PARTIAL PURIFICATION AND CHARACTERIZATION OF ANTIGONADOTROPIC ACTIVITY OF BOVINE PINEAL GLANDS

By

ROBERT WAYNE POE

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Southwest Missouri State University

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Thesis Approved:

Ja-hsin Liao

Thesis Adviser

George V. Odell

Richard J. Arts

Norman D. Durham

Dean of Graduate College

PREFACE

This study is concerned with the purification and characterization of an antigonadotropin from bovine pineal glands. The biological assay is based on the compensating increase in rate of growth of the intact ovary after removal of the one ovary. The inhibition of such compensatory growth was assumed to be due to the antigonadotropin. All purification steps were based on the assumption that the antigonadotropin has peptide characteristics. Thus, each purification step was monitored by the standard absorption wave-lengths of 280 nm and 220 nm.

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LIST OF SYMBOLS AND ABBREVIATIONS

COH	– compensatory ovarian hypertrophy
PITC	– phenylisothiocyanate
TFA	– trifluoroacetic acid
DNP	– dinitrophenyl group
g-eq	– gram-equivalent
DNS	– Dansyl group (N, N-dimethyl-1-naphthylamine-5-sulfonyl)
R_L	– ratio of migration distance of ninhydrin-positive spot and that of lysine from origin
S.E.	– standard error
NS	– not significant
t	– student's t test evaluation
p	– level of significance based on distribution of t

CHAPTER I

INTRODUCTION

In 1934 Paul Engel (1) prepared alkaline extracts of pineal glands from various species and found that when immature female rats were injected with the extract and FSH or LH the extract inhibited the action of the gonadotropin. By 1939 Engel (2) had suggested that two hormones were present in the pineal: one with inhibiting effects and the other with stimulatory effects upon the gonadotropins. This hypothesis was based upon his results and those of E. Fischer (3) and the contradictory work of Vinals (4) and Wade (1).

More recent work concerning the pineal gland with regard to endocrine and physiological functions has been documented by Reiter and Fraschini in 1969 (5), Reiter in 1972 (6) and Quay in 1974 (7). These reviews present the evidence of pineal antigonadotropic activity, but little is known of the mode of action or of the compound(s) itself.

In 1958 Lerner and colleagues (8) isolated melatonin (5-methoxy-N-acetyltryptamine) from the pineal and in 1959 (9) elucidated its structure. Wurtman (10), in 1967, was the first to attribute the antigonadotropic activity of the pineal to melatonin. Although melatonin does exhibit this property, the amount needed to elicit an antigonadotropic response of the magnitude that has been detected by various bioassays with pineal extracts excluding melatonin is much larger than the amount found in the pineal gland (11).

The compounds 5-hydroxytryptophol, 5-methoxytryptophol, 5-methoxytryptamine, serotonin and other 5-methoxy indole derivatives have received attention as being pineal hormones by McIsaac (12, 13) and others (14) because the compounds are contained in and synthesized by the pineal. There is evidence, however, that pineal extract fractions of molecular weight greater than that of indoles have antigonadotropic activity (15).

Benson (16), Orts (17), Ebels (18, 19) and others (20) have shown that pineal extracts free from melatonin exhibit biological activity on the reproductive organs. They believe that a peptide is the active component since ninhydrin-positive fractions which contain activity become inactive after incubation with proteolytic enzymes (21). Polypeptides structurally similar to oxytocin and vasopressin have been isolated from bovine and porcine pineals (22, 23, 24). Of these, arginine-vasotocin has been considered as a possible antigonadotropin (25, 26), but Benson et al. (27) has shown that it does not inhibit COH in mice, and other investigators have shown other differences between this peptide and pineal extracts (11, 23, 28, 29).

In 1963 Moszkowska first demonstrated COH inhibition with aqueous pineal gland extracts in the guinea pig (30), and in the same year Reiss et al. reported inhibition of COH in rats with a suspension of dried bovine pineal glands (31). This assay has been utilized to measure antigonadotropic activity since it depends upon a rise in gonadotropins (32, 33, 34).

Ebels (35) has reported that an aqueous sheep pineal extract inhibits COH in unilaterally ovarectomized adult mice. After separation on G-25, two low molecular weight fractions contained activity. These two

fractions were each ultrafiltered through Amicon Diaflo membranes UM-2 and UM-05. Activity was found in the residue of the UM-05 membrane which was then placed on G-10. An active component eluted well before synthetic melatonin.

In 1974 Benson, Orts and Cook (16) showed COH inhibition in aqueous extracts of bovine pineal glands. This extract was then ultrafiltered and the fraction of molecular weights less than 1000 was active. This fraction was then filtered on G-25 to exclude melatonin and extraneous protein. A low molecular weight fraction contained the biological activity. A cerebral extract was purified in the same manner and did not contain the pineal activity, providing evidence for the pineal specificity of the antigonadotropin.

Rat pineal glands (12, 36) and human pineal glands (37), when extracted and put on G-25, contain COH inhibiting activity in the same elution volume as seen with bovine pineals.

The present study was undertaken to further purify and characterize the active component of the bovine pineal gland.

CHAPTER II

MATERIALS AND METHODS

Materials

Sephadex G-25 (fine) was from Pharmacia. Analytical grade polystyrene cation exchanger (AG 50 W-x8, 200-400 mesh, $[H^+]$) was from Bio-Rad Laboratories. Sequanal grades of pyridine, PITC, TFA (anhydrous) and sodium citrate buffer (pH 2.2) and Cheng-Chin Polyamide layer sheets were supplied by Pierce Chemical Company. Dansyl chloride, thioglycolic acid, 2,4-N-DNP-L-Alanine, Blue Dextran, L-tyrosine and L-tryptophan were from Sigma Chemical Company.

Methods

Pineal Crude Extract

Fresh frozen bovine pineal glands were lyophilized and then put in five volumes of acetone with constant stirring for 30 minutes at room temperature. The residue was filtered over vacuum, stirred with one volume of acetone for 15 minutes and filtered again. The defatted glands were then dried in a vacuum oven at 50°C for 3 hours.

