BRAIN RIBONUCLEIC ACID RATIOS IN THE DEERMOUSE DURING THE ESTROUS CYCLE

by 45

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Introduction

The fact that biological changes in the mammalian female urogenital system reflect responses to variations in ovarian hormone levels is generally accepted. Stockard and Papanicolau (1917), Allen (1922) and Long and Evans (1922) demonstrated these biological or cyclic changes in the reproductive tract during the estrous cycle. Paralleling these studies was the isolation and identification of the classic estrogens: estrone (Butenandt, 1929; Doisy, Veler and Thayer, 1929), estriol (Marrian, 1929; Doisy et al., 1929) and estradiol-17B (Mac Corquodale, Thayer and Doisy, 1935; Wintersteiner, Schwenk and Whitman, 1935). Previously, Frank and Rosenbloom (1915) and Allen and Doisy (1923) had shown that a substance from the placenta and corpus luteum and the ovary elicited a physiologic response from the reproductive tract.

The level of estrogenic hormones is low during the diestrus or luteal phase of the ovary and high during the estrus or follicular phase of the estrous cycle (Long and Evans, 1922; Marrian and Parkes, 1930). Due to the classical groundwork of the aforementioned workers on the estrous cycle, cyclic changes have proved to be regular and predictable and are very convenient for correlating flucuations in endocrine activity and response of target tissues, especially between estrogen and uterus.

It has been known for sometime that the total ribonucleic

acid content or the RNA/DNA ratio of the seminal vesicle after castration (Williams-Ashman, Liao, Hancock, Jurkowitz and Silverman, 1964), of the uterus after ovariectomy (Muller, 1965 and Telfer, 1953), or of the liver after hypophysectomy or thyroidectomy (Kochakin, 1965 and Korner, 1963) in rats may be two to ten times lower than in tissues from normal animals. Administration of the appropriate hormone soon causes a return to, or even a rise above, the normal RNA level (Hamilton, Widnell and Tata, 1967a; Tata, 1967).

Talwar, Segal, Evans and Davidson (1964) have reported that target organs select the appropriate hormone from the systemic circulation, with some organs concentrating a given hormone to a greater extent than others and that the chemical identity of the hormone is not altered in these tissues. For example, estradiol-17B is rapidly taken up by the uterus and vagina (Jensen and Jacobson, 1962). The fact that the hormone localizes in specific organs and is concentrated by these organs from the systemic circulation, even when the amount of the circulating hormone is very low, suggests the possible existence of a specific "receptor" substances in target cells capable of recognizing a particular hormone. Mueller, Gorski and Aizawa (1960) have expressed this type of hypothesis with a hormone-cell receptor system whereby the hormone might serve to activate or inhibit some existing enzyme. The hormone cell receptor system in turn contributes to the improvement of the intracellular environment for protein synthesis. Similarily,

Hamilton (1963) proposed that estrogens in some way alter the cytoplasmic biochemical environment so as to facilitate or favor protein synthesis by ribosomes or that estrogen, either directly or indirectly, participates in intranuclear reactions resulting in regulation of protein synthesis. A large volume of experimental work exists and is reported by Tata's review article (1967) which indicates that the level of RNA synthesis is indeed at the ribosomal level for certain RNA's.

Furthermore, Greenman, Wicks and Kenny (1965) showed that t-RNA synthesis was stimulated by a two or three-fold increase by the steroid, testosterone, in the liver. Wicks, Greenman and Kenny (1965) also have reported that not only t-RNA synthesis was increased by steroid treatment but that increased synthesis of ribosomal RNA and the DNA-like component (mRNA) was an integral part of the process of enzyme induction in the liver. It was felt that the specificity of enzyme induction by hormones reflected a mechanism of control at the level of translation, rather than of transcription. It was concluded from these experiments that synthesis of all three RNA species, ribosomal, transfer and DNA-like (mRNA) is stimulated by steroid hormones.

Hamilton, Windell and Tata (1967a) have concluded that one of the earliest effects of estrogen in the uterus is the stimulation of nuclear RNA synthesis in vivo, and that the stimulation of synthesis of ribosomes and of ribosomal RNA is an essential feature of the early action of the hormone. This

conclusion was in agreement with an earlier demonstration (Hamilton et al., 1967b) that the rate of nuclear RNA synthesis and its transport to the cytoplasm in the uterus is more rapid during the estral than the diestral phase of the estrous cycle, when endogenous titers or levels of ovarian hormones are at the lowest level of the cycle.

The conclusions drawn from these and similar studies were that the hormone accelerates both the synthesis of all types of RNA in the nucleus and its transfer into the cytoplasm of their responsive tissues. In mammalian tissues like the liver, uterus and seminal vesicles, a sufficient dose of the hormone to the hormone-deficient animal leads to a considerable accumulation of ribosomal RNA. Also, as pointed out by Hamilton (1963); Telfer, 1953; Mueller, Herranen and Jervell, 1958; Jervell, Diniz and Mueller, 1958, there exists a mechanism of action for steroids concerned with initiation or acceleration of synthetic processes. These steroid effects on RNA synthesis are both rapid and large, and it seems reasonably safe to assume that they play a significant role in ultimate physiological shifts that occur as a result of steroid hormone treatment, and that the RNA synthesis is closely linked to the primary site of action of these hormones.

The hypothesis of Greenman et al. (1965) and that reported by Tomkins, Garren, Howell and Peterkofsky (1965) indicated that a possible role of hormone action was at the level of translation. Later, Garren, Howell and Tomkins (1965) postulated

that enzyme induction was a possible role of increased RNA synthesis. These ideas as well as other evidence reported by Tata (1967) have supported the hypothesis first described by Karlson (1963). In his article, Karlson describes the action of hormones to be at the genetic level (level of the gene). This idea was in contrast to the position taken by some that hormones were believed to control enzyme activity, as first postulated by Green in 1941. The main argument in favor of Green's hypothesis was the fact that most hormones were active at very low concentrations. The conversion of phosphorylase b into phosphorylase a by the direct action of epinephrine and glucagon was another example. In this way the hormone seemed to control the activity of a key enzyme in glycogenolysis. However, Haynes, Sutherland and Rall (1960) found that 3',5'-cyclic adenosine monophosphate (3',5'-AMP) was responsible for the conversion, thereby acting as a mediator in hormone action. Karlson and Sekeris' work (1965) with ecdysone and juvenile hormone presents seemingly convincing evidence that the mode of action is indeed at the genetic level. Much of the work being reported now is moving in this direction.

Davidson (1965) has drawn two conclusions consistent with this line of thought. First, there is no doubt that treatment with estrogenic hormones results in activation at the gene level, and that many of the well known effects of estrogen on uterine cells results from this gene activation. Second, a considerable number of genes must be activated in order to

account for the many different responses of the cells to estrogen.

During the past few years, several articles have appeared in the literature concerned with the nature of the nucleic acids in the brain. Brody and Bain (1952) studied the distribution of RNA and DNA in subcellular fractions of the whole brain of rat and rabbit and reported that RNA was located in both microsomal and nuclear fractions. Their results were confirmed by Aldridge and Johnson (1959). Interest in the metabolism of RNA in nerve tissue was stimulated by the experiments of Hyden and his collaborators. Since, both protein synthesis and nucleic acid synthesis increase during hyperplasia, the possibility that these processes may be critical for neuronal alterations which constitute the physical basis of memory, was studied by Hyden (1960). Hyden and Egyhaiz (1963, 1962), reported that increased RNA synthesis was present in nerve cells and implied that RNA may be linked with the capacity of the central nervous system (CNS) to store information which corresponded to learning. A purine-pyrimidine analysis was done on nuclear and cytoplasmic RNA of neurons and an increase in the adenine/uridene (A/U) base ratio per cell was found. They concluded that regions on the genome are activated to produce nuclear RNA with highly specific base ratios. Efforts were made by Dingman and Sporn (1961, 1962), Chamberlain, Rothchild and Gerard (1963) and Barondes and Jarvik (1964) to correlate specific functions of the CNS with the role of mRNA, but no attempt was

made to characterize such a compound in the brain. Barondes (1964) identified a DNA-dependent RNA polymerase in nuclei from the brain, and its concentration in nuclei from the cerebral cortex was reported by Bondy and Wallsch (1965) to be higher than that present in the liver nuclei. However, Barondes and Jarvik (1964) conducted experiments using Actinomycin D, which inhibits DNA-dependent RNA synthesis (Reich, Franklin, Shatkin and Tatum, 1962), and concluded that if growth is necessary for memory storage, the RNA is not from the DNA template.

The existence of rapidly-labeled RNA in brain has been shown by several authors. Kimberlin and Hunter (1965) found a rapidly-labeled RNA sedimenting in the 4-18s region of a sucrose density gradient and Berterman, Mahler, More, Dutton and Thompson (1965) eluted a rapidly-labeled RNA from a methylated albumin-kieselguhr (MAK) column. Jacob, Stevenin, Jund, Judes and Mandel (1965) obtained a rapidly-labeled RNA from rat brain. Its base composition was similar to that of rat DNA, which was sedimented in sucrose density gradients in a broad range, and eluted from a MAK column at higher salt concentrations than ribosomal RNA. Its synthesis was inhibited by actinomycin D. These results support the synthesis in rat brain of messenger type RNAs having a high molecular weight.

