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PROGRESS REPORT ON SCIENTIFIC ACTIVITIES

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## A. INTRODUCTION

This Progress Report and its Summary describe our two main lines of research, often discrete in appearance, but both directed at the solution of the single purpose of this contract.

1. Work directly related to the isolation and molecular characterization of the hypothalamic hormone LRF as well as related to its mode of action at the pituitary level to stimulate release of ovulation hormone (LH).

2. Experiments using hypothalamic peptides of native or synthetic origin, other than LRF but which we have reasons to believe to be closely enough related to LRF so that any information obtained with them will later on be of significance regarding structure--biological activity relationships, as well as mode of action of the hypothalamic hormone LRF, and directed to the devising of synthetic antagonists of LRF and their specific mode of action.

B. SUMMARY **PRIVILEGED COMMUNICATION**

1. The isolation of the hypothalamic LRF component present in the side-fractions from the (terminated) isolation program of TRF has been completed. This has yielded ca 125 micrograms dry weight material. This preparation of LRF is active in vivo to stimulate release of endogenous LH at  $\leq 1$  ng/ml incubation fluid. This is by far the most potent preparation of LRF ever obtained according to our own records and when compared to what is published in the literature.

2. Homogeneity of this preparation has been determined: The dry weight material obtained appears to consist of no less than 50-75% amino acids, the balance being most probably inert carbohydrates from the last purification step. There appears to be also a minor contaminant of the order of 1-5% in the form of some substance (free amino acid or small peptide) detectable by the dansyl reaction. The bulk of the LRF material is ninhydrin negative, Pauly positive and shows no free N-terminal by the dansyl reaction.

The amino acid composition of a 6N HCl hydrolysate of the peptide so isolated has been established to be as follows: His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1, Tyr 1, the  $\text{NH}_3$  peak suggesting the presence of an amide (C-terminal?).

3. Structure of hypothalamic LRF: We have established that the N-terminal residue of hypothalamic LRF is 2-pyrrolidone-5-carboxyl-(PCA). This has been arrived at by assessing the disappearance of biological activity of highly purified LRF following treatment with the highly specific pyrrolidone-carboxylic acid-peptidase prepared in collaboration with Fellows and Mudge; furthermore, microgram quantities of highly purified LRF have shown, by mass spectrometry using a chemical ionization probe in collaboration with Fales, fragments with  $m/e$ -84 and 129 characteristics of PCA. Moreover, treatment of

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LRF by mild HCl hydrolysis has revealed a N-terminal Glu residue following dansylation. Sequencing of LRF by the micro-Edman method is in progress.

4. Total synthesis of a series of peptide fragments containing 3,4,6 amino acids as well as 2 complete nonapeptides containing various combinations of all the amino acid residues found in LRF, have been performed and completed. All materials have been isolated in pure form and are presently used for biological and physical-chemical studies aimed at elucidating the molecular structure of LRF.

5. Biological studies in vivo and in vitro have been conducted on the highly purified LRF presently available. Recent assays indicate that in all circumstances studied so far, highly purified LRF stimulates concomitantly secretion of LH and FSH with the possibility still investigated that the threshold response for the release of FSH be somewhat higher than for the release of LH (thus raising the questions of the validity of earlier claims by others of a separate FRF, discrete from LRF). In vitro studies indicate that progestational steroids, as well as estradiol in large doses, will inhibit in part the release of LH and FSH stimulated in vitro by addition of highly purified LRF. The possibility of differential inhibition of FSH vs. LH secretion by one or a combination of the steroids is still being investigated.

6. Twenty synthetic polypeptides related to the structure of TRF (see Progress Report for their molecular structures) have been obtained and studied for their biological activity as well as antagonistic properties to TRF. Most of these analogues have been directed at modification of the C-terminal residue, only 2 being directed at the medial His residue, only 3 at the PCA N-terminal residue. All these studies indicate that C-terminal

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modified analogs of TRF do not act as antagonists to TRF, whereas some indications have been obtained for the other types of analogs. Our current hypothesis is thus that probably antagonists to LRF would most likely be obtained in an N-terminal analog of that polypeptide, especially in view of the fact that we have established that both LRF and TRF have a PCA-N-terminal residue.

7. In view of determining the best possible antagonist for LRF at the level of its target organ, i.e. the adenohypophysis, a series of studies has been undertaken using TRF as a model regarding its mode of action on the secretion of the glycoprotein TSH which is known to be closely related in structure to the glycoprotein LH. A prostaglandin-receptor for the mode of action of TRF as well as for LRF has been demonstrated using particularly the highly potent 7-oxa-13-prostanoic acid antagonist. Studies are currently underway along those lines to investigate the mode of action of TRF on the secretion and release of the various pools of TSH which have been demonstrated in pituitary cells.

8. The mass spectrometer (Varian Mat-CH5) and its computer readouts are now working routinely and are used constantly in our program (see Progress Report for limitations still present due to incomplete delivery of software by manufacturer). New techniques of derivatizations of peptides and amino acids, particularly devoted towards a microminiaturization of the methods used for volatilization of arginine are being worked out. A computer program for automatic peptide sequencing based on low resolution data is in the process of being developed.

9. The NMR spectrometer has been in full routine use for the last several months. It is constantly used for the confirmation of molecular structure of the various synthetic molecules prepared in the laboratory

(amino acid derivatives, oligopeptides).

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In summary, it would appear that our program is working pretty much according to our earlier projection as described to the Research Division of AID. My major concern pertains to the exceedingly small quantities of pure LRF that have been obtained from the large isolation program for TRF. As this progress report is written, I have thus decided that we will immediately put in effect the alternate emergency proposal which had been specified in our original application to AID, i.e. I am directing two investigators and two technicians to activate the necessary equipment and start the further extraction of approximately one-half million sheep hypothalamic fragments (available as lyophilized fragments) and conduct the necessary steps for the isolation for the LRF components of this new source of starting material.

#### C. PROJECTION FOR THE NEXT SIX MONTHS

1. It is anticipated that the full structure of an LRF active polypeptide will have been obtained including its replication by total synthesis. This LRF-active polypeptide will have been obtained from the elucidation of the complete amino acid sequence of native LRF as available presently in the laboratory. Alternatively, it will have been obtained as one of several possible combinations obtained by total synthesis of a small number of possible combinations of sequences written with the knowledge of: a) the complete amino acid composition of native LRF; and b) knowledge of a partial sequence of native LRF from the N-terminal residue combined with knowledge of the C-terminal residue. This last alternative must be envisioned if, for one of the many possible reasons, multiple cycling of the Edman degradation method did not yield the complete sequencing of native LRF in view of the exceedingly small quantities of native material available (60-70 nanomoles).