The glands were then homogenized with a glass Waring blender (model 5010) in 1.5 volumes of 2.0 N acetic acid and stirred for 1 hour at room temperature. The homogenate was centrifuged at 16300 x g for 1 hour and the supernatant was lyophilized. The residue was dissolved in 100 ml of

glacial acetic acid, stirred 45 minutes at room temperature and centrifuged at 1200 x g for 30 minutes after which the supernatant was diluted in 100 ml H_2O and lyophilized. The extract was then redissolved in 10 ml of 0.1 N acetic acid and centrifuged at 48200 x g for 1 hour. The supernatant of this step is referred to as "pineal crude extract".

Gel-filtration

System I. Sephadex G-25 (fine) (1.8 x 61 cm) equilibrated with 0.1 N acetic acid at 4°C. The eluant was monitored at 206 nm and 254 nm continuously by a LKB flow-through UV monitor (2089 Unicord III). The flow rate was 45 ml/hour and 3.7 ml fractions were collected. Fractions were pooled into 250 ml round-bottom flasks and lyophilized.

System II. Sephadex G-25 (fine) (1.55 x 94 cm) was equilibrated with 1.0% NH_4HCO_3 at room temperature. The eluant was monitored at 220 nm and 280 nm on a Perkin-Elmer Hitachi spectrophotometer; 2.1 ml fractions were collected. Blue Dextran was eluted at 81 ml and the DNP-alanine peak was eluted at 226 ml. Pyridine was eluted at 192 ml and tyrosine at 185 ml. Tryptophan eluted in a broad peak at 225 ml. Fractions were pooled into 250 ml round-bottom flasks and lyophilized.

Ion Exchange Chromotography

Analytical grade polystyrene cation exchange resin was used after washing with 2 N NaOH, H_2O, 2 N HCl and H_2O. The sample was dissolved in 0.06 M acetic acid (pH 3) and loaded onto a column of the resin in H^+ form. Elution was carried out by step-wise fashion at room temperature. Eluents were collected in separate 250 ml round-bottom flasks and lyophilized. The ion exchange systems used are shown in Table I.

High-voltage Paper Electrophoresis

The Savant flat plate model FP22A was used in all electrophoresis experiments at a voltage of 2500 V or 50 V/cm. Unless otherwise indicated, all of the electrophoretic separations were performed at about 10°C and in 30 minutes. The three solvent systems used are as follows:

System I pH 6.5 - pyridine:acetic acid:H_2O = 100:4:900

System II pH 3.5 - pyridine:acetic acid:H_2O = 10:100:890

System III pH 1.9 - formic acid:acetic acid:H_2O = 25:100:875

Whatman 3 MM filter paper was used throughout. Guide strips were stained with Cd-Ninhydrin (38), and materials on the paper were eluted with 10% acetic acid.

Dansylation and N-terminal Determination

Dansylation was carried out as described by Grey (39). The following solvent systems were used for the separations of Dansylamino acids, according to the thin layer chromatographic procedure of Woods and Wang (40) with Cheng-Chin polyamide sheets:

First dimension - H_2O:90% formic acid = 200:3 (50 min)

Second dimension - Benzene:acetic acid = 9:1 (1 hour)

OR

Heptane:butanol:acetic acid = 3:3:1 (90 min)

Protein Determination

The amount of proteins in each fraction was determined as total amino acid contents measured on an amino acid analyzer (41) after acid hydrolysis.

Acid Hydrolysis

To each dried sample to be analyzed was added 100 μl of 6 N HCl containing 0.25% phenol and 1% thioglycolic acid. The tubes (10 x 75 mm, Pyrex No. 9820) were evacuated and sealed. After 22 hours at 110°C, the acid was removed *in vacuo* and the dried sample was dissolved in sodium citrate buffer, pH 2.2, and analyzed.

COH Assay

Charles River CD-1 female mice were used in all assays. They were received at six weeks of age and were kept in light:dark 14:10 hours at 25°C and received standard laboratory chow and water *ad libitum*. All mice were between seven and eight weeks old when used in the assay. The mice were unilaterally ovarectomized under light ether anesthesia and the left ovaries weighed to the nearest 0.1 mg. A 0.1 ml IP injection of pineal extract was given to each animal in test groups and 0.1 ml saline to the control animals. The dosage of extract varies with some experiments, but 2.5 gram-equivalents* was the usual dose for each animal. The animals were terminated on post-operative day six and the remaining ovary weighed. The percentage increase in weight of the right ovary over the left ovary was given as percent (%) COH. Any inhibitory action could, therefore, give a negative % COH. Statistical comparisons were made by the student's *t* test of the mean % COH of each fraction tested. Differences in control values of different assays can in part be due to age differences of the mice used.

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*One gram-equivalent is defined as the amount of material obtained from one gram of wet whole glands.

Peptide Sequence Analysis

The Edman degradation procedure of Gray (42) was used to determine the sequence of peptides. N-terminal amino acids were, however, identified by the substractive fashion; samples were analyzed for amino acid composition after each step of Edman degradation, and the decrease in an amino acid was considered as the amino acid removed from the N-termini.

CHAPTER III

RESULTS AND DISCUSSION

Gel-filtration in Acidic Medium Followed by Ion Exchange

Experiments I through III were tabulated in Table I to show the elution conditions used. In these experiments gel-filtration was used prior to ion exchange chromatography.

The bovine pineal crude extract was gel-filtered in System I. The elution profile is shown in Figure 1. The F3, F4 and F5 fractions were assayed for COH inhibition (Table II) using dosages of 2.5 gram-equivalents and 1.0 gram-equivalent for each animal.

F4 and F5 contained activity and were purified further on paper electrophoresis at pH 6.5 and pH 1.9. The electrophorogram at pH 1.9 indicated that F5 contained large amounts of free amino acids. At pH 6.5, F4 and F5 were separated into three sections--basic, neutral and acidic--using a standard amino acid mixture as a guide strip. Each of these fractions were assayed (Table III) and activity was found in the basic and neutral regions of F4 and in the acidic region of F5. The dosages were calculated by assuming 80% recovery from elution of the paper. No activity was seen in any of the fractions at pH 1.9, possibly due to inactivation by formic acid.