Recently, Eleftheriou and Church (1967) have reported the first attempt to show some correlation between three specific brain areas and their total RNA content during the estrous cycle

of the deermouse. These results indicate that there is a significant increase in total RNA content from diestrus to estrus in all three brain areas. It was hoped that this study would permit more complete delineation of these findings and that a better understanding of total RNA content in specific brain areas could be obtained. It was felt that since steroids have been implicated in increased RNA synthesis during the estrous cycle (Hamilton et al., 1967a, b) and that the steroid, estradiol-17B, circulates in the circulatory system unchanged (Jensen and Jacobson, 1962) that additional information regarding the negative feedback mechanism that exists between the brain and the accessory sex tissues, more specifically the uterus and ovary, could be obtained. There follows a report of such a study in which deermice (Peromyscus maniculatus bairdii) brain parts (hypothalamus, amygdala, frontal cortex and cerebellum) were analyzed for their total RNA base ratios.

Materials and Methods

A total of four hundred and twenty nonfasted, adult female deermice (Peromyscus maniculatus bairdii), maintained under standard conditions (16 hr. light, 8 hr. dark, 70° temperature and food and water ad libitum) were used in experiments reported herein. All mice were at least three months of age and weighed between 13-17 grams. Animals were housed in opaque retangular cages at a rate of not more than six per cage. A normal estrous cycle was established by daily vaginal smears according to the technique of Allen (1922). Four hundred mice were segregated into eight experimental conditions of 50 mice each. In order to obtain sufficient tissue for each analysis, these 50 mice were further subdivided into 5 groups of 10 mice per group. An entire group of 50 similarly-treated animals was killed on the same day. All mice were killed by cervical dislocation and the hypothalamus (14 mg.), amygdala (26.4 mg.), frontal cortex (37.6 mg.) and cerebellum (62.4 mg.) were excised from the brain and frozen immediately in a dry-ice-ether mixture. The liver (793.6 mg.) was dissected out, gall bladder removed, and frozen immediately in the same manner. Similar brain parts (hypothalamus, amygdala, frontal cortex or cerebellum) were pooled (10/group) and transferred to a container of liquid nitrogen, frozen and reserved for later analysis. The livers also were pooled (5/group) and kept in liquid nitrogen.

Experimental conditions were as follows: estrus group and diestrus group; seven groups of ovariectomized mice further subdivided as follows: one group sacrificed at two weeks following ovariectomy; one group sacrificed at six weeks following ovariectomy; one group that received 50 ug. estradiol-cyclopentylpropionate (ECP); one group that received 50 ug. progesterone; one group that received 50 ug. ECP and 50 ug. progesterone; one group that received 200 ug. ECP; one group that received sesame oil. Steroids (in 0.1 ml sesame oil) were subcutaneously administered daily for seven days. At sacrifice on the 8th day, the ovariectomized mice were examined for complete ovariectomy. Both U-3672 progesterone and estradiol-cyclopentylpropionate were supplied by the Upjohn Company.

EXTRACTION PROCEDURES: Procedures for isolation of ribonucleic acids from mammalian tissues have been subjected to many modifications, but all are similar and utilize a buffer medium, detergent, divalent ion, phenol, a salt and an alcohol. For determination of total RNA, the procedure followed in this experiment was that of Kirby (1956). By modifying this procedure, RNA from both nuclei and cytoplasm of mammalian tissue was extracted free of DNA, protein and ribonucleases.

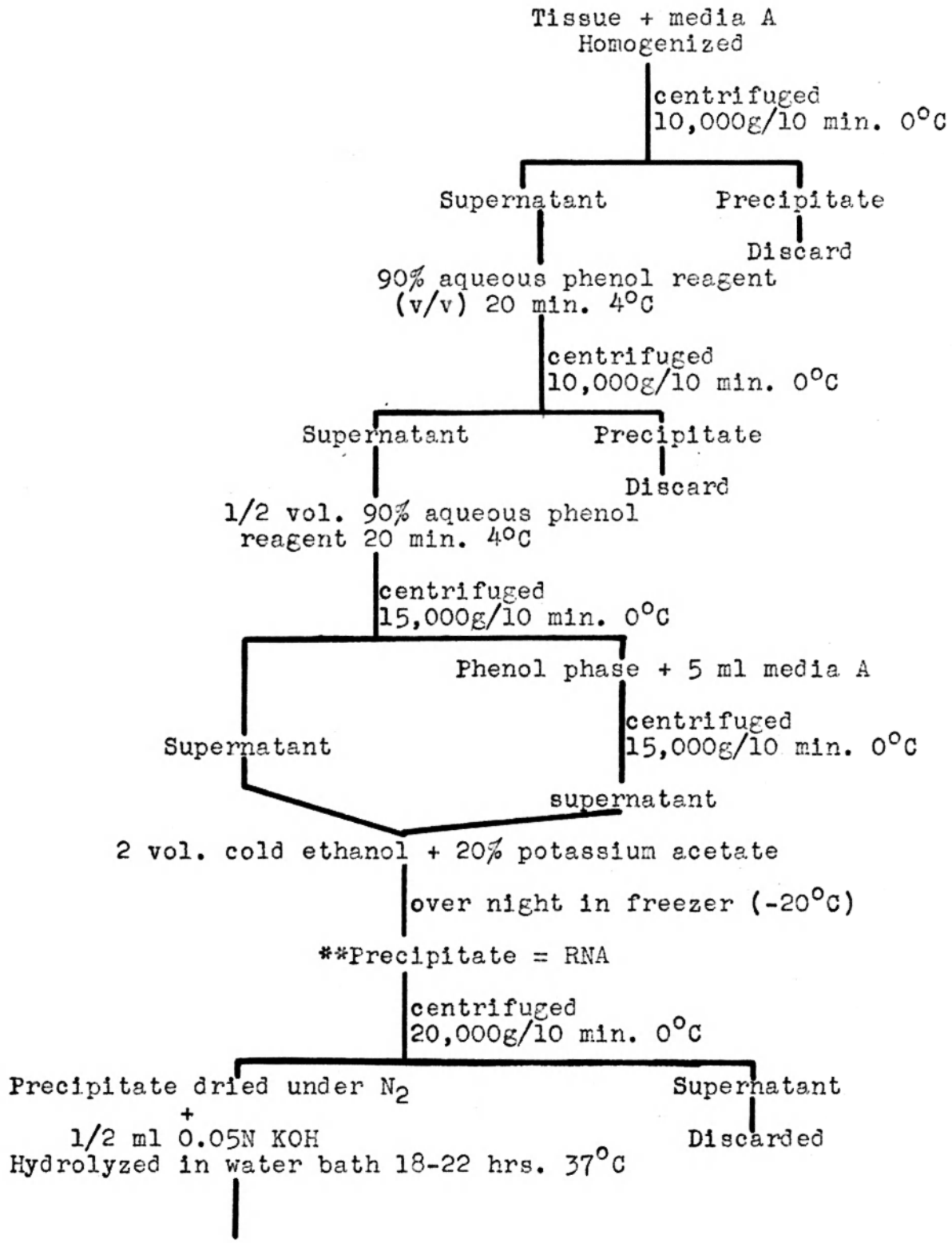
At the time of analysis, the specific tissue (hypothalamus, amygdala, frontal cortex, cerebellum or liver) was removed from the liquid nitrogen and suspended rapidly in a glass homogenizer (Potter-Elvehjem type) containing 10 ml of medium A (0.03M tris-

HCl, 0.25M sucrose, 0.001M $MgCl_2$ and 0.006M mercaptoethanol at pH 7.6). The detergents, 6% sodium dodecylsulphate (SDS), (Crestfield, Smith and Allen, 1955), and 10% sodium napthalene disulphonate (NDS), (Jacob et al., 1955), were added in amounts of 1.0 and 0.5 ml respectively, to aid in the disruption of the cells. One-half milliliter of washed bentonite was added to aid in the removal of nucleases (Petermann and Pavlovec, 1963). Each homogenate then was centrifuged for 10-15 min. at 10,000g at 0°C (Fig. 1). The supernatant was removed and diluted with an equal volume of 90% aqueous phenol reagent (90% phenol, 0.1 % 8-hydroxyquinoline (w/v) and 10% m-cresol (v/v), Hiatt, 1962) and stirred for 20 min. at 0-4°C. The precipitate was discarded. The phenol and the aqueous layer were separated by centrifugation at 10,000g for 10 min., and the treatment was repeated a second time using a 1/2 volume of the phenol reagent on the aqueous phase. The mixture was centrifuged at 15,000 x g for 20 min. and the aqueous phase removed and saved while the last phenol layer was back washed by adding 5 ml. of medium A. This mixture was stirred for 10 min. in the cold, and centrifuged at 15,000 x g (10 min.). The aqueous phase was recovered and combined with the original aqueous extract. A sufficient quantity of 20% potassium acetate and two volumes of redistilled ice cold ethanol were added to the original extract to make the final concentration 2% with respect to potassium acetate. The resulting solution was stored in the freezer (-20°C) over night.

Precipitated RNA was collected by centrifugation (20,000g) and dried under nitrogen, placed in polyethylene centrifuge tubes (2.5 in), 0.5 ml of 0.5N KOH added and incubated at 37°C for 18-22 hr. For liver samples, sometimes it was necessary to extract three or four times in order to obtain a clear aqueous phase. Precipitated polysaccharides floated to the top of the ethanol and were discarded. Liver RNA was collected and dried under nitrogen then redissolved in 5 ml of medium A. Three one milliliter portions were placed in separate polyethylene tubes and reprecipitated over night in the freezer. This procedure provided three identical aliquots/sample/group. This was done to check reproducibility for each extraction procedure.