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I feel confident that we have all the technical knowledge to produce in a record time all the peptides necessary to complete this program assuming that the Edman degradation technique has given us at least 4 residues from the N-terminus plus availability of the nature of the C-terminal residue.

2. The studies described here in their extension should lead to specific leads regarding structural modifications for synthetic analogs that would act as antagonists of TRF, to be extrapolated to antagonistic structures to LRF.

3. Further insight into the mode of action of TRF, hence of LRF, should be available at the subcellular level (of the adenohipophysial tissue) regarding the prostaglandin receptor, regarding the mode of action of steroids as part of our overall knowledge necessary for the development of LRF antagonists.

D. PROGRESS REPORT

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I. Purification of LRF.

The starting material was the concentrate of LRF obtained from the side fractions of the isolation program of TRF (300,000 sheep hypothalamic fragments see our original application to AID). Partition chromatography in the butanol:acetic acid:water system (4:1:5) using Sephadex G-25 was carried out on 2.029 mg LRF concentrate (7243 LRF Units). Because of the extreme importance of this partition chromatography, involving all of our supply of purified LRF, it was conducted on a special microcolumn (250 x 2mm) of G-.5 superfine equilibrated successively with the 2 solvent phases so that the matrix leach should be less than 5  $\mu\text{g}$ /hold up volume. It took 5 weeks to obtain one acceptable such column. To improve our method of detection of LRF without losing any material, a small amount of  $\text{I}^{125}$ -labelled LRF obtained earlier (see application to AID) had been added to the starting material for easy location by  $\gamma$ -counting.

Two distinct peaks of radioactivity were observed, however only one peak contained biological LRF activity (tubes 22 to 29). Amino acid analysis on tube 25 next to peak of biological activity (70  $\mu\text{g}$  total weight) yielded, after 6 N HCl hydrolysis, the amino acid composition His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1, Tyr 1, with enough ammonia to account for an amide (C-terminal?). Approximately 50% of the material is peptide and of the amino acids detected, 94% are the amino acids listed above. Enzyme studies were performed on the remaining material except for 1.7  $\mu\text{g}$  which is being used in in vitro experiments at present. Tube 24 had 61  $\mu\text{g}$  of material and tube 23 had 35  $\mu\text{g}$ . This will be used in sequence studies. The material so obtained is ninhydrin negative (blocked N-terminus), Pauly positive (presence of His and/or Tyr), can be labelled with  $\text{I}^{125}$  (presence of Tyr), and behaves as a single spot in a series of TLC systems.

It is biologically active in vivo at  $\leq 5$  ng/animal, in vitro at  $\leq 1$  ng/ml.

Presently, the material is kept as a frozen solution in a sealed micro-tube. All our efforts at the moment are devoted to absolutely ascertain that every single step of the methodology contemplated to establish the amino acid sequence of this LRF is working satisfactorily before the unique supply of LRF is actually entered in these experiments. To this end, we have already conducted several times complete sequencing of the nonapeptides Lys-8-, or Arg-8-vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH<sub>2</sub>), oxytocin (Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-Gly-NH<sub>2</sub>), Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), as well as the synthetic decapeptide Gly-Bradykinin. All these experiments have been conducted by using the Edman degradation method on less than 50-70 nanomoles of the peptides. These series of experiments has shown us that when working on these nanogram quantities of peptide, much of the present methodology as described in the literature has to be considerably modified and the utmost care must be taken to avoid contamination and/or oxidation at any level of the sequencing technique. As this progress report is being written, we have just started using the sequencing technique on nanomole quantities of 2 different nonapeptides containing all the amino acid residues found in native LRF and which have been synthesized in our laboratory. Only after the complete sequencing of these two model nonapeptides have been satisfactorily completed will we consider touching the unique supply of native LRF. A tetrapeptide and a pentapeptide containing the amino acid present in the 6 N HCl hydrolysate of LRF have also been synthesized, as well as two tetrapeptides PCA-His-Tyr-Pro-NH<sub>2</sub> and PCA-Tyr-His-Pro-NH<sub>2</sub> which have also been prepared. All these substances will be used as model compounds for the various methodology aimed at elucidating the structure of LRF.

If the complete sequence of the nonapeptide LRF is not obtained by the successive Edman degradation in view of the minute quantities of material with

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which the cycles will have to be performed, we are considering synthesizing all the possible combinations of peptides corresponding to the amino acid content of LRF as long as we will have established the sequence of at least 4-5 residues from and including the N-terminus and have established the identity of the C-terminus. We are well equipped to handle this large and expansive proposition with our current program of solid phase peptide synthesis.

Also, as this progress report is being written, we have started again the complete extraction procedure of approximately 1/2 million sheep hypothalamus (available from previous support by NIH AM 8290-01-06), a contingency plan which had been clearly outlined in our original application to AID. This will be carried out to the complete isolation of the LRF component present in the hypothalamic extracts. Two investigators and two technicians will work on this aspect of our program practically full time. All bioassays for LRF will be by the new method devised by our group combining bioassay and radioimmunoassay for LH and FSH in peripheral plasma; all statistical evaluation will include a complete analysis of covariance (hormone concentrations after injection of LRF, over hormone concentration prior to injection of LRF).

## II. Differentiation between LRF and FRF.

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### A. LRF on secretion of LH and FSH:

Our most highly purified preparations of LRF (isolated and assessed by their LH-releasing activity) appear to stimulate concomitant secretion of FSH and LH. It may take more LRF to achieve a significant FSH response than it does an LH response. Both 18 hr incubations and the use of fetal calf serum appear to be necessary for maximum response both to the LRF and to any steroids that may be added (see below). These surprising results observed with what would appear to be the most purified LRF ever obtained, raises, of course, the question of the significance of the many claims (McCann, Dhariwal, Schally, Martini, Jutisz) to the existence of an FRF distinct from LRF.

