Hypothalamic Control of Pituitary Gland Function

Terminal Progress Report for Research Carried out Under

AEC Contract AT(11-1)-3417

March 1, 1967 - March 1, 1972)

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Statement of Problem

Since the pioneering work of Schally in the late 1950's, it has become abundantly clear that the hypothalamus is intimately involved in the regulation of adenohypophysial cell function. Numerous books, review articles, etc., are now available which spell out, in detail, what is currently known about these regulatory mechanisms. Briefly stated, different peptides (synthesized in hypothalamic nuclei) reach the different types of pituitary cells by way of the portal vessel circulation. Once in contact with the pituitary cell membrane, a series of events occur within the cell to effect release of hormone. This regulation is amazingly specific; i.e. each hormone-producing cell type is under control of a single hypothalamic factor (hereafter referred to as releasing factor). The only apparent exception to this specificity is that of the gonadotropin releasing factor. In this case it appears that a single decapeptide controls the release of both FSH and LH.

Two of the releasing factors have now been chemically synthesized and are currently commercially available for testing. One factor, which controls the secretion of TSH, is a tripeptide. The other, which controls secretion of FSH and LH, is a decapeptide. The structure of the other releasing factors is unknown at present. The fact that these synthetic preparations are active in humans as well as laboratory animals raises many clinically significant questions; some of which center on possible new means of birth control. For a background review to this entire problem, the reader is referred to a chapter I wrote for the new edition of Best and Taylor's "Physiological Basis of Medical Practice", scheduled for publication this year (8). It was enclosed in last years' AEC progress report as document

AT(11-1)-3417-17.

In addition to the now extensive literature on releasing factors, the isolation and synthesis of pure pituitary hormones continues to be a major goal in many different laboratories. The work which we have carried out during the past 5 years under AEC support falls somewhere between those biochemical efforts indicated above, and their ultimate medical applications. Throughout the past five years, the overriding theme to our research efforts has been that of defining the sequence of intracellular events which occur in a pituitary cell after it is stimulated to release hormone. Our basic research goals were as follows:

1) to develop techniques for the separation and isolation of different pituitary cell types.

2) to study effects of hypothalamic extracts on translational and transcriptional control in the in vitro pituitary gland.

3) to investigate the problem of cell turnover in the pituitary gland.

Our feelings were (and still are) that any information gained from these experimental approaches would be helpful in understanding some of the mechanisms involved in the synthesis and release of the pituitary hormones. Those, in turn, relate to the complex physiological "loops" which ultimately assure proper function of the entire hypothalamo-hypophysial axis.

Research Goal #1: Separation of Different Pituitary Cell Types.

For over 70 years, the mammalian adenohypophysis has been largely studied using either the histological or physiological approach. In the histological approach, cells which stain differently due to their hormone contents have been described, counted and photographed after different endocrinological manipulations. (For example, the fact that castration changes a specific type of blue-staining cell provided circumstantial evidence that that cell was involved in the production of gonadotropin). While this approach served a useful purpose, its limitations have been realized in more recent years. In the second (i.e. "physiological") approach, hormone contents were measured after similar treatments. Studies in which both approaches are used have been rare indeed.

Some three years ago it became clear to us that a meaningful study of intracellular events occuring in a pituitary cell in response to stimulation by hypothalamic releasing factor would require homogenous populations of the different cell types. Accordingly we set out to develop and adapt existing cell separation methods to pituitary tissue. As might be imagined, this has proven to be a very difficult problem. However, we are encouraged by our progress to date. Much of this progress is to be found in the manuscript enclosed with this final report entitled "Enrichment of Cell Types from the Rat Adenohypophysis by Sedimentation at Unit Gravity" (7). This manuscript has passed initial review for publication in Endocrinology and a revised copy (the one submitted) is currently under consideration. The method for cell dissociation (pg. 5 of the manuscript) yields approximately 70% of the cells that make up the adenohypophysis. The description of the unit gravity cell separation method is currently done as described in this

manuscript. With this method we can now obtain cell fractions which contain over 65% somatotrophs; the primary contaminating cell type being the basophil. We currently use a second centrifugation step to separate the basophils from the somatotrophs. This involves centrifugation of the cells in the somatotroph fraction (see above) in a linear gradient of concentrated (14-28%) bovine serum albumin (BSA). Under these conditions we have discovered that the basophils band at density 1.060 ± .0007 g/cm^3; while the somatotrophs are more dense and band at 1.075 ± .0007 g/cm^3. With these 2 procedures, the second of which I developed on my sabbatical leave in Canada this year, we can now routinely obtain cell fractions which consist of over 90% somatotrophs. These results were presented at the American Federation meetings in April 1972 (19).

