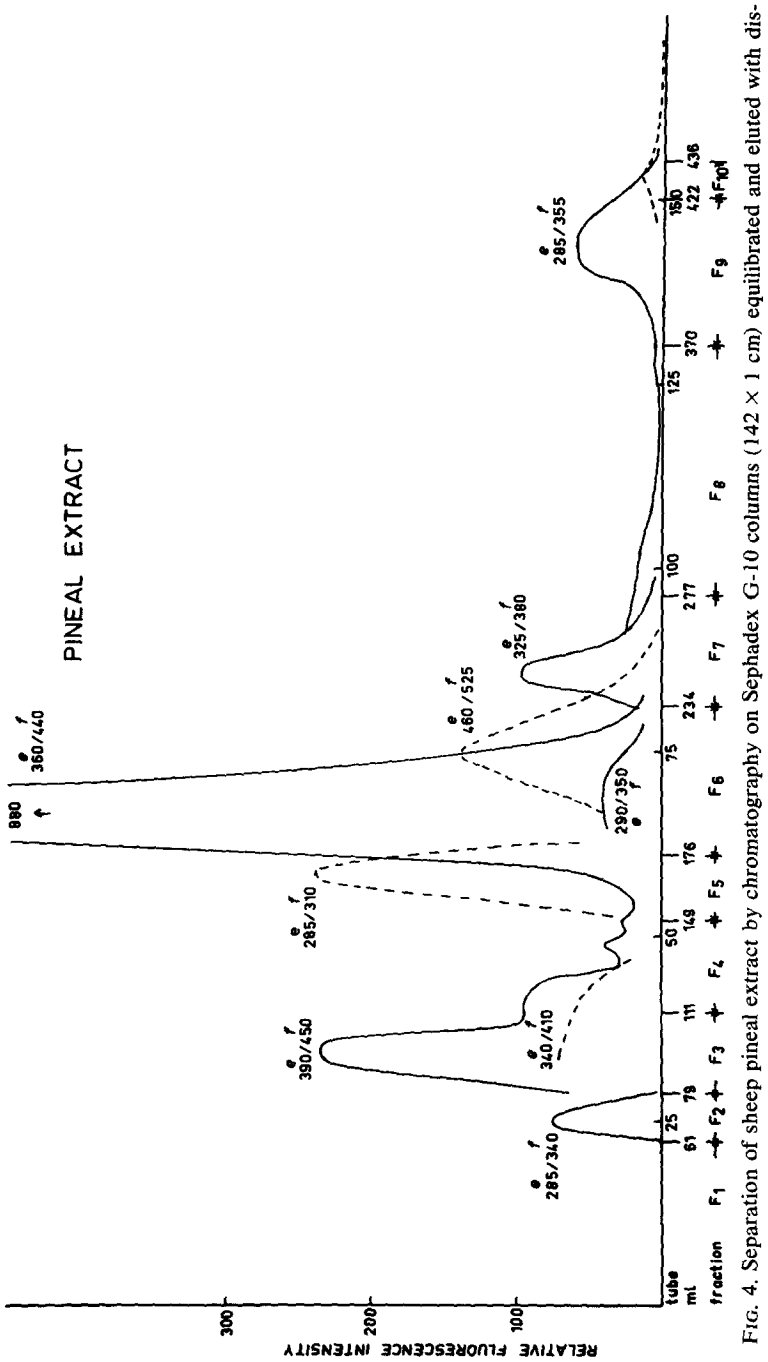

FIG. 4. Separation of sheep pineal extract by chromatography on Sephadex G-10 columns (142 × 1 cm) equilibrated and eluted with distilled water. e = excitation, f = fluorescence.---- = $\frac{e}{f}$ 285/310, $\frac{e}{f}$ 340/410, and $\frac{e}{f}$ 460/525.