Since the most significant biological activity was in the basic region of F4, the compound must bear a positive charge at pH 6.5;

therefore, the method to use cation exchanger was developed as follows.

In Experiment I (Table I) F4 was chromatographed: the COH inhibition activity was eluted with 1 M pyridine, but not with H_2O as shown in Table IV. In Experiment II F4 was chromatographed by increasing the pH from 3 to 7 and the molarity of pyridine from 0.2 to 1.0. This experiment resulted with the activity (Table V) being found in pH 5.0, 0.2 M pyridine acetate. To further purify the substance, Experiment III was carried out, and active fractions were eluted between pH 4.0 and pH 5.0, 0.2 M pyridine acetate, in 0.2 pH unit increments. The activity was found in the pH 4.4 fraction (Table VI). This fraction was further purified on paper electrophoresis at pH 6.5. The resulting paper was separated into three fractions corresponding to acidic, neutral and basic regions based on mobilities of a standard amino acid mixture. Table VII shows that the neutral fraction was active.

In another experiment, the pH 4.4 fraction was separated by paper electrophoresis at pH 6.5 and seven fractions were obtained, each of which was put on the amino acid analyzer in both hydrolyzed and unhydrolyzed forms. These analyses showed that each fraction contained peptides because unhydrolyzed samples did not show significant amounts of free amino acids on the analyzer.

Ion Exchange Chromatography Followed by Gel-filtration in Basic Medium

Because of problems of gel-formation of the crude extract in acidic medium, which made it difficult to chromatograph on G-25, it was decided to fractionate the crude extract with ion exchanger prior to gel-filtration. The bovine pineal crude extract was applied directly to the

polystyrene ion exchanger and eluted as described in Experiments IV through VI in Table I.

In Experiment IV the pH 4.4 fraction showed no activity in inhibiting COH. Since this fraction had previously contained activity, it was assumed that a stimulatory factor with respect to COH co-purifies at pH 4.4, suppressing the COH inhibition, whereas previously the G-25 purification step separated these two substances. On the basis of these observations, these two substances are of different molecular weight, but have similar charges at pH 4.4.

This non-active pH 4.4 fraction was further fractionated by paper electrophoresis at pH 6.5 for one hour. The paper was cut into seven fractions and each fraction was placed on the amino acid analyzer in hydrolyzed and unhydrolyzed forms. Many more peptides were seen in these fractions as compared to the previous pH 4.4 fraction which contained activity.

Table VIII shows the results of the COH assay of the fractions obtained from Experiment V. Two age groups of mice were used in two separate assays, and the activity appeared in the 1.0 M pyridine fraction in both assays. Previously, when gel-filtration was used prior to ion exchange, the 1.0 M pyridine fraction contained no activity. The active substance that appeared in this fraction must have been absent in the F4 fraction, and it may be of different molecular weight than that of F4. It should also be noted from Table VIII that the H_2O fraction and the pH 4.0 fraction showed significant stimulation of COH and the pH 5.0 fraction also contained inhibitory activity, though not as significant as the 1.0 M pryidine fraction.

In Experiment VI a larger amount of sample was used, and the 1.0 M pyridine fraction was gel-filtered in System II. Figure 2 shows the elution profile obtained. Table IX shows the results of the assay of different fractions from gel-filtration: FIII and FIV were active. These two fractions were then Dansylated as described by Gray (39) and individually separated further by paper electrophoresis at pH 1.9 for 2 hours. The basic spots were eluted and then hydrolyzed; the resulting Dansyl amino acids were analyzed on polyamide thin layer plates. The results showed that eluted samples were composed of a mixture of peptides. Among those N-terminals were DNS-lysine, DNS-phenylalanine and DNS-glutamic acid. FIII and FIV showed similar N-terminal DNS-amino acids.

The FIII fraction was further purified on paper electrophoresis at pH 6.5 for 45 minutes and cut into five fractions based on ninhydrin-stained guide strips of FIII and a standard amino acid mixture. Fractions 4 and 5 contained activity as seen in Table X. These fractions have similar electrophoretic mobilities to that of histidine. The most basic spot from the electrophorogram was identified as free arginine by amino acid analysis of the unhydrolyzed sample.

Fraction 4 was purified again on paper electrophoresis at pH 3.5 for 1 hour and separated into fractions A and B, which have R_L values of 0.22 and 0.18 respectively. Fraction B, but not A, was found to be active (Table XI).

Another batch of FIII from G-25 was separated on paper electrophoresis at pH 6.5 for 1 hour into 4A and 4B fractions, 250 gram-equivalents, which were hydrolyzed and placed on the amino acid analyzer. Certain amino acid residues were in integral ratios, indicative of another simple mixture of peptides. Table XII shows the amino acid contents

of these two fractions as well as those of the first and second cycles of subtractive Edman degradation of the mixture of peptides.

Fraction 4A (R_L = 0.54) probably contains a tri-peptide Phe-Gln-Lys as deduced from these procedures. It is seen that in fraction 4A Phe:Gln:Lys are in an integral ratio at the outset. After the first step of the Edman degradation, phenylalanine was completely lost. The fact that the lysine residue is slightly diminished may be due to PITC reaction with the ϵ-amino group and its subsequent cleavage upon acid hydrolysis. In the second step, glutamic acid has only reduced in half. The incomplete removal of this residue may be due to partial cyclization of glutamine (43). Glutamine is considered to be in the peptide instead of glutamic acid because of the basic nature of the peptide.

A second peptide that could be deduced from Table XII is Leu-His-Ser, but due to the unreliability of histidine residues in this procedure (44) and the accumulation of serine during Edman degradation (43), coupled with the small sample operation, the structure is not as certain as that of Phe-Glu-Lys peptide.