Once the method for the isolation of the somatotrophs was developed, we carried out a number of preliminary studies on these isolated cells. Most of the results from these studies have been presented in abstract form. Ultrastructure. It was important to determine the appearance of these cells at the electron microscope level. Our results clearly show that the cells retain excellent ultrastructural-integrity after separation. Shown in Fig. 1 is the appearance of the isolated cells at the light microscope level. Their appearance at the electron microscope level is shown in Fig. 2. The isolated cells can be incubated for 3 hours and their structure is virtually identical with that of the comparable cell type in situ. These results were recently presented at the Candaian Federation Meetings in Quebec City (20). Growth hormone content. Using a specific radioimmunoassay procedure for rat growth hormone, we have determined that the isolated somatotrophs retain growth hormone. Concentration of growth hormone in the original cell suspension is 30-40 nanograms/1000 cells. In fractions from the somatotroph region of

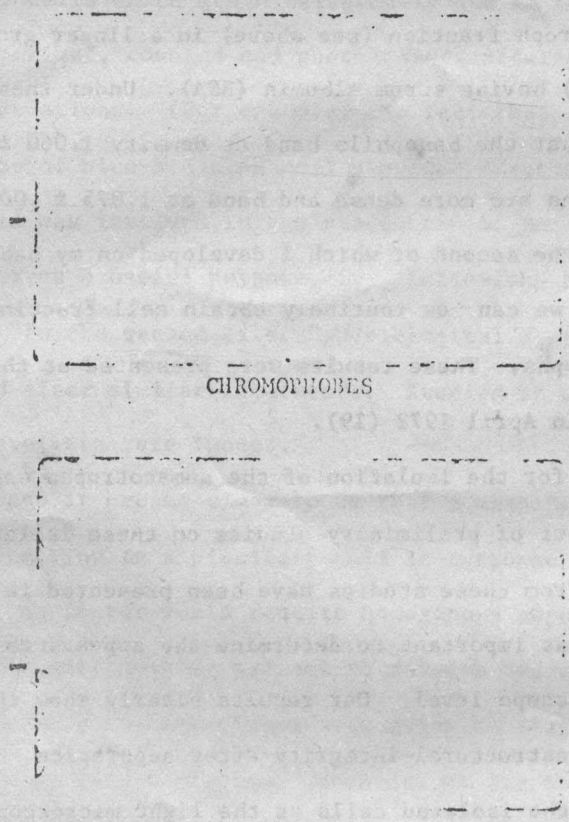


Fig. 1

Appearance of isolated cells in the a) chromophobe and b) somatotroph fractions. X430.