CONFIRMATION ANALYSIS FOR NUCLEIC ACID (RNA) EXTRACTION: In order to demonstrate that the extraction and isolation procedures utilized in this experiment were appropriate for mammalian tissues a confirmation analysis for RNA was conducted. Column chromatographic methods of Mandell and Hershy (1960) and Sueoka and Cheng (1962) utilizing methylated albumin kieselguhr (MAK) were used. Twenty grams of kieselguhr (Hyflosupercel) were boiled in 100 ml of 0.1M saline for 10 min., then cooled. The fine particles were removed by successive decantations. The column (2.5 cm x 18 cm) was composed of three layers (1, 8 and 6 gm, respectively) on top of 1/2 gm powdered cellulose. To the six grams of boiled kieselguhr, two milliliters of 1% esterified albumin were added and three milli-

Fig. 1. Flow diagram for the extraction procedure of RNA extraction from mammalian tissue.



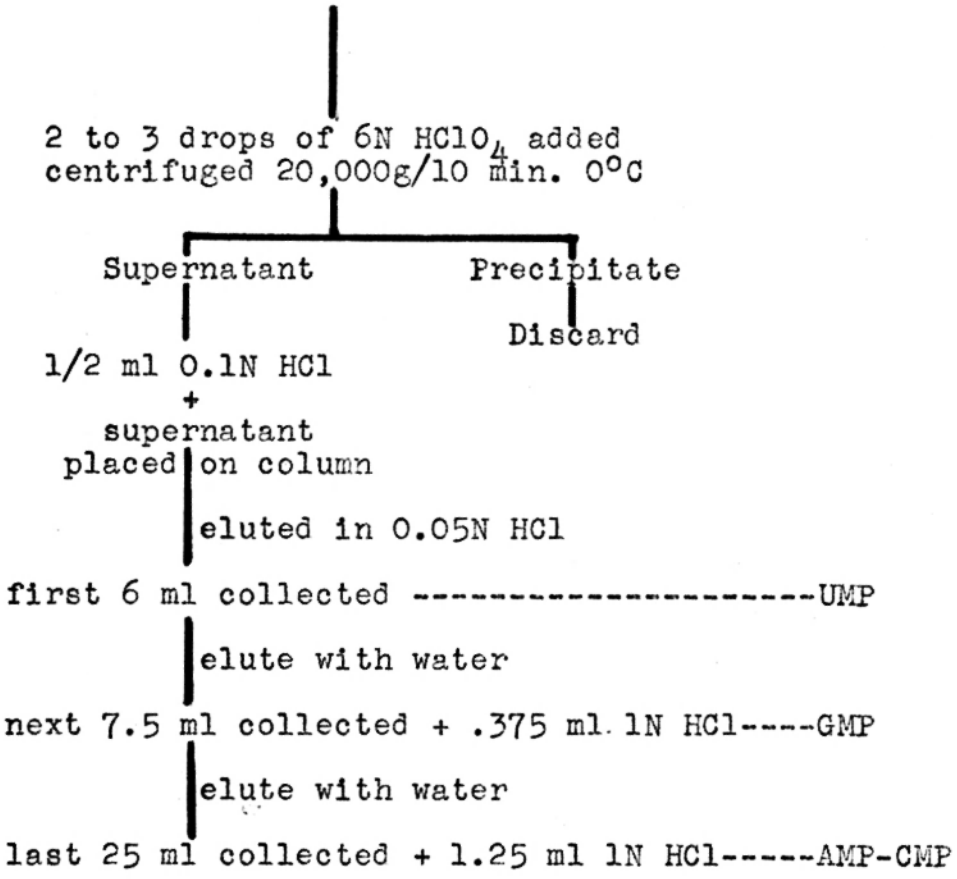
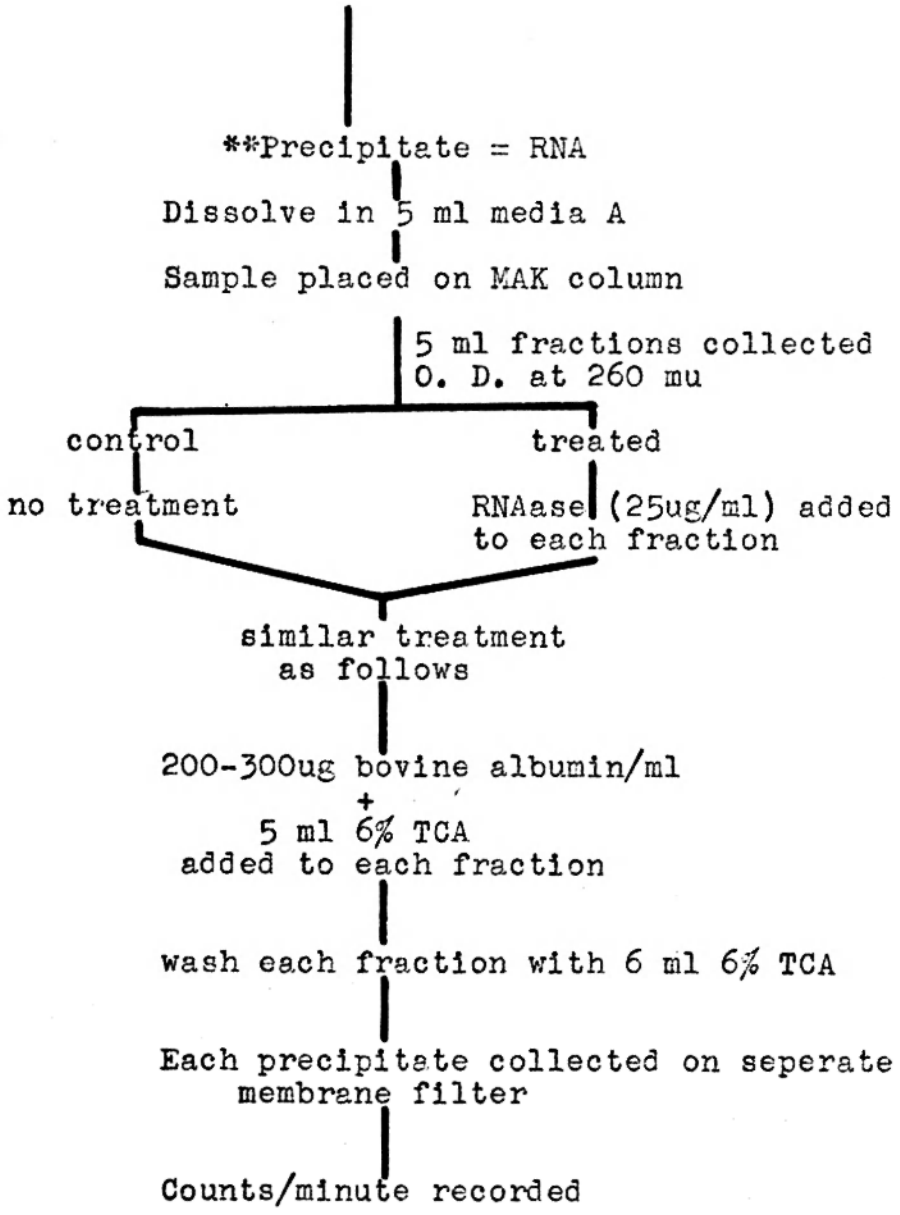

2 to 3 drops of 6N $HClO_4$ added
centrifuged 20,000g/10 min. 0°C
Supernatant
Precipitate
Discard
1/2 ml 0.1N HCl
+
supernatant
placed on column
eluted in 0.05N HCl
first 6 ml collected ----------UMP
elute with water
next 7.5 ml collected + .375 ml 1N HCl----GMP
elute with water
last 25 ml collected + 1.25 ml 1N HCl-----AMP-CMP

Fig. 2. **Flow diagram for the procedure used in MAK column analysis.



liters to the eight gram portion. The powdered cellulose was mixed in phosphate buffer (pH 6.7) and poured into the column and allowed to drain to the top. The 1, 8 and 6 gram portions are then added separately in the same manner. Pressure was used to pack the column but did not exceed 1 lb/sq. in.

Buffered saline solutions were 0.05M sodium phosphate buffer plus various amounts of NaCl. The pH of the buffer was 6.7 and although it became lower with the addition of salt, the solutions were not adjusted to 6.7, but were used as such. The buffered saline solutions were stored in the cold. Linear gradients of 0.01M to 1.0M NaCl were employed as eluting solvents.

Each of eleven deermice ranging in age from three weeks to three months were injected intraperitoneally with 40 uc of ^{32}P then sacrificed 24 hours later. Liver tissue was dissected out and total RNA extracted as previously described. The precipitated RNA dissolved in 5 ml of medium A and placed on the MAK column (Fig. 2). The sample was eluted from the column with 400 ml of gradient solutions and 5 ml fractions collected. The optical density (O.D.) of each fraction was read on a DU-2 spectrophotometer, wave length 260 mu, and plotted (Fig. 3). Each fraction then was divided into two equal aliquots (2.5 ml). One aliquot was treated with 25 ug/ml ribonuclease (Sigma) and the other aliquot not treated with nuclease. The RNA from each fraction was reprecipitated by adding 200-300 ug bovine albumin/ml and 5 ml of 6% trichloro-

acetic acid (TCA) and each fraction was poured over a membrane filter and washed with 6 ml of 6% TCA. Each membrane filter was then counted for ^{32}P activity. Results appear in Fig. 3.

BASE RATIO ANALYSIS: The technique employed for the determination of base ratios was that of Katz and Comb (1963). The columns (0.9 x 32.0 cm) were prepared by washing and decanting the fines of the resin (Dowex 50 H^+ 200 to 400 mesh 4 times cross-linked) in doubly distilled water (pH 7.6) three times and poured into the column to a height of 5.0 cm. Approximately 200 ml of 3N HCl was passed through the resin-filled column followed with water until neutral and finally washed with 20 ml of 0.05N HCl. The columns were standardized by placing 1/2 ml of a standard 2',3'-ribonucleotide mixture plus 1/2 ml of 0.1N HCl on the column and allowing it to drain to the top of the resin and collecting the fraction. One milliliter of 0.05N HCl was used to wash the sides of the column and was then collected. Five milliliters of 0.05N HCl were then added and allowed to drain to the top of the resin. This was followed by buffered doubly distilled water (pH 7.6) to provide for collection of a total of 45 one milliliter fractions. Fractions then were read on a Beckman DU-2 spectrophotometer at a wave length of 260 mu. Results of standardization are shown in Fig. 4.