### B. Effects of steroids on activity of LRF:

In several experiments, the effects of several gonadal steroids have been studied on the response to LRF. Those steroids tested were testosterone propionate (aqueous suspension), estradiol-17B, estriol, pregnenolone-SO<sub>4</sub>, and the progestagen and estradiol in combination. So far, the steroids had the same inhibitory effect on LH and FSH release at the large doses tested. The magnitude of the response may vary but the direction of the response was the same. In one experiment, however, with 4 hr preincubation, 125 μg T.P., 100 μg Preg-SO<sub>4</sub>, and 50 μg E<sub>2</sub> lowered LH levels to less than controls whereas FSH levels were higher.

As little as 10 μg of testosterone propionate (lowest dose tested) and 50 μg estradiol-17B (lowest dose tested) were able to significantly decrease the response of the anterior pituitary to LRF. The response to pregnenolone-SO<sub>4</sub> has been variable and on one occasion, reduced the LRF response with a dose of 10 μg. No synergistic effect was seen in combination with E<sub>2</sub> (50 μg of each), however, the E<sub>2</sub> was very potent alone and the pregnenolone-SO<sub>4</sub> had no effect.

### C. Dose/response to LRF:

Potency ratios (secretion of LH) to doses of 2.0, 5.0, 10.0 and 20.0 ng/ml were 1.3, 2.0, 2.5 and 2.4 times that of controls respectively in an assay with

a preincubation time of only 4 hr. Responses in terms of secretion of FSH in the same experiment and in the same fluids were 1.2, 1.7, 1.5 and 1.5 that of control, respectively. In an experiment utilizing an 18 hr preincubation and doses of 1.0, 2.5, 5.0 and 10.0 ng LRF/ml, the LH responses using adenohipophysis from 30 day old female rats were 2.4, 4.6, 6.1 and 45.7 times that of controls. The potency ratios for FSH secretion were 0.9, 1.9, 2.4, and 11.0. Adenohipophyses from 120 g male rats gave LH potency ratios of 2.5, 2.9, 3.3, and 4.8; for FSH, 1.5, 1.5, 2.9, and 4.2.

Experiments are planned to check out the responses in immature male rats. The LH/FSH responses in adenohipophyses from female rats in various stages of the cycle will be tested in the next few weeks.

These studies are important to our overall program and contract goal, inasmuch as they seem to indicate an even more important role of LRF in the overall control of the secretion of all pituitary gonadotropins than had actually be suspected.

D. Simultaneous LH-FSH radioimmunoassays:

Attempts have been made to develop an RIA which would quantitate LH and FSH at the same time using the same sample. A solid phase LH assay using I<sup>131</sup> LH as a trace coupled with a double antibody I<sup>125</sup>-FSH assay, has been tried. Problems of buffers, and pH have been worked on but cross-reaction of the FSH-Ab with LH has hampered work to date. Work is still continued on this project of methodology.

E. Plasma inactivation of LRF:

This project is a continuation of one started at Baylor. Due to overlapping confidence limits, it has been impossible to draw valid conclusions. However, it appears as if the plasma of female rats has a greater effect on LRF than the plasma of male rats. The lack of LRF available for physiological studies has hampered this project which will be renewed as soon as a synthetic LRF becomes available.

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F. Effect of LRF on prolactin secretion:

1. Normal rat pituitary cells maintained in culture secrete high amounts of LTH. We have found no effect of LRF on the secretion of LTH from these cultures. This system might be used in vitro assay for purified PIF when it is available.

2. Anterior pituitary cells cultured for 30 days were transplanted into a pneumodermal pouch of hypox rats. High levels of LTH were subsequently detected in the plasma of the recipient rats. This technique might be used as an in vivo PIF assay.

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III. Design of antagonists to releasing factors.

A. Mode of action of the releasing factors.

In view of the current thinking on the possible role of prostaglandins in c-AMP, adenyl-cyclase system as initiator of hormonal secretions, several experiments were undertaken to study a possible participation of a prostaglandin receptor at the level of the adenohipophysial tissue, in the mediation of the effects of releasing factors. Experiments were carried out to test the action of various prostaglandins on endogenous and elevated  $K^+$  mediated release of TSH, ACTH and LH. Compounds studied were 7-oxa-13-prostanoic acid (PGI),  $PGE_1$ ,  $PGE_2$ ,  $PGF_{2\alpha}$ , and  $PGA_2$ . 1) 10  $\mu$ g PGI inhibited LH secretion due to elevated  $K^+$ ; 2) 100  $\mu$ g PGI increased LH in media over controls; 3) 1  $\mu$ g  $PGE_1$  increased LH in media whereas 10  $\mu$ g of same compound decreased LH by a like amount; 4)  $PGF_2$  had no effect either on endogenous LH release or  $K^+$  mediated release at doses of 1 or 10  $\mu$ g; 5)  $PGE_2$  (10  $\mu$ g) doubled LH in incubation media and 10  $\mu$ g  $PGA_2$  decreased LH by 30%.

Furthermore, prostaglandins of the PGE series appear to release TSH and ACTH although further experiments will be required to explain inconsistencies in the response to various doses of the prostaglandins. The prostaglandin antagonist 7-oxa-13 prostanoic acid (Fried) decreases the amount of TSH and ACTH secreted in response to high potassium and inhibits the secretion of TSH in response to TRF. These experiments are presently being extended to include studies on LH and FSH secretion as well (see IIA).

B. Role of c-AMP

As part of our program investigating the role of cyclic AMP on the secretion of pituitary hormones, we studied the effect of cyclic GMP on secretion of TSH and ACTH and found no effect at doses of  $10^{-3}$  and  $10^{-2}$  M. We have asked Plenum Scientific Co. to prepare the dibutyryl derivative of

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cyclic GMP for us. If cyclic GMP were effective in releasing TSH and LH, it might explain our findings that theophylline (which retards the breakdown of cyclic GMP as well as cyclic AMP) releases TSH and LH while cyclic AMP and dibutyryl cyclic AMP do not release TSH and LH in our hands.

Assay of cyclic AMP: Using a method described by G. Gill and L. Garren (Biochem. Biophys. Res. Comm. 39, 385, 1970), we have set up an assay for cyclic AMP. We have seen, using this procedure, that incubation of rat hemi-pituitaries in  $10^{-2}$  M theophylline increased pituitary cyclic AMP. The cyclic AMP assay should be completely validated by the time cell populations suitable for the proposed studies are available (see below).