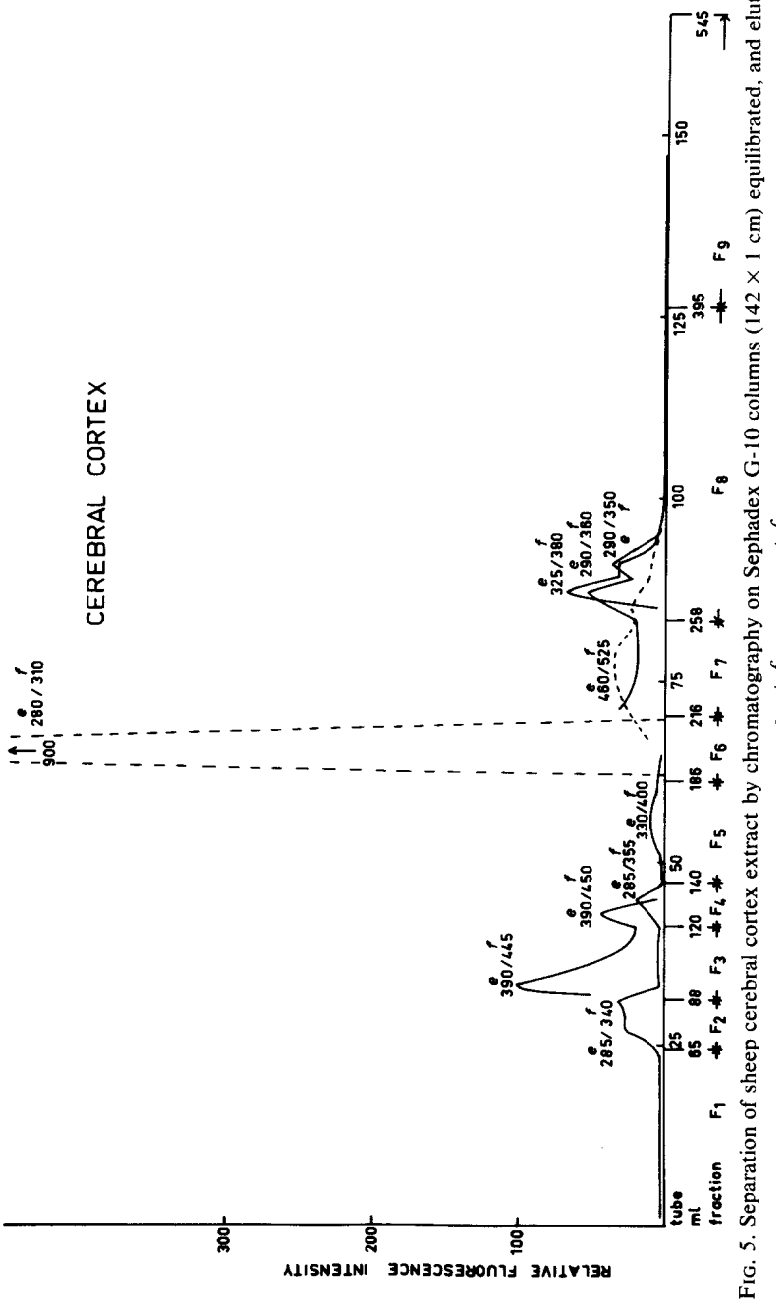

FIG. 5. Separation of sheep cerebral cortex extract by chromatography on Sephadex G-10 columns (142 × 1 cm) equilibrated, and eluted with distilled water. e = excitation; f = fluorescence. ------ = $\frac{e}{280}/\frac{f}{310}$ and $\frac{e}{460}/\frac{f}{525}$.

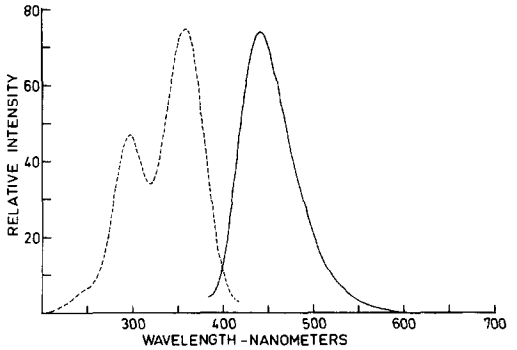

FIG. 6. Excitation (---) and fluorescence (—) spectrum of substance A in distilled water.

pH 6.5. On the nature of substance A and the class of compounds to which it belongs we can only speculate. Ultrafiltration experiments have revealed that substance A can be detected in the UM-05 filtrate, which contains generally substances with molecular weights less than 500. Structures which have excitation and fluorescence maxima comparable to those of substance A are, e.g., pteridines (Kidder and Deweij, 1968), norharman (in acid solution 0.1 *M* H_2SO_4) (Hess and Udenfriend, 1959), harmaline (in alkaline solution) (Villeneuve and Sourkes, 1966). Experiments are in progress to characterize pineal substance A. The results of biological experiments *in vitro* with substance A will be published in the near future.

CONCLUSION

Pineal glands contain biologically active components which are definitely different from melatonin.

ACKNOWLEDGMENTS

The skillful technical assistance of Mrs. A. E. M. Horwitz-Bresser, and Messrs. J. A. Leijendekkers and A. Torronteras Cabezas is gratefully acknowledged.