Fraction 4B, which contains active material, is a much too complex mixture to be analyzed at this stage of purification. A peptide with Thr-Arg sequence is fairly clear, but the remainder of the sequence is only speculated to be leucine, glutamine or both. However, whether this is the peptide that represents activity is still not clear.

The discovery of the tri-peptide in fraction 4A leaves the task of discovering its biological activity; since it is found in the pineal gland, it could function in hormonal control. The fact in fraction 4B that the amino acid composition indicates that it is a mixture of peptides lends further evidence to a peptide hormone being responsible for the antigonadotropic activity of the pineal.

TABLE I

ELUTION OF PINEAL GLAND CRUDE EXTRACTS
FROM CATION EXCHANGER**

Experiment	Sample	Column Size, cm	Eluants
I	50 g-eq F4*	0.5 x 6.0	10ml H_2O, 10ml 1 M pyridine
II	90 g-eq F4*	0.8 x 11.5	50ml each: H_2O, 0.2 M pyridine acetate: pH 3, 4, and 5; 0.5 M pyridine acetate: pH 6, 7; 1 M pyridine
III	100 g-eq F4*	0.8 x 16.0	100ml each: H_2O, 0.2 M pyridine acetate: pH 4.2, 4.4, 4.6, 4.8 and 5.0
IV	200 g-eq crude extract	0.8 x 19.5	200ml each: H_2O, 0.2 M pyridine acetate: pH 4.0 and 4.4
V	195-300 g-eq crude extract	1.90 x 2.75	75ml H_2O, 50ml pH 4.0', 100 ml pH 5.0', 75ml 1 M pyridine
VI	600-700 g-eq crude extract	1.90 x 4.0	75ml H_2O, 75ml pH 4.0', 125ml pH 5.0', 75ml 1 M pyridine

* gram-equivalents of the F4 fraction from gel-filtration, System I
' 0.2 M pyridine acetate

** In Experiments I through III active samples were chromatographed by gel-filtration in System I; whereas, in Experiment VI they were chromatographed in System II. In Experiments I through III, gel-filtrations were carried out prior to ion-exchange; in Experiment VI the process was reversed.

TABLE II

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY
OF GEL-FILTRATION SYSTEM I FRACTIONS

Fractions	Mean % COH ± S.E.	t	p
Assay I (2.5 g-eq*)			
Saline	54.4 ± 9.18		
F3	48.0 ± 8.05	0.530	NS
F4	21.4 ± 6.80	2.90	<0.010
F5	25.8 ± 6.28	2.58	<0.025
Assay II (1.0 g-eq*)			
Saline	51.4 ± 4.00		
F3	30.2 ± 5.30	3.21	<0.005
F4	8.0 ± 4.50	7.23	<0.001

* g-eq designates gram-equivalents injected IP/animal

TABLE III

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF F4 AND F5 ELECTROPHORESIS FRACTIONS

pH	Fraction		R_L*	Mean % COH ± S.E.	t	p
	Saline			61.9 ± 12.4		
6.5	F4	1	1.08 to 0.19	19.8 ± 11.2	2.52	<0.025
		2	0.19 to (-0.08)	26.9 ± 10.1	2.20	<0.050
		3	-0.08 to (-1.14)	49.8 ± 6.0	0.88	<0.400 (NS)
	F5	1	0.89 to 0.20	10.4 ± 21.9	2.05	<0.100 (NS)
		2	0.20 to (-0.09)	37.2 ± 11.2	1.47	NS
		3	-0.09 to (-1.09)	15.4 ± 10.9	2.81	<0.025
1.9	F4	1	0.94 to 0.58	48.1 ± 30.5	0.42	NS
		2	0.58 to 0.07	57.0 ± 12.2	0.28	NS
		3	0.07 to (-0.14)	76.1 ± 15.7	0.71	<0.500 (NS)
	F5	1	1.14 to 0.52	58.1 ± 11.9	0.22	NS
		2	0.52 to 0.31	49.1 ± 18.1	0.58	NS
		3	0.31 to (-0.10)	30.3 ± 10.4	1.95	<0.100 (NS)

(44-day-old mice were used)

* R_L = ratio of migration distance of ninhydrin-positive fraction and that of lysine from origin.

TABLE IV

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS OF F4 AND F5 FROM EXPERIMENT I

Fraction	Number of mice	Mean % COH ± S.E.	t	p
Saline	15	66.3 ± 10.9		
F4 H_2O	8	48.7 ± 11.7	1.10	<0.400 (NS)
F4 Pyridine	12	30.9 ± 13.4	2.05	<0.100 (NS)
F5 H_2O	12	69.2 ± 10.8	0.19	NS
F5 Pyridine	11	66.3 ± 18.0	0.00	NS

(Dosages were calculated to be 3.0 gram-equivalents.)

TABLE V

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS OF F4 FROM EXPERIMENT II

Fraction	Number of mice	Mean % COH ± S.E.	t	p
Assay I				
Saline	9	21.4 ± 4.7		
pH 3*	10	42.2 ± 13.4	1.46	<0.200 (NS)
pH 4*	10	36.8 ± 6.9	1.84	<0.100 (NS)
pH 5*	10	13.7 ± 6.8	0.93	<0.400 (NS)
pH 6°	10	47.8 ± 14.3	1.75	<0.100 (NS)
pH 7°	10	34.0 ± 7.0	1.49	<0.200 (NS)
1 M pyridine	10	34.7 ± 11.0	1.11	<0.400 (NS)
Assay II				
Saline	7	46.3 ± 9.6		
pH 3*	7	41.4 ± 9.0	0.37	NS
pH 5*	8	38.0 ± 8.8	0.64	NS

* = 0.2 M pyridine acetate

o = 0.5 M pyridine acetate

TABLE VI

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS OF F4 FROM EXPERIMENT III

Fraction	Number of mice	Mean % COH ± S.E.	t	p
Saline	8	29.2 ± 2.7		
F4 pH 4.2	6	31.6 ± 4.5	0.457	NS
4.4	8	13.9 ± 7.5	1.92	<0.100 (NS)
4.6	9	25.2 ± 3.8	0.869	NS
4.8	9	39.7 ± 3.4	2.42	<0.050
5.0	7	27.7 ± 10.1	0.144	NS

TABLE VII

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF F4 FROM ELECTROPHORESIS AT pH 6.5

Fraction	R_L*	Number of mice	Mean % COH ± S.E.	t	p
Saline		9	44.8 ± 5.7		
F4 (4.4) 1	1.06 to 0.16	7	34.6 ± 5.2	1.32	NS
2	0.16 to (-0.06)	9	28.8 ± 3.5	2.42	<0.050
3	-0.06 to (-1.09)	7	39.2 ± 2.9	0.888	NS

(1 = basic; 2 = neutral; 3 = acidic fraction)

R_L* = ratio of migration distance of ninhydrin-positive fraction and that of lysine from origin.