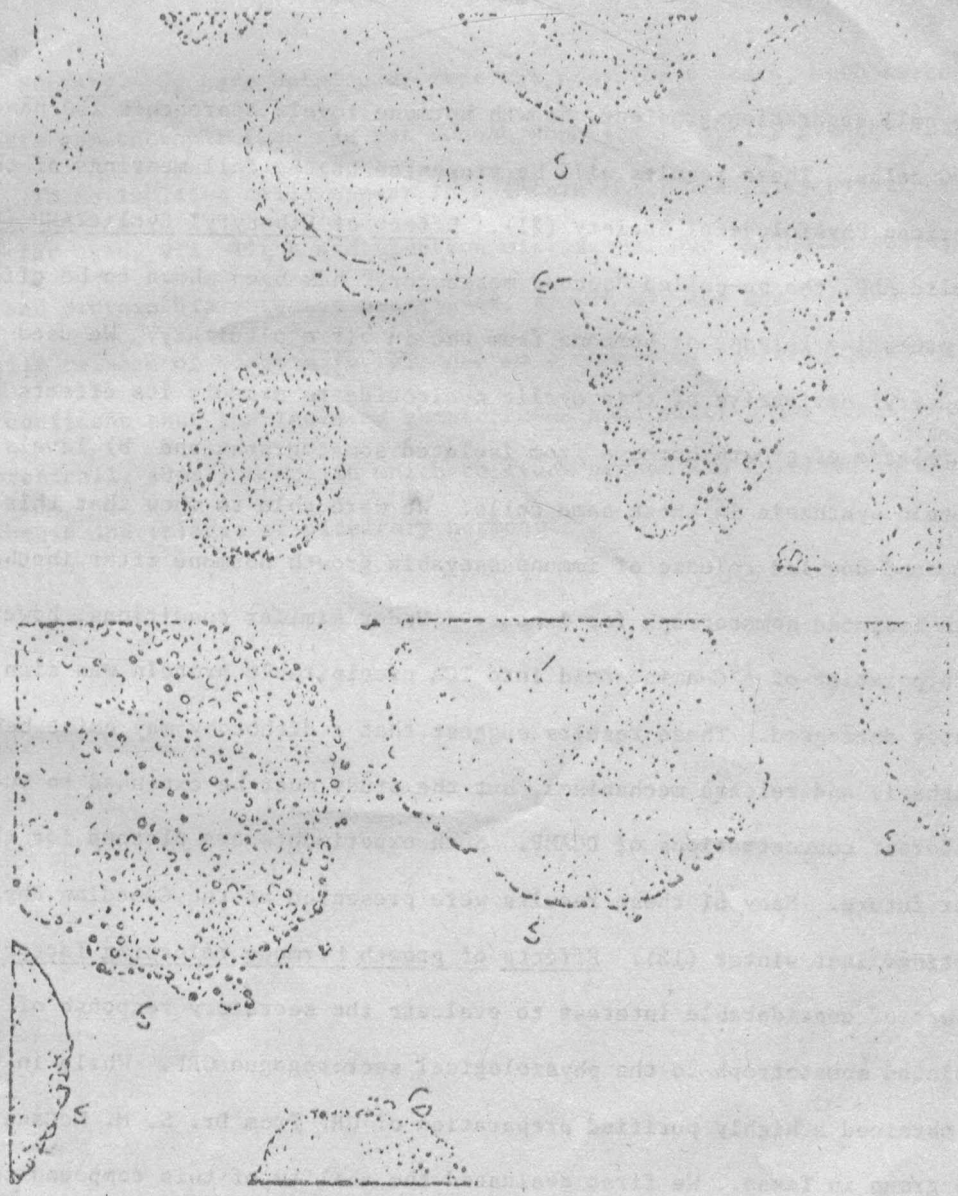


Fig. 2

Appearance of cells in the original suspension (top) and 70% somatotroph fraction (bottom). Cells are identified on the basis of secretion granule size. S = somatotroph. G = gonadotroph.

the cell separation gradient, growth hormone levels approaches 250 nanograms/ 1000 cells. These results will be presented at the fall meetings of the American Physiological Society (21). Effect of Dibutyryl Cyclic AMP (DCAMP). Cyclic AMP, the so-called "second messenger," has been shown to be effective in promoting release of hormone from the in vitro pituitary. We used the dibutyryl derivative of this cyclic nucleotide to measure its effects on a) release of growth hormone from isolated somatogrophs and b) levels of protein synthesis in these same cells. We were able to show that this compound doubled release of immunoassayable growth hormone after incubation with isolated somatotroph for 1 hour. Under similar conditions; however, incorporation of ^{14}C-amino acid into TCA precipitable protein was significantly decreased. These results suggest that a dichotomy may exist between synthesis and release mechanisms, but the study must be extended to include different concentrations of DCAMP. Such experiments are planned for the near future. Many of these results were presented at the Canadian Physiology Meetings last winter (18). Effects of growth hormone releasing factor (GRF). It was of considerable interest to evaluate the secretory response of the isolated somatotroph to the physiological secretogogue GRF. While in Canada we obtained a highly purified preparation of GRF from Dr. S. M. McCann and his group in Texas. We first evaluated the ability of this compound to promote release of growth hormone from the in vitro pituitary. In three separate experiments we then used concentrations of 16, 50 and 100 microliters of this material to test its effectiveness in causing release of growth hormone from the isolated somatotrophs. The samples from these experiments are in the freezer waiting to be assayed. If positive results are obtained, we feel that this could well be the ideal model to use in future studies investigating intracellular mechanisms of hormone release. I will continue to collaborate with Dr. Kraicer in this exciting area of research.