The hydrolyzed RNA was neutralized (pH 5-8) by adding 6N $HClO_4$ dropwise, centrifuged at 20,000g and the supernatant poured into 5 milliliter glass tubes and 1/2 ml 0.05N HCl added.

Fig. 3. Results of MAK column chromatography. Extracted ^{32}P labelled RNA was separated by the column and 5 ml fractions collected. Fractions were divided into two equal portions with one set receiving RNAase (25ug/ml) the other receiving no RNAase. RNA was reprecipitated with TCA on separate membrane filters and cpm recorded. Optical density for each fraction was read at 260 mu and plotted.

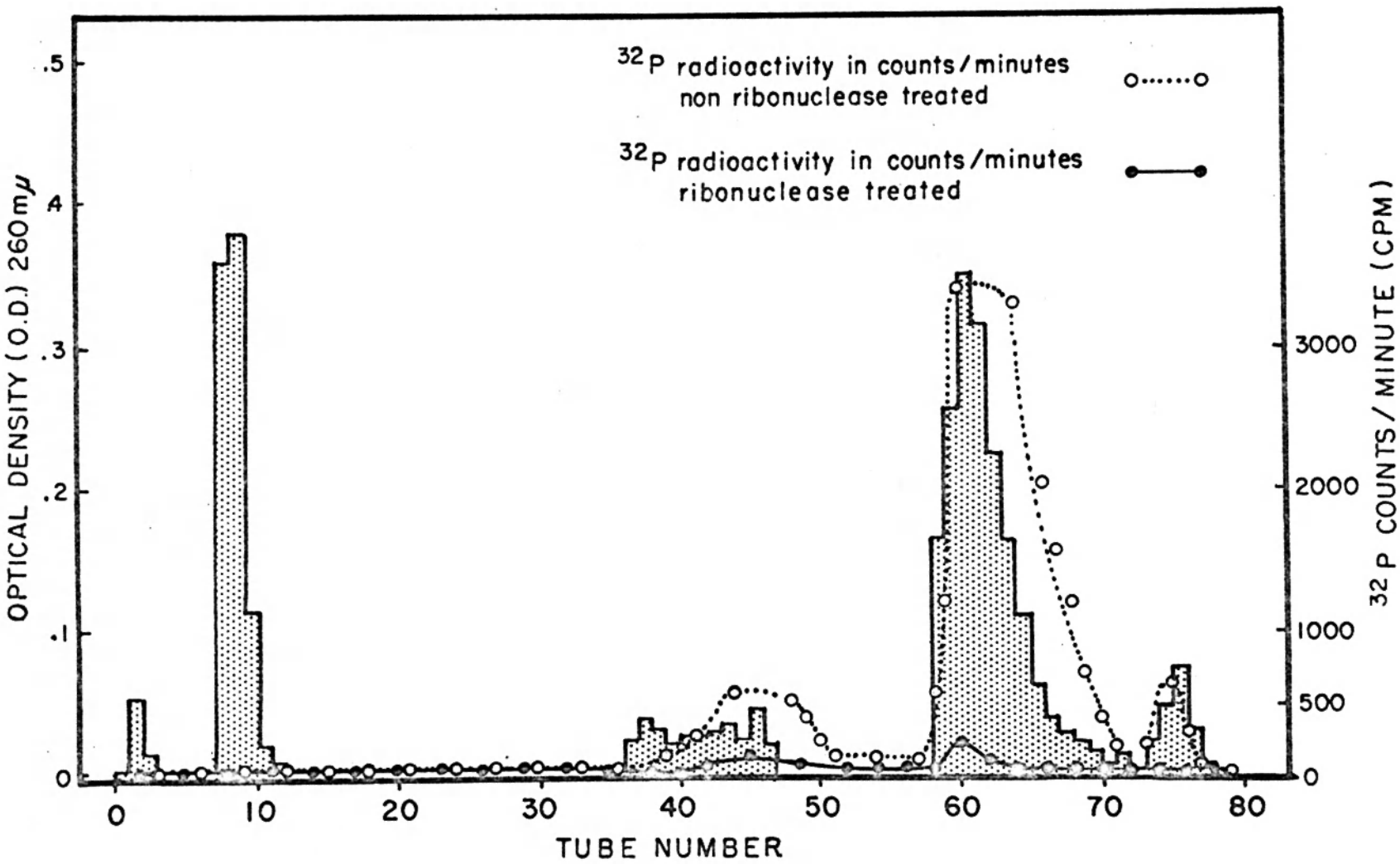
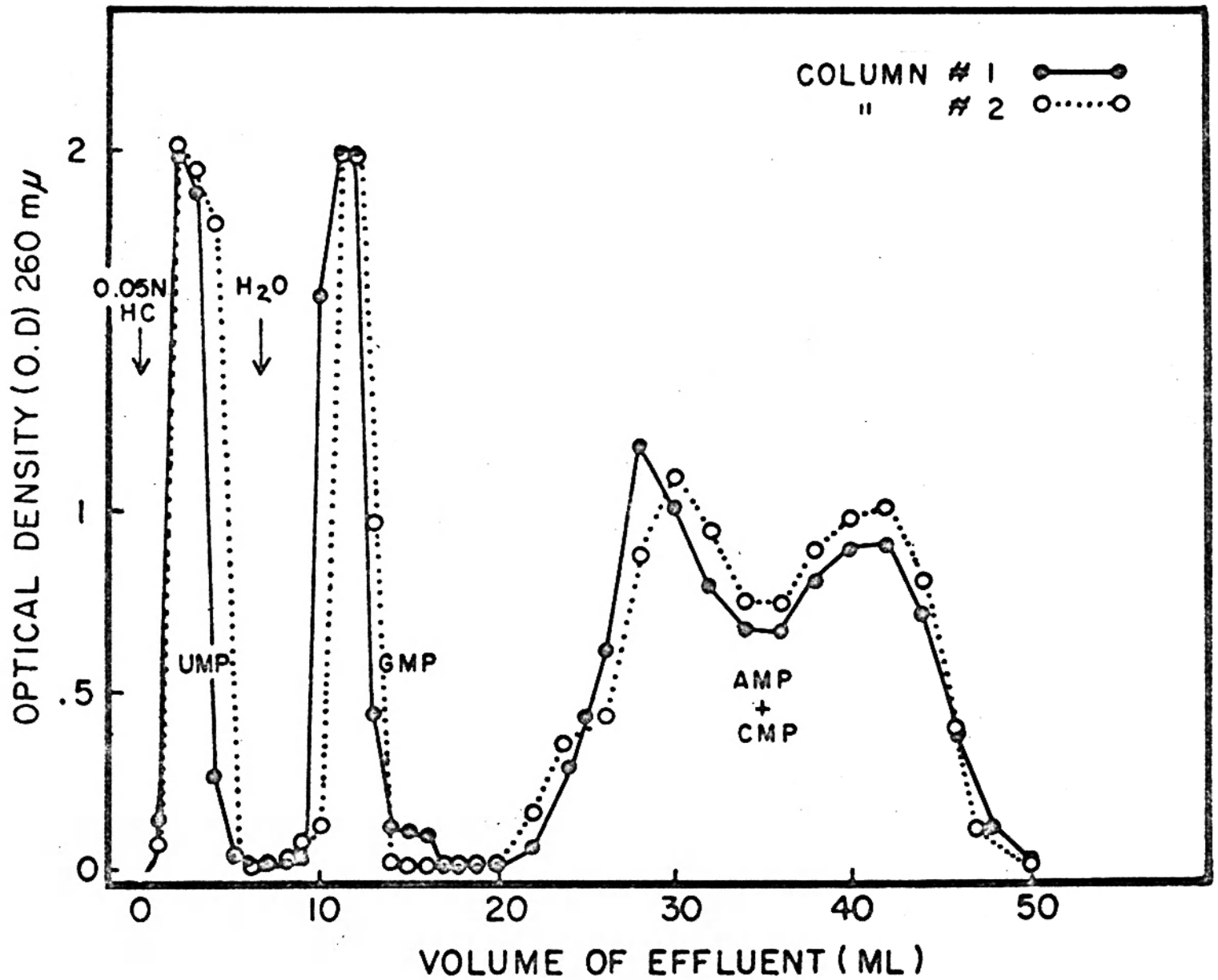

^{32}P radioactivity in counts/minutes non ribonuclease treated
^{32}P radioactivity in counts/minutes ribonuclease treated
OPTICAL DENSITY (O.D.) 260mμ
.5
.4
.3
.2
.1
0
^{32}P COUNTS/MINUTE (CPM)
3000
2000
1000
500
0
0
10
20
30
40
50
60
70
80
TUBE NUMBER

Fig. 4. Results of column chromatography standardization on hydrolyzed RNA. Each nucleotide or nucleotide combination was eluted from the Dowex columns in specific fractions. These were collected and their optical density values determined.