TABLE VIII

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS OF PINEAL CRUDE EXTRACT FROM EXPERIMENT V

Fraction	Number of mice	Mean % COH ± S.E.	t	p
Assay I*				
Saline	10	44.7 ± 4.37		
H_2O	8	52.4 ± 1.93	2.66	<0.025
4.0	6	52.3 ± 5.99	1.02	NS
5.0	9	30.6 ± 4.62	2.22	<0.050
1 M	10	21.1 ± 5.77	3.27	<0.005
Assay II≠				
Saline	9	68.8 ± 13.3		
H_2O	9	109 ± 12.5	2.52	<0.025
4.0	9	95.8 ± 11.9	1.83	<0.100
5.0	8	35.7 ± 11.6	1.88	<0.100
1 M	9	31.8 ± 8.1	2.07	<0.050

* = 38-day-old mice

≠ = 45-day-old mice

TABLE IX

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS FROM GEL-FILTRATION SYSTEM II

Fractions	Number of mice	Mean % COH ± S.E.	t	p
Saline	8	64.8 ± 12.3		
1 M I	10	64.0 ± 10.3	0.050	NS
II	10	50.8 ± 13.2	0.777	NS
III	10	40.4 ± 6.4	1.76	<0.100
IV	10	39.0 ± 7.0	1.82	<0.100
V	10	46.5 ± 13.2	1.01	NS

TABLE X

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS FROM ELECTROPHORESIS AT pH 6.5

Dosage, g-eq*	Fraction	R_L**	Number of mice	Mean % COH ± S.E.	t	p
	Saline		9	63.5 ± 10.8		
1.0	1M-III-1	1.06 to 0.83	9	53.4 ± 13.3	0.590	NS
2.5			9	47.3 ± 18.3	0.764	NS
1.0	1M-III-2	0.83 to 0.67	9	57.8 ± 12.3	0.349	NS
2.5			9	54.5 ± 14.1	0.508	NS
1.0	1M-III-3	0.67 to 0.59	9	38.6 ± 11.5	1.58	NS
2.5			8	74.7 ± 12.5	0.768	NS
1.0	1M-III-4	0.59 to 0.49	8	51.8 ± 13.3	0.684	NS
2.5			8	29.3 ± 6.2	2.75	<0.025
1.0	1M-III-5	0.49 to 0.13	9	71.7 ± 11.4	0.522	NS
2.5			7	23.1 ± 10.0	2.74	<0.025

* g-eq = gram-equivalents injected IP/animal

**R_L = ratio of migration distance of ninhydrin-positive fraction and that of lysine from origin.

TABLE XI

COMPENSATORY OVARIAN HYPERTROPHY (COH) ASSAY OF FRACTIONS FROM ELECTROPHORESIS AT pH 3.5

Dosage	Fraction	R_L**	Number of mice	Mean % COH ± S.E.	t	p
0.1 cc	Saline		9	40.1 ± 12.4		
2.5 g-eq*	A	0.22	10	40.0 ± 9.6	0.006	NS
2.5 g-eq*	B	0.18	8	20.0 ± 9.4	1.29	NS

** R_L = ratio of migration distance of ninhydrin-positive spot and that of lysine from origin.

* g-eq = gram-equivalents injected IP/animal

TABLE XII

SUBTRACTIVE EDMAN DEGRADATION
OF FRACTION 4A

	4A hydrozylate, 10^{-8} moles	1st Edman Cycle hydrozylate, 10^{-8} moles	2nd Edman Cycle hydrozylate, 10^{-8} moles
Asp	0.00	7.00	11.38
Thr	7.20	2.76	5.10
Ser	33.2 (0.4)*	21.1 (0.5)*	19.1 (0.4)*
Glu	84.8 (1.0)*	46.8 (1.0)*	24.0 (0.5)*
Gly	20.9	15	31.3
Leu	32.8 (0.4)*	7.8 (0.2)*	9.4 (0.2)*
His	32.7 (0.4)*	24 (0.5)*	21.3 (0.5)*
Lys	89.9 (1.1)*	32.4 (0.7)*	23.5 (0.5)*
Arg	45.3 (0.5)*	59.7 (1.3)*	21.6 (0.5)*
Phe	77.8 (0.9)*	0 (0.0)*	0 (0.0)*

* = ratio to glutamine

Summary of the Edman Degradation in Table XII:

	Peptide 4A-I			Peptide 4A-II		
	Phe	Gln	Lys	Leu	His	Ser
Composition	0.9	1.0	1.1	0.4	0.4	0.4
Edman Degrad. (Step I)	0.0	1.0	0.7	0.2	0.5	0.5
Edman Degrad. (Step II)		0.5	0.5		0.5	0.4