C. Methods of developing availability of homogeneous pituitary cells preparations.

A major stumbling block in all our studies on the mode of action of LRF is the considerable heterogeneity of the normal pituitary tissue (i.e., the presence in one mm<sup>3</sup> of pituitary tissues of thousands of somatotrophs, prolactin cells, and only a few gonadotrophs and thyrotrophs).

Considerable effort must be and is presently expended in trying to obtain homogeneous pituitary cell populations which could be studied just like a bacterial culture medium. To this effect, the following studies have been conducted:

1. Studies of secretion of pituitary hormones from dispersed pituitary cells. We have found that incubation of anterior pituitary cells prepared by trypsinization of anterior pituitary tissue will secrete TSH when stimulated by TRF. However, the magnitude of the increase in the amounts of TSH secreted in response to TRF is smaller than the increase observed with hemi-pituitaries. Another difference is that T<sub>4</sub> pretreatment which inhibits the response to TRF by hemi-pituitaries, has no effect on the response to TRF by

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acutely dispersed cells. Dibutyryl cyclic AMP was ineffective in stimulating secretion of TSH from dispersed pituitary cells as well as normal hemipituitaries, possibly indicating that the lack of effect of dibutyryl cyclic AMP on TSH secretion is not due to the inaccessibility of the thyrotrophs.

2. Studies on cultured pituitary cells. Completely dispersed anterior pituitary cells have been distributed equally among several tissue culture dishes and maintained for varying periods of time. Daily treatment of cultured rat pituitary cells with TRF for up to 30 days results in an increase in the TSH activity of the media as measured by bioassays. No TSH was detected in the cells of either control or TRF treated tissue. It was necessary in that experiment to collect media that contained 7 days' secretion in order to detect TSH by the bioassay. Therefore, due to possible differences in TSH stability inside and outside of the cell, we cannot conclude that TRF was increasing the rate of synthesis of TSH.

A similar experiment with control, TRF and  $5 \times [K^+]$  treatments has been underway for three weeks. New methodology will allow us to determine the rate of TSH synthesis in these cells.

Several different lines of TSH secreting tumors are being maintained in tissue culture. We are presently studying the nature of the materials secreted by these tumors and determining if TRF, cyclic AMP, prostaglandin antagonists, prostaglandins, high  $[K^+]$ , etc. are capable of modifying the rate of synthesis or secretion of any of these materials (see below).

3. Several proposed experiments will require using tissue with no extracellular space. Due to the problems encountered with the acutely dispersed cells, the feasibility of using short term (48-72 hr) tissue culture of pituitary cells is being investigated. If this system appears adequate, it will be used for calcium flux studies, and for determining if TRF-bound to large

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impermeable molecules can still release TSH. This would add to the evidence that TRF is acting at the plasma membrane level. Similar studies are to be conducted with LRF.

4. Studies monitoring metameters of cell function other than specific hormone synthesis or secretion. It would be of great interest to determine whether specific releasing factors, such as LRF or TRF, or specific inhibitory peripheral hormones, for example gonadal steroids or thyroxine, have an effect on cyclic AMP or prostaglandin levels, on  $Ca^{++}$  or  $Ka^{+}$  activity, on the plasma membrane potential or on the rate of RNA or protein synthesis in the specific cells involved. The fundamental obstacle to investigations of this type is the fact that the anterior pituitary is a heterogeneous organ containing several cell types of which only the thyrotrophs, a small percentage of the total, would be expected to respond to their specific stimulus, LRF or TRF, or to their specific inhibitor, steroids or thyroxine.

In order to circumvent this problem, three basic approaches are being taken:

a) A technique for separation of dispersed cells--a method described by Hymer (J. Cell. Biol. 47, 94a, 1970), modified by G. Grant. Preliminary results indicate that it will be possible to separate the TSH containing cells from 80-90% of the other anterior pituitary cells.

b) Specific killing of anterior pituitary cells by cytophilic antibodies. Antisera are being produced in rabbits against mouse anterior pituitary cells. The antisera will then be adsorbed with TSH secreting tumor cells in order to eliminate those antibodies against all non-specific pituitary cell antigens and those specifically against thyrotroph antigens. The antisera might then be used to lyse all of the cells except the thyrotrophs. The antisera raised to this date are pituitary cytophilic, however, the titers

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are not yet high enough to warrant proceeding to the adsorption step.

c) Another approach to obtaining a high population of thyrotrophs is to carry out these studies on TSH secreting tumors on pituitaries from chronically thyroidectomized donors. Several different generations of TSH tumors are being maintained in culture. Earlier experience with an old tumor line (transplanted many times) indicated that TRF did not increase the secretion of biologically active TSH from these tumors. TRF did release TSH from dispersed cells obtained from a  $G_0$  tumor. For that reason, we have thyroidectomized 200 mice which should start yielding  $G_0$  tumors by March 1971. These  $G_0$  tumors should contain a high proportion of thyrotrophs and, therefore, would be suitable for many proposed studies.

D. Role of releasing factors in the biosynthesis and secretion of pituitary hormones.

At the moment, it seems best to approach this problem with TRF, readily available, thus studying secretion of TSH. We have every reason to believe that results can be extrapolated to LRF and LH.

TSH secretion will be examined initially in TSH secreting mouse pituitary tumors to obtain a material actively secreting TSH growing in tissue culture. To this end, three Farth TSH pituitary tumors have been established in culture. Preliminary experiments carried out have been established in culture. Preliminary experiments carried out have been concerned with pool sizes and equilibrium rates of TSH inside and outside the cell, and with the nature of the secreted protein material. Double labeling experiments indicate that the secreted protein is composed of both newly labeled and previously labeled material with previously labeled protein being secreted at a linear rate for about 10 hours. The protein secreted by the tumors appears to be biologically inactive but immunologically normal. Examination of the secreted tumor

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protein on SDS-acrylamide gel electrophoresis demonstrated 3 proteins, only 2 of these are immunologically precipitated corresponding to  $\alpha$  and  $\beta$  TSH chains. The mobility of these TSH subunits on SDS gels indicates that a large size discrepancy exists between the expected (12,000) and apparent (25-30,000 and 40,000) Molecular Weights. This is likely to be due to the presence of carbohydrate moieties on the subunits as reported in the case of immunoglobulins. The addition of carbohydrate to the subunits provides a useful handle for the study of synthesis of complete TSH molecules. Further examination of this problem is obviously merited.