COLUMN # 1
" # 2
OPTICAL DENSITY (O.D) 260 mμ
2
1
.5
0
0.05N HC
H2O
UMP
GMP
AMP + CMP
0
10
20
30
40
50
VOLUME OF EFFLUENT (ML)

Each sample then was placed on a column which had been washed previously with 20 ml of 0.05N HCl and allowed to drain to the top of the resin. The sample was washed down with one milliliter of 0.05N HCl and allowed to drain to the top of the resin. The first six milliliters of the HCl effluent were collected (Fig. 4) and the remainder discarded. Doubly-distilled water (pH 7.6) was allowed to pass through the column at a flow rate of not more than 1 ml/minute. Care was taken not to disturb the resin when water was added. The first 7.5 ml were collected (Fig. 4) and brought to 0.05N concentration by the addition of 1.0 N HCl. The next 25 milliliters (Fig. 4) of water passing through the column were collected and also brought to 0.05N HCl concentration. The three fractions contained UMP, GMP and AMP-CMP, respectively.

DETERMINATION OF RELATIVE MOLE PERCENT OF NUCLEOTIDES (uM/100uM nucleotide): The three fractions uridine-monophosphate (UMP), guanosine-monophosphate (GMP) and adenine monophosphate-cytidine monophosphate (AMP-CMP) were read on a Beckman DU-2 spectrophotometer at their respective maximal absorbancy wave length (UMP, 260mu; GMP, 257mu; AMP-CMP, 257 and 279mu). The A_{279}: A_{257} ratio in 0.05N HCl for AMP is 0.238 and for CMP, 2.32. The absorbancy due to each of these nucleotides in the mixture was calculated using the following equations (Loring, 1955):

$$x = \frac{2.32(A_{257}) - A_{279}}{2.08}$$

$$y = A_{279} - 0.238x$$

where x is the absorbancy at 257 mu due to AMP alone, y is the absorbancy at 279 mu due to CMP alone, and A is absorbancy (O.D.). Once the optical density of each nucleotide was determined, the relative mole percent was found by dividing each nucleotide by its particular extinction coefficient, multiplying by the dilution factor (fraction volume), adding the total and finding the percentage.

EXAMPLE:

$$x = \frac{2.32(A_{257}) - A_{279}}{2.08} = \frac{2.32(.170) - .145}{2.08} = .11990$$

$$y = A_{279} - .238x = .145 - .238(.11990) = .11647$$

Base	O.D.	Extinction Coefficient	Dilution (fraction)	Product	Micromoles /uM of RNA
UMP	.144	9,600	6.000	$.900 \times 10^{-4}$	10.90
GMP	.276	11,800	7.875	1.851 "	22.33
AMP	.1199	14,900	26.250	2.096 "	32.66
CMP	.1165	13,000	26.250	2.819 "	34.14
				8.256	

STATISTICAL TREATMENT: All data were treated statistically by means of a two way analysis of variance on all mean values found for the nucleotide base percent. Also, the rank order interaction test, Newman-Kules test (Winer, 1962), was used to determine which base was affected the most within groups by the various experimental treatments. In addition, standard deviations also were calculated for all mean values.

Results

QUALITATIVE ANALYSIS: The ribonucleic acid obtained by cold phenol extraction and alcohol precipitation from deermouse brains presented an absorption curve typical of nucleic acids (Fig. 5). The maximum absorption for the extracted RNA was found to be at 258 mu and the minimal absorption at 230 mu. The 280mu/260mu ratio was equal to 0.486, thus demonstrating the purity of the product. These results agree well with those reported by Popa, Cruceanu and Lacatus (1966) who found a maximal absorbance of mouse brain RNA at 258 mu, minimal absorbance at 234 mu and the 280mu/260mu ratio equal to 0.47. Further supporting evidence that RNA was isolated actually from deermouse brain areas, such as the hypothalamus, amygdala, frontal cortex and cerebellum was obtained by using MAK column chromatography. The extracted brain sample was fractionated by the MAK column in a typical pattern as reported by Popa et al. (1966), Mandell and Hershy (1960) and Sueoka and Chang (1962). Furthermore, treatment with ribonuclease and uptake of radioactive phosphorus (^{32}P) showed no RNA activity after the addition of the enzyme.

QUANTITATIVE ANALYSIS: By two-way analysis of variance (Table 2), it can be seen that all treatments had a significant effect in all the specific brain areas tested, and that significance was exhibited by the experimental treatments for each nucleotide (UMP, GMP, AMP and/or CMP). The Newman-Kules procedure is a

Table 1. Mean values of nucleotides found for each brain area following experimental treatment. Standard deviations are given.

Treatment	N	Nucleotide	Hypothalamus	Amygdala	Frontal Cortex	Cerebellum
Estrus	50	UMP	26.92±3.78	24.58±2.45	19.23±1.27	18.25±2.14
		GMP	44.07±0.48	41.60±4.03	39.39±1.69	54.59±3.53
		AMP	12.22±2.04	12.88±1.86	16.06±2.19	11.07±2.11
		CMP	19.13±0.72	19.47±1.51	22.70±1.87	15.20±2.30
Diestrus	50	UMP	23.97±1.48	25.56±2.30	27.49±2.02	32.69±0.36
		GMP	40.18±2.21	41.01±2.58	38.15±4.75	38.36±2.65
		AMP	14.39±1.47	14.61±2.06	16.07±2.10	14.49±0.69
		CMP	20.96±0.94	20.20±2.11	16.05±3.14	14.40±2.31
Ovariectomy 2 weeks	50	UMP	31.22±2.87	28.90±1.91	29.12±1.20	37.70±4.70
		GMP	37.71±1.40	39.33±1.98	38.12±3.37	40.17±4.45
		AMP	17.37±4.30	13.38±1.67	14.48±1.19	13.51±2.74
		CMP	12.99±3.19	18.37±1.68	19.17±1.31	16.39±2.46
Ovariectomy 6 weeks	50	UMP	19.90±1.74	18.78±1.27	23.61±1.70	17.68±2.14
		GMP	38.67±1.93	38.92±1.59	12.29±3.37	37.76±1.40
		AMP	18.05±0.57	18.10±0.90	27.36±2.21	19.41±0.57
		CMP	24.70±1.41	25.33±1.36	36.74±1.87	25.35±0.71
Replacement 200ug ECP	50	UMP	35.28±5.29	30.16±0.45	32.07±3.01	20.73±0.91
		GMP	37.91±3.88	40.13±1.86	40.65±1.25	39.45±1.01
		AMP	12.77±0.93	18.02±0.82	14.10±1.40	16.73±0.81
		CMP	11.80±0.62	11.69±1.54	17.04±1.99	23.08±0.76
Replacement 50ug ECP	50	UMP	30.06±0.98	22.87±1.59	23.52±4.17	27.07±3.12
		GMP	37.91±0.97	34.72±2.67	39.56±4.29	41.09±2.35
		AMP	12.64±2.32	17.11±1.43	24.08±0.55	15.65±2.25
		CMP	20.14±2.08	22.79±0.90	10.78±1.58	20.23±4.60
Replacement 50ug progest.	50	UMP	22.87±1.65	20.54±0.97	18.89±1.19	19.96±0.87
		GMP	34.46±1.27	35.01±1.52	35.91±1.70	34.14±0.56
		AMP	19.10±1.69	18.75±1.38	19.30±0.70	20.12±1.16
		CMP	23.87±0.86	25.59±1.26	25.90±1.65	26.00±0.54
Replacement 50ug progest. plus 50ug ECP	50	UMP	16.70±2.82	17.10±1.00	17.20±1.46	15.95±1.23
		GMP	35.71±0.30	37.20±0.80	38.13±1.80	37.16±3.04
		AMP	20.42±1.23	19.40±0.84	19.15±0.33	20.44±0.84
		CMP	27.19±1.30	26.76±0.78	26.39±0.26	25.56±1.81
sesame oil controls	20	UMP	22.91±0.62	24.96±1.36	26.29±0.87	31.01±2.21
		GMP	40.10±2.74	42.75±3.67	39.10±2.97	39.96±4.01
		AMP	15.14±1.62	13.95±0.76	16.19±1.09	12.91±0.82
		CMP	21.85±2.00	18.34±2.01	18.42±1.36	16.12±0.54

Fig. 5. Comparison UV absorption curves of a standard RNA preparation and RNA extracted from deer-mouse brain (hypothalamus). Low reading at 234mu and high reading at 258mu.