REFERENCES

BALEMANS, M. G. M., EBELS, I., AND VONK-VISSER, D. M. A. (1970). Separation of pineal extracts on Sephadex G-10. I. A spectrofluorimetric study of indoles in a cockerel pineal extract. *J. Neurovisc. Relat.* **32**, 65–73.

BENSINGER, R., VAUGHAN, M., AND KLEIN, D. C. (1973). Isolation of a non-melatonin lipophylic antigonadotropic factor from the bovine pineal gland. *Fed. Proc. 32/3*, part I, 225.

BENSON, B., MATTHEWS, M. J., AND RODIN, A. E. (1971). A melatonin-free extract of bovine pineal with antigonadotropic activity. *Life Sci.* **10**, 607–612.

BENSON, B., MATTHEWS, M. J., AND RODIN, A. E. (1972). Studies on a non-melatonin pineal antigonadotrophin. *Acta Endocrinol.* **69**, 257–266.

CITHAREL, A., EBELS, I., L'HERITIER, A., AND MOSZKOWSKA, A. (1973). Epiphyseal–hypothalamic interaction. An *in vitro* study with some sheep pineal fractions. *Experientia* **29**, 718–719.

EBELS, I., MOSZKOWSKA, A., AND SCÉMAMA, A. (1965). Etude *in vitro* des extraits épiphysaires fractionnés. Résultats préliminaires. *C. R. Acad. Sci.* **260**, 5126–5129.

EBELS, I., BALEMANS, M. G. M., AND VERKLEIJ, A. J. (1972). Separation of pineal extracts on Sephadex G-10. II. A spectrofluorimetric and thin layer chromatographic study of indoles in a sheep pineal extract. *J. Neurovisc. Relat.* **32**, 270–282.

EBELS, I., BALEMANS, M. G. M., AND TOMMEL, D. K. J. (1972). Separation of pineal extracts on Sephadex G-10. III. Isolation and comparison of extracted and synthetic melatonin. *Anal. Biochem.* **50**, 234–244.

EBELS, I., BENSON, B., AND MATTHEWS, M. J. (1973). Localization of a sheep pineal antigonadotropin. *Anal. Biochem.* **56**, 546–565.

FRASCHINI, F., MESS, B., PIVA, F., AND MARTINI, L. (1968). Brain receptors sensitive to indole compounds: Function in control of luteinizing hormone secretion. *Science* **159**, 1104–1105.

HESS, S. M., AND UDENFRIEND, S. (1959). A fluorometric procedure for the measurement of tryptamine in tissues. *J. Pharmacol. Exp. Ther.* **127**, 175–177.

KIDDER, G. W., AND DEWEIJ, V. C. (1968). A new pteridine from tetrahymena. *J. Biol. Chem.* **243**, 826–833.

MCISAAC, W. M., TABORSKY, R. G., AND FARRELL, G. (1964). 5-Methoxytryptophol: Effect on estrus and ovarian weight. *Science* **145**, 63–64.

MATTHEWS, M. J., AND BENSON, B. (1973). Inactivation of pineal antigonadotrophin by proteolytic enzymes. *J. Endocrinol.* **56**, 339–340.

MOSZKOWSKA, A., AND EBELS, I. (1971). The influence of the pineal body on the gonadotropic function of the hypophysis. *J. Neurovisc. Relat. Suppl.* **X**, 160–176.

MOSZKOWSKA, A., SCÉMAMA, A., LOMBARD, M. N., AND HÉRY, M. (1973). Experimental modulation of hypothalamic content of the gonadotropic re-

leasing factors by pineal factors in the rat. *J. Neural. Transm.* **34,** 11–22.

REITER, R. J., AND FRASCHINI, F. (1969). Endocrine aspects of the mammalian pineal gland: A review. *Neuroendocrinology* **5,** 219–255.

SORRENTINO, S., JR., AND BENSON, B. (1970). Effects of blinding and pinealectomy on the reproductive organs of adult male and female rats. *Gen. Comp. Endocrinol.* **15,** 242–246.

VILLENEUVE, A., AND SOURKES, T. L. (1966). Metabolism of harmaline and harmine in the rat. *Rev. Can. Biol.* **25,** 231–239.

WURTMAN, R. J., AXELROD, J., AND CHU, E. W. (1963). Melatonin, a pineal substance effect on the rat ovary. *Science* **141,** 277–278.

ZURBURG, W., AND EBELS, I. (1974). Separation of pineal extracts by gel filtration. I. Isolation from sheep pineals of a substance with special fluorescence characteristics. *J. Neural. Transm.* **35,** 117–124.