The study of TRF mode of action will be approached as follows:

We plan to use  $^3\text{H}$ -TRF of high specific activity and bind it to pituitary cells and/or membranes. This technique will provide two important handles, A) an assay of TRF by competition for  $^3\text{H}$ -TRF, and B) a binding constant of TRF for the cell surface. This latter number will provide the basis for a study of the many analogs of TRF which we now have in the lab and possibly the results of competition experiments will tell us, in conjunction with data on biological potency, the functional parts of the molecule.

A second series of experiments will investigate whether TRF induces release of TSH from normal pituitary cells via the stimulation of cyclic nucleotide synthesis (for discussion purposes referred to as c-AMP only (See above). This has been studied on pituitaries which have been dispersed with trypsin into individual cells and fractionated on a BSA gradient à la Hymer and Evans). The most rapidly migrating cells appear to contain the majority of the thyrotrophs but only 20% of the pituitary cells. The level of stimulation of c-AMP in this population upon TRF stimulation will be tested.

E. Structure/activity studies on synthetic analogs of TRF.

We are already expending considerable efforts at studying synthetic analogs of TRF as possible antagonists (of TRF), our rationale here being that results obtained with this series of compounds could be extrapolated to similar problems relating to LRF.

The following synthetic peptide analogs of TRF have been obtained and tested for their agonist and antagonist properties:

1. Modifications of the C-terminus: PCA-His-OMe (I); PCA-His-Pro-N (Et)<sub>2</sub> (II); PCA-His-Pro-N (III); PCA-His-azidine-NH<sub>2</sub> (IV); PCA-His-Pyrrolidine (V); PCA-His-Pro-N (CH<sub>3</sub>)<sub>2</sub> (VI); PCA-His-Morpholine (VII); PCA-His-N  (VIII); PCA-His-Pro-anilide (IX); PCA-His-Pro-NH-(CH<sub>2</sub>)<sub>2</sub>OH (X); PCA-His-Pro-Tyr (XI); PCA-His-Pro-Tyr-NH<sub>2</sub> (XII); PCA-His-Pro (OH)-NH<sub>2</sub> (XIII); PCA-His-Leu-NH<sub>2</sub> (XIV).
2. Modifications of the N-terminus: Pro-His-Pro-NH<sub>2</sub> (XV).
3. Modifications of the medial His-residue: Pro-His-(1 N-CH<sub>3</sub>)-Pro-NH<sub>2</sub> (XVI)

All compounds as above were used here only in their pure form; the structures were confirmed by high resolution NMR (Jecolco PS-100) and mass spectrometry (Varian-Mat CH-5). Compounds I, XI, and XVI were synthesized in our laboratories; other analogs were synthesized by the Parke-Davis Research Labs, Ann Arbor, Michigan.

Bioassays: All substances were quantitatively assayed for TRF activity in vivo using pure synthetic TRF (50,000 U/ug) as reference standard. After the proper range of dose to be used had been determined in pilot experiments, the final assay design was that of a complete 4-point assay with no less than 5 replicate/dose, the interval of dose being equal for standard and unknown and usually equal to 3.0. All 4-point assays were calculated by factorial analysis after log<sub>10</sub> transformation and covariance adjustment. For studies of possible antagonistic properties (to TRF) a classical 2 x 2 factorial design was used.

The synthetic analogs of TRF described above were shown to possess the following specific activities (TRF U/ag): (I): 0, no biological activity at doses up to 200  $\mu$ g; (II): 20; (III): 300; (IV): 800; (V): 100; (VI): 250; (VII): 80; (VIII): 20; (IX): 10000; (X): 10000; (XI): <2.5; (XII): <2.5; (XIII): >50; (XIV): >50; (XV): 5; (XVI): 20. Compounds (XV), (XVI), and (XI) were tested for antagonistic properties to TRF; they showed no inhibition of TRF activity at ratios of  $1 \times 10^3$  or  $4 \times 10^3$  molar excess.

It would thus appear that none of the modifications of the C-terminal Proline--other than removing it altogether (PCA-His-OMe has no biological activity), or making it the Copenultimate residue (PCA-His-Pro-Tyr-OH or PCA-His-Pro-Tyr-NH<sub>2</sub> have no activity)--destroyed completely the biological activity. None of these compounds appeared to be potent antagonists of TRF at the doses tested. The next series of synthetic analogs as possible antagonists will be directed to modifications of the N-terminus which appears to be much more susceptible to changes leading to alterations of biological activity.

Additional insight of the structure activity relationships of TRF will be gained if labelled TRF can be found to bind to intact cells or a membrane fraction of disrupted cells. The displacement of this binding by TRF analogs will allow us to measure the binding constants of the various analogs for the TRF receptor. With an appreciation of the site(s) on the TRF molecule determining the affinity of TRF for its receptor and the site(s) responsible for intrinsic activity, we will be able to better suggest what analogs of TRF might have antagonistic activity. So far, we have been unable to detect binding of low specific activity <sup>3</sup>H-TRF to dispersed cells in vitro. Higher specific activity TRF will be required to test the feasibility of this method.

IV. Program of synthesis of peptides.

PRIVILEGED COMMUNICATION

A. Solid phase synthesis:

We have completed the construction of several Merrifield-type synthesizers, construction of a vacuum system for handling anhydrous HF; the synthesis of several peptides has also been achieved and purification procedures for oligopeptides have been devised for routine use.

The peptides synthesized include several TRF analogs and two trial peptides of the LRF composition. The TRF analogs include the following resin peptides:

1) PCA-Gly-Pro  $\nu$  resin; 2)  $\text{CH}_3\text{CH}_2\overset{\text{O}}{\parallel}\text{C}$  Gly-His-Pro  $\nu$  resin; 3) GLN-His-Pro  $\nu$  resin; 4)  $\text{N}^\alpha$  acyl-GLN His Pro  $\nu$  resin. Peptides #2) - #4) include changes at the N-terminus of the TRF peptide. Peptide #2), a propionylated Gly-His-Pro peptide, is almost a structural isomer of native TRF. The other two PCA analogs are the glutaminy and  $\text{N}^\alpha$ -acyl-glutaminy PCA analogs. Except for the glutaminy analogs, none of these peptides should provide difficulties in obtaining pure products. The purification of these peptides will be undertaken as soon as the HF train is operating efficiently enough to remove the peptides from the resin.