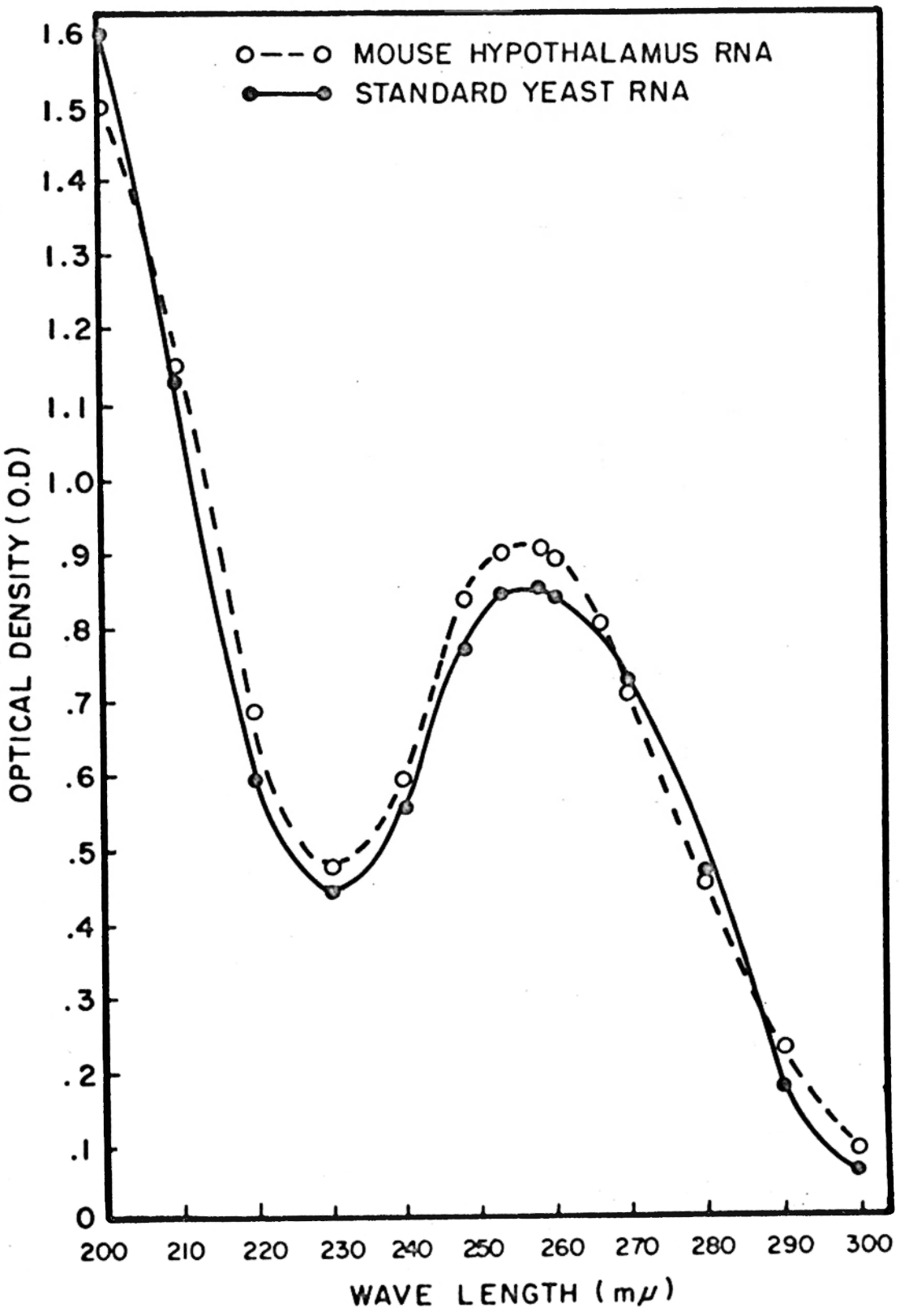

MOUSE HYPOTHALAMUS RNA
STANDARD YEAST RNA
OPTICAL DENSITY (O.D)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
200
210
220
230
240
250
260
270
280
290
300
WAVE LENGTH (mμ)

Table 2. Statistical treatment showing the results of the two-way analysis of variance procedure.

Nucleotides	Source of Variation	Mean Square	Degrees of Freedom	F ratio	P
UMP	Treatment Combinations	58.96	3	27.17	.00001
	Between main groups	558.31	7	257.29	.00001
	Interaction	76.29	21	35.16	.00001
	Sampling error	2.17	155		
GMP	Treatment Combinations	174.37	3	55.88	.00001
	Between main groups	289.75	7	92.86	.00001
	Interaction	148.84	21	47.70	.00001
	Sampling error	3.12	155		
AMP	Treatment Combinations	68.33	3	48.95	.00001
	Between main groups	158.27	7	113.40	.00001
	Interaction	32.09	21	22.99	.00001
	Sampling error	1.39	155		
CMP	Treatment Combinations	27.53	3	17.21	.00001
	Between main groups	478.96	7	299.35	.00001
	Interaction	82.71	21	51.69	.00001
	Sampling error	1.60	155		

rank order interaction analysis and shows which base was affected the most by the various experimental treatments. This particular type of analysis was carried out for each nucleotide and each treatment on all specific brain parts. Table 3 is an example of how the test was carried out and Table 4 is a summary of the results of all tests for each nucleotide following each experimental treatment.

The uridine monophosphate (UMP) ratio or base percent, calculated according to the procedures mentioned in the methods section, was seen to be at a maximum (35.28±5.29) in the hypothalamus after ovariectomy with a two-week atrophy period and followed by 200ug ECP replacement therapy and minimal (16.70± 2.98) after 50ug ECP + 50ug progesterone replacement therapy (Table 1). Similarly, the same trend was found to be true of the amygdala and frontal cortex, that is 30.16±0.45 and 32.07± 3.01 %, respectively, after 200ug ECP administration and minimal (17.10±1.00, 17.20±1.46 %, respectively) after estrogen-progesterone (50ug) treatment. The cerebellum exhibited maximum UMP content (37.70±4.70) after two week ovariectomy and minimal (15.95±1.23), like the other three brain areas, after the estrogen and progesterone (50ug) treatment.

Guanosine monophosphate (GMP) percent was the highest during two of the normal cyclic periods of the estrous cycle and non-significant from each other, that is, estrus and diestrus in the hypothalamus (44.07±0.48, 40.18±2.21 %), amygdala (41.60± 4.03, 41.10±2.58) and frontal cortex (39.39±1.69, 38.15±4.75 %)

Table 3. Model table of the Newman-Keuls procedure. Bases are ranked in order and the differences found between means. The base, CMP and the brain part, hypothalamus.

Order	1	2	3	4	5	6	7	8
Treatments in order of ranking	h	d	g	b	f	a	c	e
	27.19	24.70	23.87	20.96	20.14	19.13	12.99	11.80
		2.49	3.32	6.23	7.05	8.06	14.20	15.19
			0.83	3.74	4.56	5.57	11.71	12.90
				2.91	3.73	4.74	10.80	12.07
					0.82	1.83	7.97	9.16
						1.01	7.15	8.34
							6.14	7.33
								1.19

Truncated range	2	3	4	5	6	7	8
$q_{.95}$ (r, 155)	2.80	3.36	3.69	3.92	4.10	4.24	4.36
$q_{.95}$ (r, 155) nMS_{error}	3.53	4.23	4.65	4.94	5.17	5.34	5.49

a= estrus
b= diestrus
c= 2 wk. ovariectomy
d= 6 wk. ovariectomy
e= 200ug ECP
f= 50ug ECP
g= 50ug progesterone
h= 50ug ECP + progesterone

Table 4. Summary of results on the Newman-Kules procedure. Nucleotides are listed separately with the brain areas tested. Each letter(s) is given in ranking order of its effect on the nucleotide and is significantly different from the other letter(s) in the same rank. Sesame oil controls were not significantly different from the diestrus group and are not listed.

Nucleotide	Ranking order of base effect			
UMP				
Hypothalamus	e*	c,f	a,b,d,g	h
Amygdala	e,c	b	f,a	g,d,h
Frontal cortex	e,c	b	f,d	a,g,h
Cerebellum	c	b	f,e	a,d,g
GMP				
Hypothalamus	a,b		c,d,e,f,g,h	
Amygdala	a,b,c,d,e,h		f,g	
Frontal cortex	a,b,c,e,f,g,h		d	
Cerebellum	a		b,c,d,e,f,g,h	
AMP				
Hypothalamus	h,g,d,c	a,b,e,f		
Amygdala	h,g,d,e,f	a,b,c		
Frontal cortex	d	f	g,h	a,b,c,e
Cerebellum	h,g,d	e	b,c,f	a
CMP				
Hypothalamus	h,g,d	f,b	a,c,e	
Amygdala	h,g,d	f	b,a,c,e	
Frontal cortex	d	h,g	c,e	a,b,f
Cerebellum	h,g,d,e	f,b,a,c		

*a= estrus
b= diestrus
c= 2 wk. ovariectomy
d= 6 wk. ovariectomy
e= 200ug ECP
f= 50ug ECP
g= 50ug progesterone
h= 50ug ECP + progesterone

while the cerebellum was high (54.59±3.53) during estrus. Also, the (G+C/A+U) ratio, calculated from the mean values in Table 5, was greatest (2.37) in the cerebellum during estrus. The lowest base percent was found to exist after ovariectomy and 50ug progesterone administration in the hypothalamus (34.46±1.27), amygdala (35.01±1.52) and the cerebellum (34.14±0.56).

The hypothalamus, amygdala and cerebellum were found to exhibit their maximal AMP base percent, 20.42±1.23, 19.40±0.84 and 20.44±0.84, respectively, after ovariectomy, followed by 50ug progesterone plus 50ug replacement therapy. Also the same brain areas responded to ovariectomy and progesterone (50ug) in a manner similar to progesterone + estrogen (50ug) treatment with no significant difference between the former and latter treatments: hypothalamus (19.10±1.69), amygdala (18.75±1.38) and cerebellum (20.12±1.16). The value for AMP was found to be at its lowest during the estrus period of the cycle in all four brain areas: hypothalamus (12.22±2.04), amygdala (12.88±1.86), frontal cortex (16.06±2.19) and cerebellum (11.07±2.11).

It may be of some interest that CMP, the base pairing counterpart of GMP, was affected in the opposite manner after the various treatments. Where GMP was high during estrus in the hypothalamus, amygdala and cerebellum and low after 50ug progesterone replacement, CMP was high after 50ug progesterone treatment and low during estrus in the same brain areas. Also, the same trend was seen to exist for the other base pairing partners. Adenosine monophosphate was maximal during ECP plus

Table 5. The G+C/A+U ratios found to exist for the specific brain areas after experimental treatments.

Treatments	Hypothalamus	Amygdala	Frontal Cortex	Cerebellum
Estrus	1.61	1.63	1.41	2.37
Diestrus	1.59	1.52	1.24	1.11
2 wk. ovary*	1.04	1.36	1.31	1.10
6 wk. ovary	1.66	1.74	0.96	1.70
200ug ECP	1.03	1.07	1.24	1.67
50ug ECP	1.35	1.43	1.06	1.28
50ug progest.	1.38	1.28	1.62	1.50
50ug progest. + 50ug ECP	1.69	1.75	1.77	1.72

Table 6. The A/U ratios found to exist for the specific brain areas after experimental treatments.