Summary. We have developed, over the past three years, methods to isolate somatotrophs from the rat adenohypophysis in purity greater than 90%. These isolated cells appear to maintain their viability by all criteria thus far used, viz. light and electron microscopy, dye exclusion tests, RNA and protein biosynthetic capacities, growth hormone content and finally release of hormone in response to a number of secretagogues. We are confident that the isolated somatotrophs will offer a new (and theoretically ideal) model in which to study mechanisms involved in the synthesis and release of pituitary hormone.

Research Goal #2: Effects of hypothalamic extracts on translational and transcriptional control in the in vitro pituitary gland.

In vitro Incubation Method. The system that we have used to monitor the effects of hypothalamic extracts (HE) on a number of intracellular events in the pituitary is virtually identical to the one used by all other workers in the field. It has been known for the last 10-15 years that addition of crude acid extracts of fresh rat hypothalamic tissue to anterior pituitary glands maintained in vitro will cause significant release of all pituitary hormones (except prolactin) into the incubation medium. This simple technique has provided a major portion of the experimental evidence for the existence of the releasing factor molecules. In all of our experimental work, hypothalamic extracts (HE) have been prepared from fresh hypothalami by homogenization in 0.1NHCl, centrifugation, neutralization of the clear supernatant and subsequent use in the incubation system. This system involves incubation of single (or half) pituitary glands in 1.5 ml of Medium 199 or Krebs-Ringer bicarbonate buffer, both maintained at pH 7.3-7.4 by incubation at 37C in a Dubnoff Metabolic Shaker with constant gasing to provide an atmosphere of 95% O_2 and 5% CO_2. Controls for these experiments utilize an identical extraction procedure on fresh cerebral cortical tissue (CE). Using this system, a number of interesting findings relating to the phenomenon of hormone release have emerged from numerous laboratories over the last ten year period. Some of the more important are a) over a certain limited range, hormone release is related to dose of HE; b) there is an absolute requirement for Ca^{++} in the incubation medium, release will not occur under conditions where $[Ca^{++}]$ is low (e.g. EGTA); c) elevation of K^{+} to 5 times its normal physiological concentration