The "LRF" peptides synthesized include the following: 1) PCA-His-Pro-Tyr-Leu-Gly-Arg-Ser-Gly  $\nu$  resin; 2) His-Ser-Arg-Pro-Gly-Gly-Glu-Leu-Tyr  $\nu$  resin. "LRF" peptide #1) has been taken through several trial purification procedures, and the best one was BAW (butanol:acetic acid:water) (4:1:5). Chromatograms show seven peaks, the major peak giving amino acid analyses corresponding to the synthesis as carried out. The two tetrapeptides PCA-His-Tyr-Pro-NH<sub>2</sub> and PCA-Tyr-His-Pro-NH<sub>2</sub> have also been prepared.

During the above synthesis, several techniques have been worked on for improving and testing the purity and homogeneity of products in the synthesis of peptides by solid-phase. These include acylation by several reagents in

PRIVILEGED COMMUNICATION

order to terminate chains which are unreactive to acylation by amino acid derivatives. Reagents include acetic acid, benzoic acid, and others. This should improve homogeneity and simultaneously provide a means of separating contaminating shorter peptides from final products.

B. Peptide synthesis by "wet" procedures:

1. Synthesis of the oligopeptide PCA-His-Pro-NH<sub>2</sub>: The compound N(α), N(i<sub>m</sub>)-dicarbobenzoxyhistidylprolyl-p-nitrophenyl-ester was prepared in 71% yield, but was difficult to obtain analytically pure. Removal of blocking groups and coupling of carbobenzoxy-PCA, followed by deprotection and ammonolysis of the active ester, produced TFF in 5% overall yields. Various attempts to increase the yield of the second coupling step were unsuccessful.

- a) N(α), N(i<sub>m</sub>)-dicarbobenzoxyhistidine (Di Z-His) + HBr·Pro-  

$$\text{O} \begin{array}{c} \text{O} \\ \text{NO}_2 \end{array} \xrightarrow[\text{DCC, EtOAc}]{\text{N-methylmorpholine (NEM)}} \text{Di Z-His-Pro-}$$

$$\text{O} \begin{array}{c} \text{O} \\ \text{NO}_2 \end{array} \quad 71\% \text{ yield};$$
- b) Di Z-His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$   $\xrightarrow[\text{Dioxane}]{\text{HBr}}$  2HBr·N(i<sub>m</sub>) Z-His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$  93% yield.
- c) Z-PCA + 2HBr·N(i<sub>m</sub>) Z-His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$   $\xrightarrow[\text{DCC, EtOAc}]{\text{NEM}}$   

$$\text{Z-PCA-N(i}_m\text{)-Z-His-O} \begin{array}{c} \text{O} \\ \text{NO}_2 \end{array} \quad 25\% \text{ yield}$$
- d) Z-PCA-N(i<sub>m</sub>)His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$   $\xrightarrow{\text{HBr}}$  PCA-N(i<sub>m</sub>)His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$
- e) PCA-N(i<sub>m</sub>)Z-His-Pro-O $\begin{array}{c} \text{O} \\ \text{NO}_2 \end{array}$   $\xrightarrow{\text{NH}_3}$  PCA-His-Pro-NH<sub>2</sub>

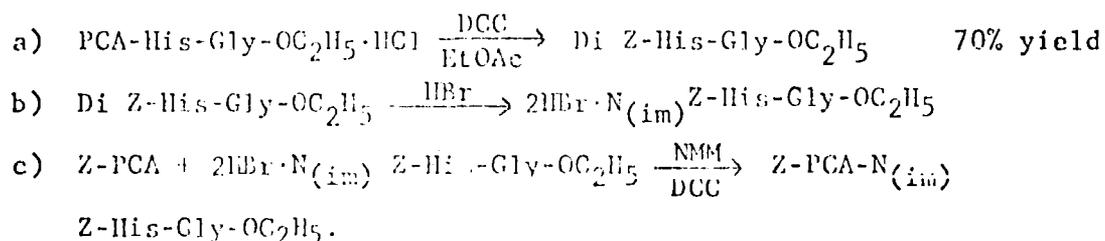
The coupling of dicarbobenzoxyhistidine to proline amide gave rise to several products. Attempted purification of the desired product caused further decomposition as evidenced by TLC. Products of this reaction have not been identified further.

- a) Di Z-His + Pro-NH<sub>2</sub>  $\xrightarrow[\text{DMF}]{\text{DCC}}$  Di Z-His-Pro-NH<sub>2</sub>
- b) Di Z-His-Pro-NH<sub>2</sub>  $\xrightarrow{\text{HBr}}$  2HBr·N(i<sub>m</sub>) Z-His-Pro-NH<sub>2</sub>
- c) Z-PCA + 2HBr·N(i<sub>m</sub>) Z-His-Pro-NH<sub>2</sub>  $\xrightarrow[\text{DCC}]{\text{NEM}}$  Z-PCA-N(i<sub>m</sub>) Z-His-Pro-NH<sub>2</sub>
- d) Z-PCA-N(i<sub>m</sub>) Z-His-Pro-NH<sub>2</sub>  $\xrightarrow{\text{H}_2}$  PCA-His-Pro-NH<sub>2</sub>

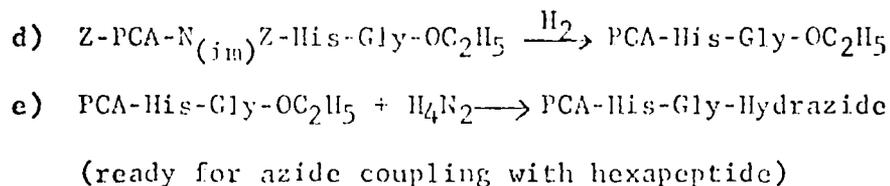
2. Synthesis of PCA-His-Pro-NH<sub>2</sub> by the Studer-Gillessen procedure:

The necessary neutralization of histidine methylester dihydrochloride calls for use of sodium methoxide and this method has been used. However, an easier resin neutralization of the histidine methylester dihydrochloride has been accomplished and the desired product verified by mass spectroscopy, NMR, and purity by TLC, and used in the Studer-Gillessen procedure.