TABLE XIII

SUBTRACTIVE EDMAN DEGRADATION
OF FRACTION 4B

	4B hydrozylate, 10^{-8} moles	1st Edman Cycle hydrozylate, 10^{-8} moles	2nd Edman Cycle hydrozylate, 10^{-8} moles
Asp	16.9	14.4	16.9
Thr	31.2 (1.2)*	10.9 (0.4)*	7.72 (0.4)*
Ser	63.0 (2.3)*	56.8 (1.9)*	33.3 (1.6)*
Glu	27 (1.0)*	30.1 (1.0)*	21.4 (1.0)*
Gly	27.7	36.9	36.5
Met	4.61 (0.2)*	3.30 (0.1)*	1.64 (0.1)*
Ile	7.67 (0.3)*	4.66 (0.2)*	4.26 (0.2)*
Leu	33.0 (1.2)*	24.5 (0.8)*	16.0 (0.8)*
Tyr	42.4 (1.6)*	32.1 (1.1)*	17.0 (0.8)*
Phe	12.3 (0.5)*	2.52 (0.1)*	1.68 (0.1)*
His	51.9 (1.9)*	36.2 (1.2)*	23.6 (1.1)*
Lys	57.9 (2.1)*	27.2 (0.9)*	14.1 (0.7)*
Arg	28.8 (1.1)*	25.4 (0.8)*	1.66 (0.1)*

* = ratio to glutamine

Summary of the Edman Degradation in Table XIII

Peptide 4B-I

	Thr	Arg	(Leu	Gln)
Composition	1.15	1.06	1.22	1.00
Edman Degrad. (Step I)	0.36	0.84	0.81	1.00
Edman Degrad. (Step II)		0.08	0.75	1.00

TABLE XIV

SUMMARY OF PURIFICATION OF ANTIGONADOTROPIC PEPTIDE FROM PINEAL GLAND

Amount Analyzed, g-eq*	Sample	Total Free Amino Acids, nmoles	Free Amino Acids, nmoles/g-eq*	Purification (fold)	Purification Step
0.13	crude	570.7	4259		
20.0	1M	1162	58.1	73.3	cation exchange
20.0	1M-III	97.17	4.86	876	G-25
20.0	1M-III-4	2.16	0.108	39440	Elect.6.5
50.0	1M-III-4B	0.83	0.017	250500	Elect.3.5

* g-eq = gram-equivalents injected IP/animal

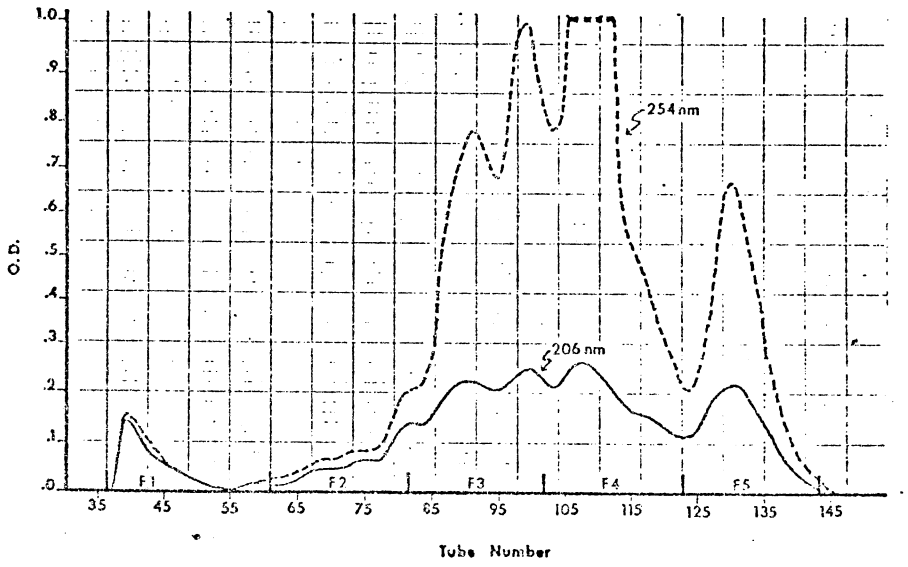


Figure 1. The Elution Profile of the Pineal Crude Extract from the Gel-filtration System I.