Treatments	Hypothalamus	Amygdala	Frontal Cortex	Cerebellum
Estrus	0.45	0.52	0.83	0.60
Diestrus	0.60	0.57	0.58	0.44
2 wk. ovary	0.56	0.46	0.50	0.35
6 wk. ovary	0.91	0.96	1.15	1.09
200 ug ECP	0.36	0.60	0.44	0.80
50ug ECP	0.42	0.74	1.02	0.58
50ug progest.	0.83	0.91	1.02	1.00
50ug progest. + 50ug ECP	1.22	1.13	1.11	1.28

*ovary = ovariectomy

progesterone (50ug) and progesterone (50ug) alone treatments in the hypothalamus, amygdala and cerebellum, as was CMP during the same kinds of treatment. Another similarity noted was that CMP was maximum in all four brain areas: hypothalamus (24.70± 1.44), amygdala (25.33±1.36), frontal cortex (36.74±1.86) and cerebellum (25.35±0.71), after six weeks ovariectomy and was not significantly different from the ECP plus progesterone (50ug) treatments, or the 50ug progesterone treatment in the hypothalamus (27.19±1.30, 23.87±0.86), amygdala (26.76±0.78, 25.95±1.26) and cerebellum (25.56±1.81, 25.00±0.54), respectively.

There are some interactions that seem to appear often and might be of some significance. In Table 5 it can be seen that the ovariectomized animals allowed to atrophy for six weeks and those treated with ECP plus progesterone (50ug) did not differ significantly in their G+C/A+U ratio in the hypothalamus (1.66, 1.69), amygdala (1.74, 1.75) and cerebellum (1.70, 1.72). Whether this is an indication or reflection of the two treatments having similar effects on the mouse brain is not known, and can not be stated as such. The A/U ratio, Table 6, also can be seen to reflect the same trend. In many of the effects of the experimental treatments which have been pointed out, the hypothalamus, amygdala and cerebellum have responded to the experimental treatments in a similar manner. And, in all cases, the hypothalamus and amygdala react to experimental conditions in a similar manner. Since these two brain areas have been shown to be closely related in function and make up part of the

limbic system, their responses are not entirely surprising and results in turn tend to support the experimental design.

Discussion

There is ample evidence to indicate the importance of the hypothalamus in the control of reproduction in mammals. Two reviews by Harris and Donovan (1966) cite evidence that neural tissue and circulating hormones regulate production or release of tropic hormones. Although most studies have concentrated on the hypothalamus, and there is convincing evidence that this area is of primary importance in controlling hypophyseal function, other areas of the brain also have been implicated. Recent studies have indicated that a part of the rhinencephalic-limbic system, known as the amygdala, is of importance to gonadotropic and corticotropic function (Shealy & Peele, 1957; Alonso-deFlorida & Delgado, 1958; Yamada & Greer, 1959). Several hypothalamic functions have been shown to be influenced by the rhinencephalon or limbic lobe of the brain. Kling (1965) and Yamada *et al.* (1959) have reported that lesions in the amygdaloid area affect the secretion of gonadotropins. Sawyer (1960) found that ovulation could be induced by stimulating the amygdala and suggested that this may represent part of a feedback system of ovulating hormone (LH) and oxytocin to shut off further neural activation of the pituitary. Egger(1967) has shown that there are discrete interconnections between the hypothalamus and the amygdala.

It now is quite evident that estrogen exerts both a stimulatory and inhibitory action on the secretion of pituitary

gonadotropins. The majority of the recent work supports a hypothalamic "receptor" site for the negative feedback of gonadal steroids. The presence of discrete hormone sensitive areas in the brain has been established for the rat (Lisk, 1960), rabbit (Davidson & Sawyer, 1961a), dog (Davidson & Sawyer, 1961b) and cat (Harris, Michael & Scott, 1958). Investigations by Flerko and Szentagothai (1957) have shown that rats with anterior hypothalamic lesions exhibited an inhibition in the secretion of follicle-stimulating hormone (FSH) but had a very small inhibitory effect after estrogen administration. They concluded that estrogens affected the gonadotropic function of the hypophysis through a nervous mechanism. It was assumed that the functional state of nervous elements, localized somewhere in the region of the paraventricular nuclei, is modulated by the blood level of estrogens and thus regulates the secretion of gonadotropic hormones.

Experiments using hormone implants can aid in locating possible sites of hormone effects, but can not prove the hypothesis of hormonal feedback. It must be shown that hormones from the blood stream arrive at the hypothetical site of action and are taken up there. Michael (1962) placed stereotaxic implants of native estrogens (^{3}H-estradiol) and synthetic estrogens (^{14}C-diethyl-stilbesterol) in the brain of rats. By autoradiographic studies he showed that certain cells in the hypothalamic region were selectively sensitive to estrogens, and that the cells from that part of the neuronal apparatus

which were sensitive to the hormone mediated the expression of sexual behavior. Pfaff (1965) has reviewed cerebral implantation and autoradiographic studies of sex hormones, and more recently (1968), he has reported the uptake of ^{3}H-estradiol in the brain of ovariectomized rats. Uptake of the labeled estrogen 1/2 hour after injection was found to be one and one-half times as great in the hypothalamus, olfactory and limbic structures as uptake in other brain regions. Two hours after injection, uptake still was high in the limbic system and hypothalamus, but had fallen in the olfactory bulb and non-limbic areas. The time course of uptake in the brain cells (hypothalamus and limbic system) resembled that seen in the uterus and vagina.

Eleftheriou and Church (1967) have shown that the total RNA content of the hypothalamus, amygdala and frontal cortex increased from diestrus to proestrus in the deermouse. During estrus the hypothalamic and frontal cortical RNA content did not change significantly, but decreased below that of diestrus in the amygdala (187.4±10.2 to 166.7±7.4).

Results reported herein show that the guanine + cytidine/adenine + uridine (G+C/A+U) ratio of RNA increased from diestrus to estrus in the same brain areas as those reported by Eleftheriou and Church (1967) plus an interesting, and somewhat disturbing, increase in the cerebellum. The fact that the G+C/A+U ratio decreases below diestrus after two week ovariectomy can be explained. Harris et al. (1966) have shown that FSH

and LH increase in the pituitary after ovariectomy in the rat. However, the increase does not reach its peak in FSH for over two weeks at which time LH begins increasing, reaching a maximum level a month or more later. Karlson (1963) first postulated that hormones affect the cell and that this effect is manifested at the genetic or gene level. Since that time there have been numerous reports (hydrocortisone, Garren et al., 1964; steroid hormones, Tomkins et al., 1965; estrogen, Gorski et al., 1965; and estrogen, Hamilton et al., 1967) indicating that hormones do indeed affect the cell at the genetic level by increasing RNA and protein syntheses. Now, it could be possible that there is a continuous build up of FSH and LH in the pituitary taking place since there is no circulating estrogen and therefore no negative feedback to the hypothalamus and amygdala areas which have been shown previously to be sensitive to estrogen. This increase would be accomplished by an increase in the neurohumoral releasing factors, FSH-RF and LH-RF via the synthesis of their respective RNA's. In other words, with the absence of estrogen there is no control of the synthesis of the neural transmitters, resulting in a build up of FSH and LH in the pituitary.

Flerko and Bardos (1966) feel that the pituitary gonadotropic function might have a duel neural control. The first level of control involves the "hypophysiotropic mechanism" found in the "hypophysiotropic area" which produces and releases, at the basal level, FSH-RF and LH-RF inhibiting factors. This

system, however, is not capable of enhancing or inhibiting the release of said factors according to changing requirements. This would indicate that other brain areas or neural structures may modulate cells producing these factors. Cells that are concerned with synthesis or secretion of FSH- and LH-releasing factors are concentrated in the preoptic and anterior pituitary area as well as the hypothalamus and limbic system. Since this is true, it could be possible for estrogen to selectively stimulate these brain areas at the genetic level (DNA) whereby estrogen would, in some way, affect a cause and effect mechanism on the increased synthesis of RNA, which in turn would influence production of releasing factors and/or inhibiting factors. By the same token, the absence of estrogen would not effect the synthesis of said factors.

Since no circulating estrogen is present, it may be that hormonal stimulation of specific brain areas results in increased synthesis of releasing factors and, therefore, a build-up of FSH and LH in the pituitary. If this hypothesis is true, then the effect of replacement therapy treatments can be at least partially explained. With 200ug ECP replacement therapy, we see the results indicate a negative feedback system in the hypothalamus especially when the G+C/A+U ratios are compared to those at two-weeks after ovariectomy. There is virtually no difference in this ratio. The amygdala has actually decreased in this ratio. After 50ug ECP administration, however, we see a positive feedback where the synthesis of RNA increases

in both the hypothalamus and amygdala. Progesterone, which has been reported by Harris et al. (1965) to affect the build-up of LH, causes an increase in the G+C/A+U ratio when compared to the two-week atrophy period but not when compared to normal levels. Following the administration of progesterone (50ug) primed with estrogen (50ug), there is evidence that a synergistic action between the two hormones results in an increase in the G+C/A+U ratio to the level found during the estrus period of the estrous cycle.

The mechanism of feedback control and possible effect at the genetic level poses an interesting question. Stimulation of protein synthesis can be accounted for in a variety of ways. A higher concentration of intra-cellular amino acids or the removal of some inhibitory mechanism may result in higher yields of protein. Moreover, when a specific protein or enzyme is to be synthesized, the stimulus must be specific, that is, it must intervene somewhere in the chain leading from DNA via m-RNA to ribosomes producing a particular protein. Such systems, that is, the production of specific proteins in response to hormonal stimulation, have been proposed for the production of insect hormone, ecdysone (Karlson, 1963), and tryptophan pyrolase and tyrosine transaminase in the liver after steroid induction (Tomkins et al., 1965).