3. Synthesis of a nonapeptide containing the known amino acids of LRF: The tripeptide PCA-His-Gly-hydrazide was prepared for coupling with a hexapeptide. The second step (e.g. Z-PCA + N<sub>(im)</sub> Z-His-Gly-OC<sub>2</sub>H<sub>5</sub>) was highly solvent dependent. The reaction gave a 70% yield in acetonitrile, but no product in ethyl acetate.



Step c) didn't work in EtOAc, gave 70% yield in acetonitrile.



The protected dipeptide active ester (Z-(O benzyl) Tyr-Pro-O~~NO~~<sub>2</sub>) was prepared for coupling with Leu-Ser-Gly-NH<sub>2</sub>.

Carbobenzoxynitroarginine and nitroarginine methylester were prepared. A small amount of Z-(O benzyl) Tyr-Pro-O~~NO~~<sub>2</sub> was coupled to nitroarginine methylester to show the possibility of this reaction.

4. Preparation of PCA-Pro-Tyr-OCH<sub>3</sub>: The blocked peptide, Z-PCA-Pro-(O benzyl) Tyr-OCH<sub>3</sub> was prepared and confirmed by mass spectroscopy. The deprotected peptide is apparently unstable and purification procedures are still in progress. This peptide is to be used for permethylation studies.

PRIVATE ONLY COMMUNICATION

5. Attempted synthesis of  $N(\alpha)$ , Boc,  $N_G$ ,  $N_G$  dicarbobenzyloxy arginine.

This should be an excellent derivative since the Boc group can be removed leaving the Z groups intact and the guanidino groups intact. The tricarbobenzyloxy arginine was prepared, but the removal of the alpha carbobenzyloxy group was unsuccessful. The Boc group can be removed leaving Z groups for full protection of guanidino group.

- a) Z-Cl + Arg tri-Z-Arg
- b) Tri-Z-Arg  $\xrightarrow{SOCl_2}$   $N_G, N_G$ -Di-Z-Arg
- c) Boc azide + Di Z-Arg  $\longrightarrow$   $N(\alpha)$  Boc,  $N_G, N_G$ , Di Z-Arg.

6. Preparation of benzhydrylamine resin for solid-phase synthesis of various polypeptides.

- a) Benzoylation with benzoyl chloride;
- b) Reduction to formyl amine with  $NH_4OOC$ ;
- c) Hydrolysis to free amine

CHN analysis on benzoylated resin, and benzhydryl amine resin indicated 0.86 meq N had been introduced. Coupling of Boc-Pro with the resin introduced 0.43 moles Pro per gram of resin.

7. Synthesis of L-PCA-D-His-L-Pro-NH<sub>2</sub>D-His: This is still in progress; D-His (obtained commercially) may not be optically pure as measured in the polarimeter.

V. Computer Programming.

PRIMARY DATA COMMUNICATION

Our computer activities seem to fall into two general areas - those concerning the use of the 1050 terminal with the Baylor 360/50 system, and those concerning the Varian 620/i computer, dedicated to the CH-5 mass spectrometer.

Looking at the 1050/360/50 work first, EXBIOL and the programs associated with it are already debugged and running well; only minor changes have been made to cut out some unnecessary lines of printed output.

The IMMUNO program had some more serious problems but has subsequently been debugged and the output rearranged and reduced for clarity and brevity. At this point, we are discussing the possibility of dividing it into two sections. The first section would take the data and store it in a data set. This should eliminate the need for reentering the data from the long paper tapes when the system goes down, there is line trouble, or other error conditions occur. The second section would operate on the data already stored, proceeding with the same calculations IMMUNO does now. However, we hope to include a few new options that we feel are desirable and rearrange the program slightly in order to make it more flexible for future use in other assays.

Meanwhile, we have been investigating computer facilities in our more immediate geographical area which might be able to accommodate our present and future needs. So far, we have not found one with an operating system that would be compatible with the programs already written, would have remote terminal and paper tape input capabilities, and be within our local calling area. The survey will continue considering the various solutions available and the changes they would require along with any new possibilities that may arise.

In the area concerning the Varian 620/i facilities, the initial efforts involved becoming acquainted with the overall system of the mass spectrometer and more specifically, the operation of the Varian computer. Fortunately, a

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two-week course was available at Varian Data Machines in late October 1970 which covered its operation, DAS (assembly language), machine language and Fortran programming, a possible operating system, and use of various peripheral equipment. We have been working on a program to analyze the data on paper tape from the amino acid analyzer. We are also developing a program to look for differences in masses found in a spectrum that might indicate specifically interesting fragments. Eventually, we plan to develop a program that will sequence a peptide from low resolution mass spectrographic data.

VI. Physical chemistry, Mass spectrometer, nuclear magnetic resonance spectrometer.

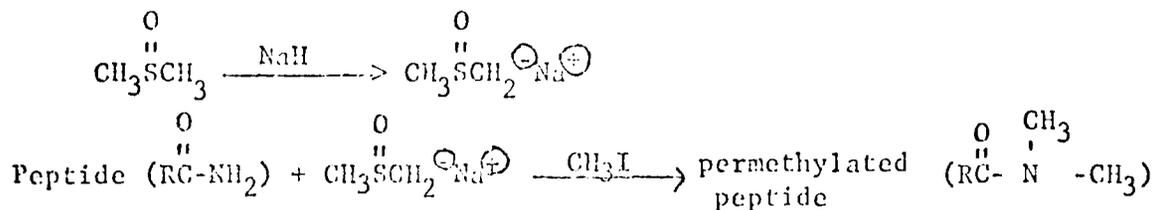
PRIVILEGED COMMUNICATION

The mass spectrometry laboratory had to be completely installed. All the equipment has been tested; we are satisfied with the performance of all components, with the exception of the Peak Matching Unit, the GC line-of-sight inlet system, and the Library Encoding and Spectrum Averaging Program. The Peak Matching Unit, according to the Varian service engineer (November 1970) will be adjusted to its proper working condition as soon as he receives instructions from the factory research center. The line-of-sight inlet will be installed and demonstrated by Varian engineers when the mass spectrometer is moved to its new location next July; its performance is not an absolute requisite for the progress of our present program. Varian-Mat delivered what they called the Library Encoding and Spectrum Averaging Program on December 21, 1970. However, the service engineer had difficulty in running this program. As a result, we are still holding Varian responsible for the delivery of a workable Library Encoding and Spectrum Averaging Program as specified in the purchase contract.