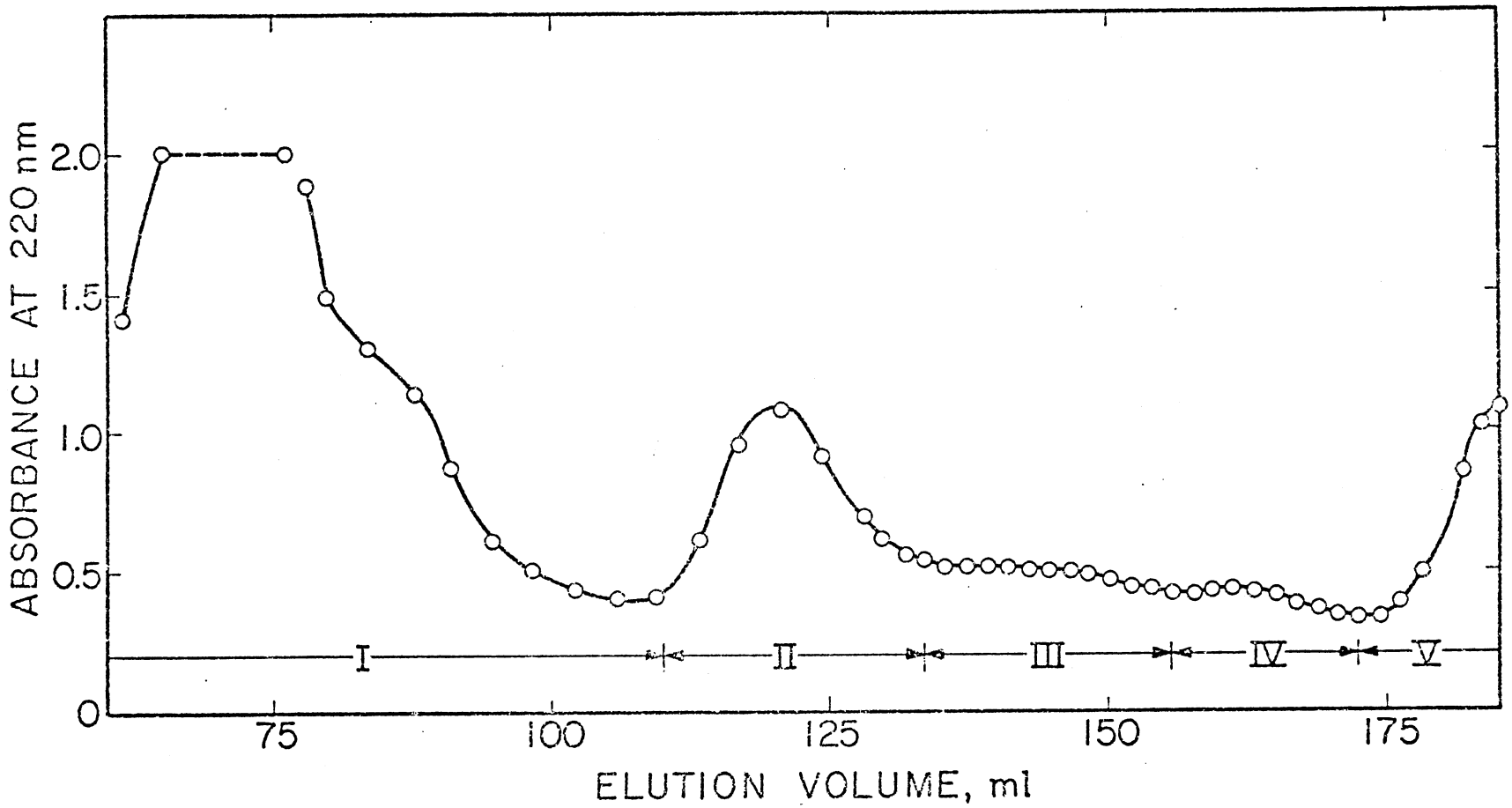
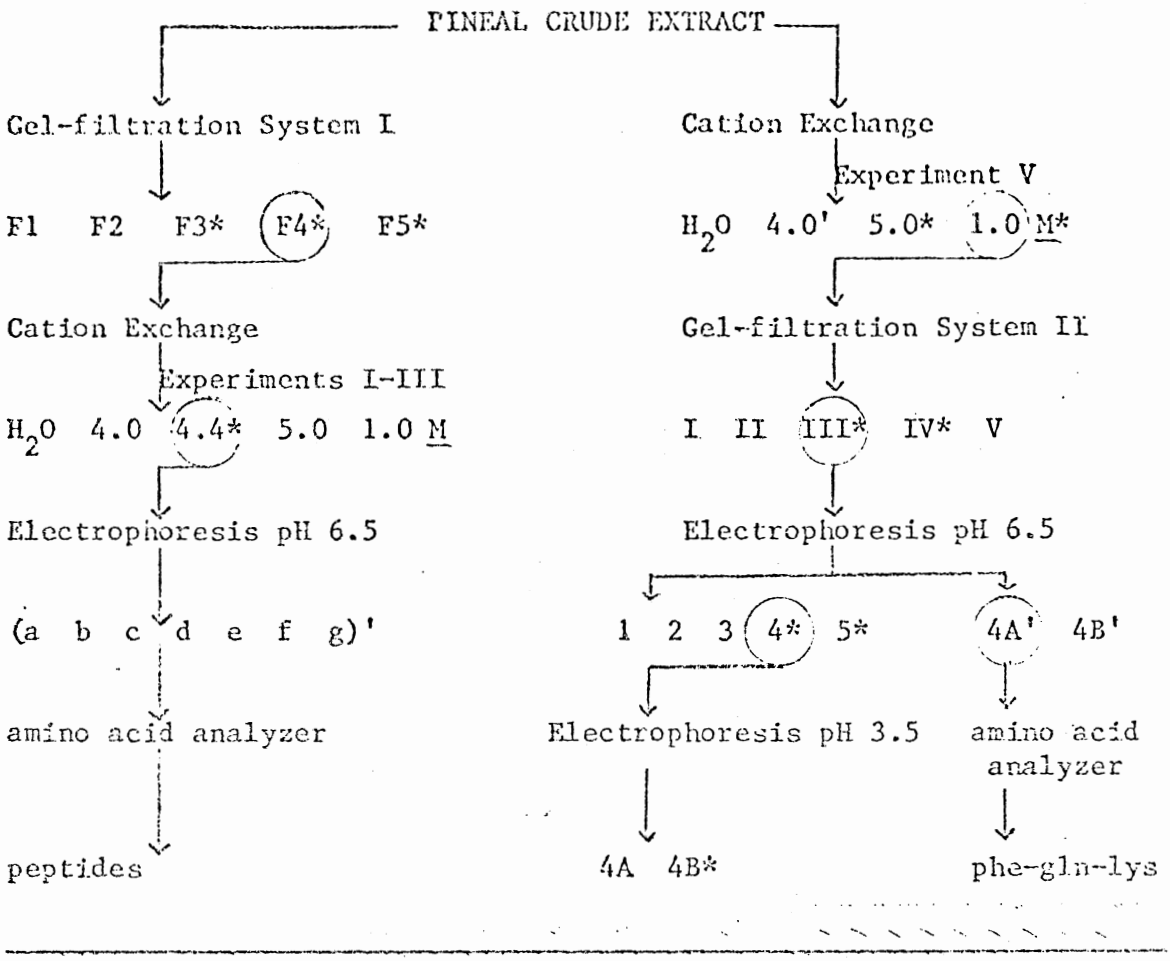


Figure 2. Elution Profile of the 1.0 M Pyridine Fraction from Gel-filtration System II.



* contains COH inhibiting activity
O fraction purified by next step
' not assayed for activity

Figure 3. Purification Summary

CHAPTER IV

SUMMARY

Partial purification and characterization of the antigonadotropic activity of bovine pineal glands was attempted by tracing the activity through each purification step by means of a bioassay (COH). After an acetic acid extraction procedure, the pineal glands were gel-filtered on G-25 in acidic medium and then purified further by cation exchange. The active fraction contained a mixture of peptides.

The extract was then placed on cation exchange prior to gel-filtration which was in basic medium. The active fraction was separated further by high-voltage paper electrophoresis of which an active fraction and another closely migrating fraction, which was in a more purified state, were degraded by the Edman method.