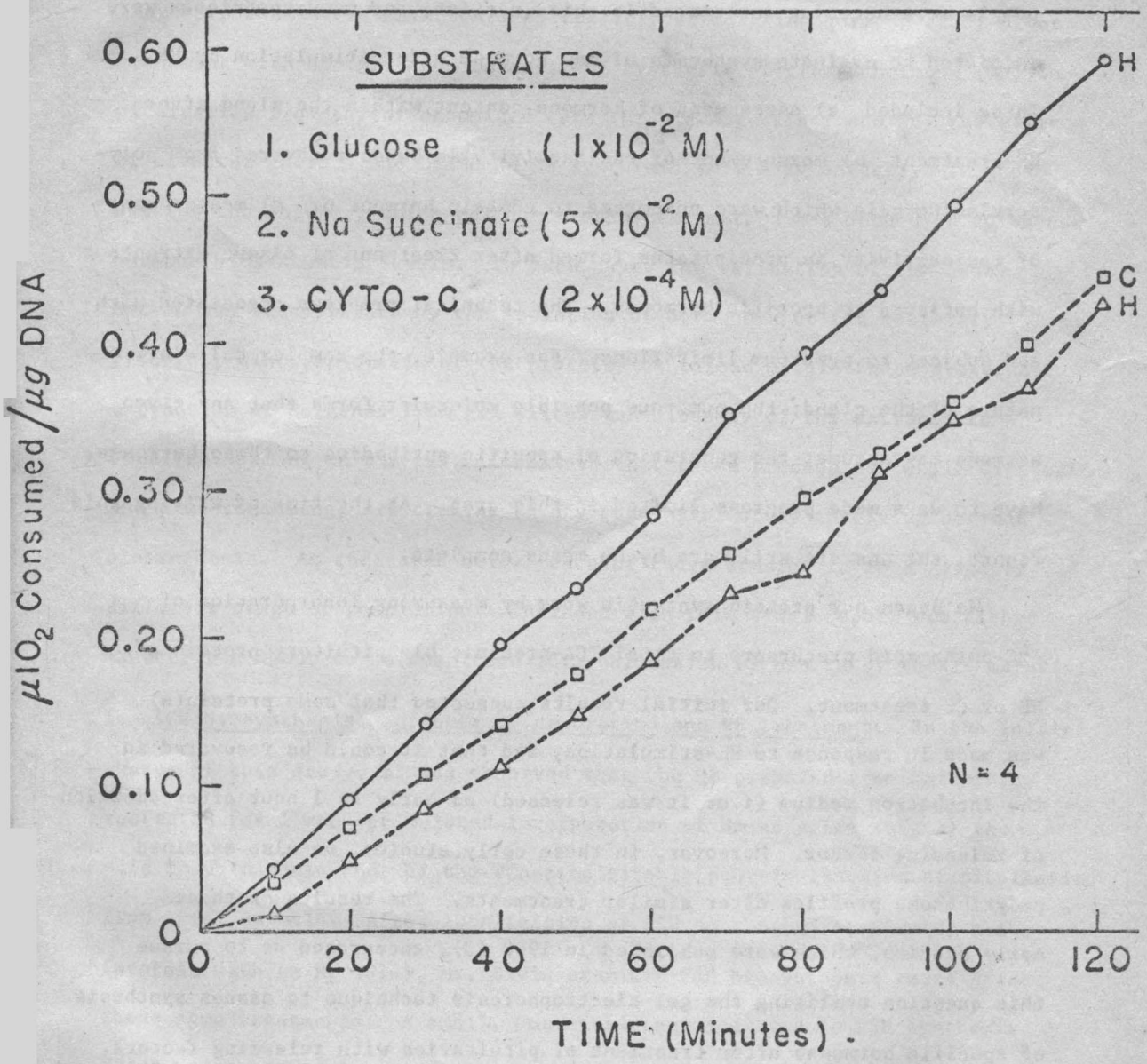
(i.e. 30 mM) in the incubation medium will cause hormone release (presumably making the cell membranes "leaking") and d) addition of low concentrations of dibutyryl cyclic AMP (DCAMP) to the medium causes significant hormone release. From these observations, the following current working hypothesis on the mechanism of releasing factor action has emerged: a releaser binds to a receptor site on the target cell membrane. Adenyl cyclase is closely linked to this receptor site. Binding of the releaser promotes activation of the cyclase system which leads to increased levels of cyclic AMP; this in turn promotes activation of several protein kinases. It is not yet clear what proteins are phosphorylated by this enzyme; some may be of the ribosomal variety, others may be in the stored hormone. By mechanisms which are as yet undefined (perhaps through contraction of microtubular elements), secretory granules undergo exocytotic processes leading to hormone release. While much of this work has recently emerged from Labrie's laboratory (see e.g. J. Biol. Chem. Dec. 1971); the above scheme by no means represents the entire story.

Especially unclear are questions relating to synthesis of new hormone, or activation of a "prohormone" form after stimulation by HE. The work described in the section of the report deals largely with questions relating to this particular point. Our basic approach has been that of adding labeled precursor amino acids (e.g. C^{14}-leucine) to the incubation medium; allowing incorporation to proceed (i.e. uptake of label into the gland as well as incorporation into a TCA-precipitable protein or hormone); and an estimation of the effect of HE on hormone synthesis (usually evaluated by polyacrylamide gel electrophoresis). The kinds of responses that we have obtained are briefly discussed in the following sections.

Effects of HE on General Intracellular Metabolic Events

O_2 consumption. It has been shown by several investigators that the HE-induced release of hormone from pituitaries incubated in the presence of respiratory and/or metabolic inhibitors is completely blocked by these agents. A number of years ago we measured the rate of O_2 consumption by pituitary halves incubated in medium containing HE or CE. In four separate experiments the respiratory rate in HE-treated glands was significantly higher than the corresponding control tissues treated with CE or neutralized HCl (Graph 1). Although this result was not published, it is of interest because it shows that a general parameter of metabolic activity, viz. O_2 consumption, is increased when pituitary cells are stimulated to release hormone. With the availability of purified somatotrophs, it would be of interest to repeat these experiments.