While we were testing the equipment, we have also been compiling mass spectral data of amino acids and peptides. All the peptide and amino acid samples run in our mass spectrometer were either made in our own laboratory or supplied by D. Nicolaides (Parke-Davis Research Labs).

Since most underivatized peptides have very low volatility, another major project has been to devise means to increase the volatility of the peptides so that evaluable spectra can be obtained in the mass spectrometer. So far, we have been successful in using the permethylation technique to increase the volatility of some peptides. The permethylation method that we used was adapted from S. Lande et al. (Biochem. Biophys. Res. Comm., 1970). The procedure

involves the preparation of sodium methylsulfinyl methide from sodium hydride and dimethyl sulfoxide, and adding it to a solution of the peptide in dimethyl sulfoxide followed by methyl iodide.



We have been using a tripeptide, PCA-Leu-Ala-amide as the standard for refining our permethylation technique and we were able to scale the reaction successfully down from 100  $\mu\text{g}$  to 10  $\mu\text{g}$  and still obtained excellent spectra from our mass spectrometer.

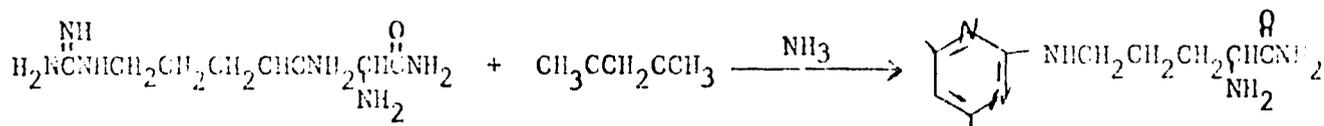
The significance of the permethylation reaction in increasing the volatility of a peptide was demonstrated by permethylating synthetic Tyr-Pro-Leu-Ser-Gly-amide. The underivatized peptide did not show a molecular ion or any peak in the molecular ion range whereas the permethylated compound clearly showed the molecular ion.

The permethylation reaction, however, does not give a clean product when the peptide contains histidine because the molecular ion is composed of the permethylated compound as well as ions with one, two, or three extra methyl groups. This discrepancy is due to the fact that the  $\text{CH}_2$ -group of the histidine is quite acidic. As a result, the two hydrogens could be replaced by methyl groups in the permethylation reaction, and thus would account for the appearance of one or two extra methyl groups on the molecular ion. Methylation of the second nitrogen on the histidine ring would account for the molecular ion plus three methyl groups. Permethylation experiments performed with histidine methyl ester are currently being undertaken to verify this hypothesis.

In the general area of increasing the volatility of peptides, we have been trying to prepare a volatile derivative of arginine. So far, we have been

RESEARCH COMMUNICATION

successful in converting one milligram of arginine amide to a volatile pyrimidyl derivative using acetylacetone and ammonia. However, we have difficulty in scaling the reaction down to the microgram quantity and we are currently working on this problem.



We are also working on the development of a computer program that will subtract one mass from another in a mass spectrum, starting with the highest mass. This is just a subtraction program which we can do in our Varian 620/i computer. The idea is to start from the highest mass and subtract the successive masses from it. When the last (lowest) mass is subtracted, we then move to the next highest mass and do the subtractions again, and so on. The difference should correspond to the mass of a neutral fragment that splits off from the molecular ion or its fragment ions. Recognizing all the possible neutral fragments, one can test for the molecular ion as well as reconstruct the whole molecule.

The high resolution NMR (Jeolco JMN PS-100) spectrometer is now in full routine use. This includes: a) Frequencies and field sweep recording; b) internal and external lock; c) double resonance under each mode of recording; d) variable temperature; and 3)  $^1\text{BF}$ .

We have also performed preliminary studies on application of the Overhauser effect in NMR spectroscopy for determination of the spatial configuration of peptides. These techniques were applied to all the synthetic compounds available in the laboratory leading to the recording and interpretation of a large number of NMR spectra of protected and unprotected amino acids and peptides as a means of control of identity and/or of purity.

THE SALK INSTITUTE  
for  
BIOLOGICAL STUDIES  
February 8, 1971

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AID Contract No. AID/csd-2785

ADMINISTRATIVE REPORT

June through December 1970 - 7 Months

EXPENDITURES

<u>Category</u>	<u>Total 3 Year Budget</u>	<u>Total Expended</u>
Salaries & Wages	\$ 796,750	\$ 40,849
Benefits	165,665	7,051
Travel & Transportation	7,500)	
Subsistence Expense	7,500)	1,740
Contractor Acquired Property	455,963	246,460
Consumable Material & Supplies	256,900	56,129
Computer Terminal & Hardware	75,000	6,085
Other Direct Costs	<u>39,250</u>	<u>12,237</u>
Total Direct Costs	\$1,804,528	\$370,551
Overhead @93.5 S&W	<u>450,000</u>	<u>38,194</u>
TOTAL COSTS	<u>\$2,254,528</u>	<u>\$408,745</u>

AID Contract No. AID/csd-2785  
ADMINISTRATIVE REPORT

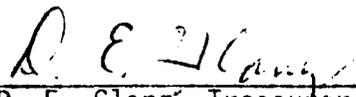
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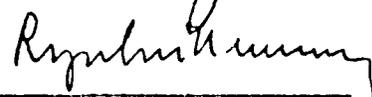
PERSONNEL

<u>Name</u>	<u>Title</u>
Dr. Roger Burgus	Senior Research Associate
Dr. Geoffrey Grant	Postdoctoral Fellow
B. Dorscht	Administrative Assistant
R. Kaiser	NMR Technician
T. Ewing	Lab Technician
E. Raines	Lab Technician
R. Smith	Lab Technician
P. Wilson	Lab Technician
C. Garrison	Lab Assistant
C. Rivier	Jr. Lab Assistant
D. Mensch	Secretary - 1/2 Time
P. Bock	Lab Aide - Part time

NIH Supported through December 1970

Dr. W. Vale	Research Associate
Dr. M. Amoss	Research Associate
Dr. T. Dunn	Research Associate
Dr. N. Ling	Research Associate
Dr. J. Rivier	Research Associate
Dr. M. Monohan	Postdoctoral Fellow
A. Pitzer	Computer Programmer
H. Anderson	Lab Technician
M. Butcher	Lab Technician
A. Ereneau	Lab Technician
C. Otto	Lab Technician

  
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D. E. Glanz, Treasurer

  
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Principal Investigator