Relevant here is the concept of genetic repressors proposed by Jacob and Monod (1961) to explain the induction of enzymes. According to these authors, enzyme synthesis is under

dual control, that is, molecular organization is controlled by structural genes through the synthesis of m-RNA while the rate of m-RNA production is controlled by operator genes. The activity of these genes is in turn regulated by the so-called repressor substances, which inhibit or block gene action. These repressors can be inactivated by certain metabolites, the "inducers", thus leading to enzyme synthesis (induction of enzymes). Another way to look at this would be to propose that in the absence of an inducer (hormone and in this case estrogen), the repressor would block the transcription of the genetic information and keep the hormone-sensitive tissue (specific brain cells) in an atrophied or "nearly-inhibited," de-sensitized state. The uptake of the hormone by the tissue by means of the "receptor" would release the repression of RNA synthesis. It then may be that the action of estrogen and/or progesterone in specific brain areas is regulated in a manner similar to this type of mechanism. Since we know that all somatic cells of the body have exactly the same number of chromosomes and genetic make up, then why do some cells respond to certain stimuli differently from other tissues or cells? Perhaps one reason may be masking of part of the genome in some manner. This would leave the genome or parts of the genome free to produce substances (proteins) that are not masked. Estrogen might remove or depress the masking of the genome to produce the correct protein for neurohumors.

This brings us to the point of trying to say something

concerning the kind or type of RNA whose induction results in production of a specific protein. Most studies indicate that the greatest change may occur in ribosomal RNA. Greenman et al. (1965), however, found that there was a turnover of all three types of accepted RNA. An increase in the GC content indicated increase in r-RNA and an increase in the AU content indicated increased m-RNA. Since the design of this experiment, and the technique employed for RNA extraction did not differentiate between various types of RNA, one can not make a statement as to the nature of RNA(s) isolated. Statistical analysis indicates that the four nucleotides (UMP, GMP, AMP, CMP) were affected differentially by the treatments and that there was an effect of base/brain part/treatment. Two-hundred micrograms of estrogen affect the ratio of UMP to the greatest extent and this is expressed in the hypothalamus, amygdala and frontal cortex. Also, the amygdala, frontal cortex and cerebellum exhibit the maximum UMP ratio after two week ovariectomy which does not differ significantly from the 200ug replacement therapy. The least effect on the UMP ratio was seen after the administration of ECP plus progesterone (50ug) and was exhibited in all brain areas. If one compares this finding with the overall G+C/A+U ratio and the finding that 200ug of estrogen exhibited no effect, and that the ECP + progesterone administration had the greatest effect, one might conclude that the nucleotide UMP is not needed to any great extent over the normal levels of the nucleotide in the synthesis of more or a "new"

RNA.

Guanosine monophosphate was seen to have its highest ratio during estrus and diestrus in the hypothalamus, amygdala and frontal cortex. Most of the other treatments did not effect the amygdala and frontal cortex. Again, the lowest value or ratio of GMP was found after ECP plus progesterone administration. It seems, therefore, that the existing levels of GMP (diestrus or control) in the brain was similar to that found during estrus and diestrus. These nucleotide ratios seem to indicate that GMP is not used in any great quantities above this level for the increased synthesis of RNA after ECP + progesterone administration. In three instances, it is seen that the hypothalamus, amygdala and cerebellum are all high in the AMP and CMP after ECP + progesterone, progesterone, and six week ovariectomy experimental periods. The frontal cortex is seen to be high in these same components after the sixth week of ovariectomy. It would seem then that the increased synthesis of total RNA in deermouse brain (Eleftheriou & Church, 1967) is a result of an increase in RNA rich in AMP and CMP with no apparent increase in the ratios of GMP and UMP. Whether this increase in AMP and CMP reflects the nature of the neurohumors of gonadotropins or the increase is a result of synthesis of a particular type of RNA responsible for these molecules is not certain since the m-RNA or r-RNA have not been isolated for these moities.

The reasons for the cerebellum reacting the way it did to

various treatments is not clear at this time. Originally it was proposed that the cerebellum is not involved in steroid metabolism and, therefore, could be used as an inner or double control. However, it is seen to respond to the treatments in a manner similar to the hypothalamus and amygdala. As mammals evolved, the cereberal hemisphere moved posteriorly and enlarged in size and function and became more and more involved in intergration of nervous function. The cerebellum is considered to be the center of muscular coordination and equilibrum and controls those events below the level of consciousness. Also, this area is involved in intergrating nerve impluses originating from the body. Perhaps the cerebellum is involved with the mechanism of sex perception and/or psychic heat. It was found that during estrus the G+C/A+U ratio was highest in the cerebellum and was not approximated by any of the various replacement treatments.

Recently, Eleftheriou, Desjardines and Pattison (unpublished data) concluded an experiment, using the rabbit, similar to that reported in this paper. Results show trends similar to those reported herein. Since the rabbit is a spontaneous ovulator one would not expect the estrus level and replacement level of estrogen to affect base ratios differently if the same general mechanism of action exists in the rabbit as that proposed in the deermouse. Indeed, it was found that the hypothalamus, amygdala, frontal cortex and cerebellum did not differ in G+C/A+U ratios in normal controls and estrogen replacement

therapy. Also, the spinal cord did not change in the G+C/A+U ratio throughout the experimental treatments. This may indicate that the spinal cord is below the level of intergration of sex and hormone interactions whereas the other brain areas studied are most certainly involved.

In summary, results reported herein indicate that the hypothalamus, limbic structures and cerebellum of deermouse brain are in a negative feedback system, thus accounting for the action of the gonadotropins and that the locus of action of estrogen and/or progesterone may be at the level of the gene. Estrogen may operate on a receptor-repressor mechanism whereby the correct sequence of events leads to the synthesis of FSH-RF, LH-RF or some other neurohumor. It is proposed that events leading to production of these proteins are controlled by RNA synthesis and further, that this RNA may be rich in the nucleotides AMP and CMP.

Summary

Total RNA extracted from the brain (hypothalamus, amygdala, frontal cortex and cerebellum) of female deermice was subjected to base ratio analysis after various experimental treatments. It appears that after the source of estrogen and progesterone has been removed (ovariectomy) there is no chemical message to the brain that would cause the decreased production of FSH and LH. By the same token, replacement therapy causes an increase in the nucleotides that approaches that occurring during estrus. It may well be that estrogen and progesterone affect the brain, primarily the hypothalamus and amygdala, by causing the increased synthesis of a neurohumoral agent(s) (FSH-RF, LH-RF) which inhibits production of gonadotropins. In any event, there is an increase in the total RNA in deermouse brain areas and data supports the implication that there is an increased need for AMP and CMP. It would seem, therefore, that estrogens and progesterone, affect brain cells at the genetic level and that the increase in RNA is due to an increase in the nucleotides, AMP and CMP. The role of the frontal cortex and cerebellum in this system is not clear at this time. Perhaps there are steroid sensitive cells in these areas that can perceive and integrate the sex-steroid response and prepare the organism for ensuing events.

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BRAIN RIBONUCLEIC ACID RATIOS IN THE DEERMOUSE DURING THE ESTROUS CYCLE

by

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Abstract

Total ribonucleic acid (RNA) was extracted by cold phenol method from four brain areas: hypothalamus, amygdala, frontal cortex and cerebellum, taken from female deermice (Peromyscus maniculatus bairdii). A total of 420 adult female mice were segregated into eight test groups: estrus, diestrus, 2 week ovariectomy, 6 week ovariectomy, replacement therapy groups of progesterone (50ug), ECP (50ug), ECP (200ug), ECP plus progesterone (50ug) and sesame oil controls. Animals received hormone injections subcutaneously each day for seven days and were killed on the eighth day. Tissue samples were immediately frozen in liquid nitrogen and reserved for further analysis. RNA from each brain area was extracted, hydrolyzed and a base ratio analysis preformed.

The results show that the ratio of each base was significantly different in all experimental groups. Uridine monophosphate (UMP) was seen to be maximum in the hypothalamus, amygdala and frontal cortex after 200ug ECP replacement therapy and minimal after estrogen-progesterone (50ug) treatment. The cerebellum UMP was maximal after two weeks of ovariectomy and minimal after estrogen-progesterone administration. Guanosine monophosphate (GMP) was highest during two of the normal cycling periods, that is, estrus and diestrus in all four brain areas. The cerebellum exhibited maximum base percent of GMP (54.59± 3.53) and the highest G+C/A+U ratio (2.37) during the same

period. Low values of GMP in the hypothalamus, amygdala, and cerebellum were found after progesterone treatment. Both AMP and CMP were seen to exhibit maximum base percent in the hypothalamus, amygdala and cerebellum after progesterone and estrogen-progesterone administration. The same two bases were lowest during the estrus and diestrus periods. In many cases the hypothalamus, amygdala and cerebellum responded similarly and in all cases the hypothalamus and amygdala exhibited identical responses. A number of inter-reactions between treatments and bases occurred.

Enough evidence now exists to establish firmly that a feedback mechanism operates between the brain (hypothalamus and pituitary) and the ovary. Results reported herein indicate that the feedback mechanism for estrogen and progesterone may be controlled by specific cells in the hypothalamus and amygdala, and that the hormone(s) might effect these cells at the genetic level. Whether transcription or translation was effected or if even a "new" species of RNA is responsible for the operation of the system is not known. It seems, however, that the possibility of an RNA synthesis rich in AMP and CMP might play an important role in the hormone response. The role of the frontal cortex and cerebellum may be that of perception and integration of the sex-steroid response.