RNA synthesis. About the time this contract began, it was generally believed that many hormones acted, as a primary event, through activation of transcriptive mechanisms within the nucleus of the target cell. Accordingly, we studied the characteristics of RNA metabolism in the incubated pituitary, especially after stimulation by HE. Although we were able to show that a) RNA synthesis was taking place in the tissue and b) new ribosomes were being made during the 4 hours incubation, we were unable to find any alteration in the rate of RNA synthesis after administration of HE *in vitro* or *in vivo*. These results, which were published in 1967 (1), strongly indicated that formation of new RNA molecules were not required for releasing factor action. Since 1967 the results of other investigators would argue in favor of this generalization.


SUBSTRATES
1. Glucose (1x10^{-2}M)
2. Na Succinate (5x10^{-2}M)
3. CYTO-C (2x10^{-4}M)
H
C
H
N = 4
0.60
0.50
0.40
0.30
0.20
0.10
0
0
20
40
60
80
100
120
$\mu l O_2$ Consumed / μg DNA
TIME (Minutes)

General Protein Synthesis. After these RNA studies were completed, we turned our attention to another possible site of releasing factor action, viz. translational control at the polyribosome level. About this time many people were becoming interested in this question, and many techniques were initiated to evaluate synthesis of new hormone after stimulation by HE. These included a) assessment of hormone content within the gland after HE-treatment b) measurement of radioactivity in bands recovered from polyacrylamide gels which were purported to contain hormone or c) measurements of radioactivity in precipitates formed after treatment of tissue extracts with antisera to specific hormones. The technical problems associated with and subject to numerous limitations. For example, the complex cellular nature of the gland; the numerous possible molecular forms that any given hormone may assume; the generation of specific antibodies to these hormones, have to date made progress limited in this area. At the time of writing this report, the answers still are by no means complete.

We began our protein synthesis work by measuring incorporation of ^{14}C-amino acid precursors to total TCA-precipitable pituitary protein after HE or CE treatment. Our initial results suggested that some protein(s) was made in response to HL-stimulation, and that it could be recovered in the incubation medium (i.e. it was released) as early as 1 hour after addition of releasing factor. Moreover, in these early studies, we also examined polyribosome profiles after similar treatments. The results of these early studies, which were published in 1968 (3), encouraged us to pursue this question utilizing the gel electrophoresis technique to assess synthesis of specific hormones after treatment of pituitaries with releasing factors. These studies are summarized in the next section.

Effects of HE on Synthesis of Specific Pituitary Hormones.

Over the past three years, my graduate students have studied patterns of hormone synthesis after numerous physiological treatments. Specifically, they have examined a) synthesis of follicle stimulating hormone (FSH) after treatment with HE (Mr. John Davis); b) synthesis of growth hormone (GH) in pituitaries from thyroidectomized rats (Mr. Ed Augustine) and c) the effects of divalent cations and DCAMP on GH synthesis (Mr. Gary Snyder). In these studies the polyacrylamide gel electrophoretic method has been used to assess hormone biosynthetic levels. In each case, the validation of the assay method was accomplished by the isolation of hormone-specific secretory granules, electrophoresis of the proteins contained within these granules; extraction of hormones from gel slices and bioassay of the extracts to localize hormone on the gel. I believe that these procedures, while difficult and time-consuming, are crucial to any meaningful study involving hormone biosynthesis. As indicated below, each of these studies is in a slightly different stage of completion. Included with this final report are either manuscripts about to be submitted for publication or drafts of thesis material.

I. FSH biosynthesis: effects of castration and HE treatment. In the initial phases of this study, it was observed that the HE prepared from male rats castrated for 1 week stimulated incorporation of amino acids into a) the acid soluble pools and b) the TCA-precipitable protein fraction of pituitaries from these animals. After localization of FSH on the polyacrylamide gel (protein with an R_f .614), Mr. Davis examined FSH biosynthesis rates after these same treatments. A small, but consistent increase in FSH synthesis rates was observed in glands coincubated with HE. These data were presented at the AIBS meetings two years ago (13) and provide the basis for the working