The active fraction is still not purified enough to determine the sequences of the peptides which are present. A Phe-Gln-Lys peptide was identified in a fraction separated from the activity by paper electrophoresis. The biological activity of this peptide remains to be determined.

A SELECTED BIBLIOGRAPHY

(1) Wade, N.J. (1937) Endo., 21, 681.

(2) Engel, P. (1939) Endo., 25, 144.

(3) Fischer, E. (1938) Arch. Internat. Pharmacodynamie, 49, 340.

(4) Vinals, E. (1935) Fo. congr. Nac. de med. Rosario, 3, 287.

(5) Reiter, R.J., and Fraschini, F. (1969) Neuroendo., 5, 219.

(6) Reiter, R.J. (1972) in Reproductive Biology, H. Baling and D. Glasser, eds., p. 71. Excerpta med., Amsterdam.

(7) Quay, W.B. (1974) in Pineal Chemistry, I.N. Kugelmass, ed. Charles C. Thomas, Springfield, Illinois.

(8) Lerner, A.B., Case, J.D., Takahashi, Y., Lee, T.H., and Mori, W. (1958) J.A.C.S., 80, 2587.

(9) Lerner, A.B., Case, J.D., and Heinzelman, R.V. (1959) J.A.C.S., 81, 6084-6085.

(10) Wurtman, R.J. (1967) in Neuroendocrinology, L. Martini and W.F. Ganong, eds., p. 19. Academic Press, New York.

(11) Benson, B., Matthews, M.J., and Rodin, A. (1972) Acta Endo., 69, 257-266.

(12) McIsaac, W.M., Farrell, G., Taborsky, R.G., and Taylor, A.N. (1965) Science, 148, 102.

(13) McIsaac, W.M., Taborsky, R.G., and Farrell, G. (1964) Science, 145, 63.

(14) Fraschini, F., Mess, B., Piva, F., and Martini, L. (1968) Science, 159, 1104.

(15) Orts, R.J., Benson, B., and Cook. B.F. (1974) Acta Endo., 76, 438.

(16) Benson, B., Matthews, M.J., and Rodin, A. (1971) Life Sci., 10, 607-612.

(17) Orts, R.J., and Benson, B. (1973) Life Sci., 12, 513-519.

(18) Ebels, I., Moszkowska, A., and Scemama, A. (1965) Compt. Rend. Acad. Sci., 260, 5126.

(19) Ebels, I. (1967) Biol. med., 56, 395.

(20) Moszkowska, A., Kordon, C., and Ebels, I. (1971) in Ciba Foundation Symposium on the Pineal Gland, G.E.W. Wolstenholme and J. Knight, eds. J. and A. Churchill, London.

(21) Matthews, M.J., and Benson, B. (1973) J. Endocr., 56, 339-340.

(22) Pavel, S. (1965) Endo., 77, 812-817.

(23) Cheesman, D.W., and Fariss, B.L. (1970) Proc. Soc. exp. Biol. Med., 133, 1254-1256.

(24) Milco, S.M., Pavel, S., and Neacsu, C. (1963) Endo., 72, 563-566.

(25) Vaughan, M.K., Vaughan, G.M., and Klein, D.C. (1974) Sci., 186, 938-939.

(26) Pavel, S., and Petrescu, S. (1966) Nature, 212, 1054.

(27) Benson, B., Matthews, M.J., and Smith, M.L. (1973) in The Pineal Gland: Proceedings of a Symposium, D.C. Klein, ed. Raven Press, New York (in press).

(28) Moszkowska, A., and Ebels, I. (1968) Experientia, 24, 610-611.

(29) Moszkowska, A., and Ebels, I. (1970) J. Neuro-visc. Rel., 31, 83.

(30) Moszkowska, A. (1963) Ann. Endocr., 24, 215.

(31) Reiss, M., Mauer, I., Sideman, M.B., Davis, R.H., and Plichta, E.S. (1963) J. Neurochem., 10, 851.

(32) Benson, B., Correntino, S., and Evans, J.S. (1969) Endo., 84, 369-374.

(33) Rameriz, V.D., and Sawyer, C.H. (1974) Endo., 94, 475-482.

(34) Welschen, R., and Dullaart, J. (1974) J. Endocr., 63, 421-422.

(35) Ebels, I., and Benson, B. (1973) Anal. Biochem., 56, 546-565.

(36) Orts, R.J., Benson, B., and Cook, B.F. (1974) Life Sci., 14, 1501-1510.

(37) Matthews, M.J., Benson, B., and Rodin, A. (1971) Life Sci., 10, 1375-1379.

(38) Dreyer, W.H., and Bynum, E. (1967) Methods in Enzymology, 11, 37.

(39) Gray, W.R. (1972) Methods in Enzymology, 25, 121-138.

(40) Woods, K.R., and Wang, K.I. (1967) Biochim. Biophys. Acta, 133, 369-370.

(41) Liao, T.H., Robinson, G.W., and Salnikow, J. (1973) Analytical Chemistry, 45, 2286-2288.

(42) Gray, W.R. (1972) Methods in Enzymology, 25, 333-344.

(43) Konigsberg, W. (1972) Methods in Enzymology, 25, 326-332.

(44) Schroeder, W.A. (1972) Methods in Enzymology, 25, 298-313.

VITA

Robert Wayne Poe

Candidate for the Degree of

Master of Science

Thesis: PARTIAL PURIFICATION AND CHARACTERIZATION OF ANTIGONADOTROPIC ACTIVITY OF BOVINE PINEAL GLANDS

Major Field: Biochemistry

Biographical:

Personal Data: Born in San Antonio, Texas, July 15, 1951, the son of Mr. and Mrs. Ralph Poe.

Education: Graduated from Parkview High School, Springfield, Mo., in June, 1969; received the Bachelor of Science degree in Chemistry from Southwest Missouri State University in August, 1974; completed requirements for the Master of Science degree at Oklahoma State University in December, 1976.

Professional Experience: Served as a research assistant at Oklahoma State University from 1974 to 1976; served as a teaching assistant from 1975 to 1976.