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NO DESIGN DOCUMENT

(Project summaries/abstracts developed from

PRO AGs, PIOTs, and PARs)

Proj. 9320518 (3)
PD-AAF-904

PROJECT SUMMARY

14p

Project No. 932-518

Project Title : Development of Inhibitors of the MH-Releasing Factor as Contraceptive Agents

Proposed Contractor : The Salk Institute, San Diego, California (a non-profit organization)

Principal Investigator : Roger Guillemin, M.D., Ph.D., presently Professor of Physiology, Baylor College of Medicine, Houston, Texas; starting June 1, 1970, Resident Fellow, The Salk Institute, San Diego, California

Duration : Three years (June 1, 1970 - May 31, 1973)

Estimated Cost : Total $2,254,550

Funding Required FY	70 -	$2,254,550
	71	0
	72	0

Action Officers: TA/POP, R. T. Ravenholt, M.D.
TA/POP/R, J. Joseph Speidel, M.D.

I. Background

Development of improved techniques of fertility regulation is still of crucial importance to the solution of population problems in the LDC's.

The Salk Institute has recently decided to devote a considerable part of its resources to establish an extensive program in the field of Reproductive Biology in order to participate in the efforts to find a solution to population problems. The area of reproductive biology that appeared most attractive to the Scientific Committee of the Salk Institute in terms of its present status of achievements, potentially rapid breakthrough and also considerable appeal to present members of the Institute in view of current and future programs of research was that of neuroendocrinology.

Dr. Roger Guillemin, presently Professor of Physiology and Director of the Laboratories for Neuroendocrinology at the Baylor College of Medicine, Houston, Texas, and several senior members of his present staff will move to the Salk Institute by July 1, 1970, to play a leading role in this overall program.

This proposal to develop a new contraceptive is based on the fact that a part of the brain, called the hypothalamus, controls secretions of the anterior pituitary gland (adenohypophysis). The anterior pituitary secrets hormones called gonadotropins (e.g. LH or luteinizing hormone and FSH follicle-stimulating hormone) which in turn control the endocrine activities of the reporductive organs necessary to initiate and maintain pregnancy.

The brain and the anterior pituitary are linked by chemical agents called releasing factors. This proposal is concerned with determining chemical structure of one of these releasing factors, luteinizing hormone releasing factor (LRF) which ultimately controls endocrine processes necessary to pregnancy. When the chemical make up of LRF is determined, it will be possible to attempt synthesis of chemicals which interfere with its activity and therefore prevent conception and possibly disrupt early pregnancy. There is a good reason to believe that these chemicals will be active orally effective on a once-a-month basis and will have few of the systemic side effects of the present oral contraceptives.

II. Description of Proposed Project

It is proposed: 1) to isolate the LH and FSH gonadotropin releasing factors and establish their molecular structure; 2) to synthesize these molecules as well as a series of various analogs with LRF activity; 3) to synthesize analogs of LRF with anti-LRF activity as inhibitors of the secretion of LH, thus acting as antifertility agents; 4) to initiate clinical studies with these substances.

III. Significance of the Project to A.I.D. Objectives

Presently available contraceptive techniques still have many limitations when used in the developing countries. Those most satisfactory for LDC's are oral contraceptives and IUD's. Both methods require some degree of medical supervision. Oral contraceptive require forethought and regular use. Need for continuing maintenance of adequate supplies of the medication continuing motivation to use them and side effects have resulted in high rates of discon-

tinuance of the medication. Experience with the IUD shows similar high rates of discontinuance because of expulsions and medical side effects. The national program in Korea is not atypical, of over 1.5 million IUD insertions fewer than 600,000 remain in situ. Therefore, new methods of fertility regulation, of greater simplicity to use and greater patient acceptability are of importance to the solution of LDC population problems. A convenient non-toxic contraceptive would both promote use by facilitating use by those with lower levels of motivation for family planning and mitigate the necessity for expensive medical and public health infrastructure to administer family planning programs.

The widescale use of abortion in all societies even where it is illegal and therefore dangerous points up the importance of developing methods which are effective after conception.

IV. Relevance of Project to Existing Knowledge

It is now well established that the ultimate control/regulation mechanism of the secretion of all adenohypophysial hormones reside in the hypothalamus in the form of the hypothalamic releasing factors, a series of neurohormones secreted by cells of the hypophysiotropic area of the hypothalamus. This concept applies to the control of the secretion of the gonadotropins. There is good evidence for the existence of a substance of hypothalamic origin called LH-releasing factor (LRF), while the existence of an FSH-releasing factor (FRF) as a specific entity is still open to question. Of major significance for the relevance of the program presented is the now well accepted fact that the feedback mechanism between gonadal steroids (of the type used in current oral progestative anti-fertility agents) and secretion of pituitary gonadotropins is for the largest part, if not exclusively, at the level of the hypothalamus (and/or higher brain centers) rather than on the adenohypophysial tissue as had been originally hypothesized even though one locus of action is not exclusive of the other -- this being probably related to the plasma levels of circulating steroids and/or hypothalamic hypophysiotropic factor. Thus the ultimate mechanisms in the release of LH and FSH hence triggering of ovulation and maintenance of a functional corpus luteum (both necessary for pregnancy) is in the hypothalamic gonadotropin releasing factors.

V. Description and Evaluation of Methodology

A. Isolation of LRF

a) **Bioassay**. Essential to any program of purification and

isolation of the releasing factors is availability of reliable, reproducible, simple and specific bioassays. For the last 2½ years, Dr. Guillemin's group has worked to develop a simple, highly sensitive, highly specific bioassay for LRF; this is now available: the method uses as a test animal, male rats. Levels of (murine) LH are measured in plasma samples by a solid-phase radioimmunoassay (RIA). A covariance analysis is applied on the LH levels, pre- and post-injection to determine statistical significance of any increase; all statistical computations are run on digital computers. Thus there is a highly reliable in vivo assay for LRF that can be used as a routine method to assess the purification of hypothalamic LRF.

b) Starting materials for purification and isolation of LRF. Dr. Guillemin has saved and accumulated from the isolation program on TRF (TSH-releasing factor), side fractions to the TRF zone at the first step of purification corresponding to the crude extract of approximately 1.5 million sheep brains. Thus there are adequate quantities of starting material.

c) Purification and isolation of LRF; determination of its molecular structure. The crude extract is obtained by organic solvent extraction of the powder of lyophilized fragments of sheep hypothalamus, followed by ultrafilitration under nitrogen pressure, through diaflo membranes further purified by ion exchange chromatography. The subsequent stages of purification will be high voltage column electrophoresis followed by multiple steps of partition column chromatography.

Assuming that LRF is a relatively simple polypeptide, determination of its molecular structure will be approached by methods with which the laboratory is well acquainted (amino acid analysis following alkaline or acid hydrolysis, enzymatic hydrolysis, the dansyl method of Gray & Hartley, the Edman degradation method). Efforts will be directed at obtaining low and high resolution mass spectra of natural and/or derivatized LRF, as well as IR, UV and nuclear magnetic resonance (NMR) spectra of the isolated material. When amino acid sequences can be observed or reasonably suspected, the laboratory will engage in the synthesis of the pertinent structures. If, as in the case of TRF, one can postulate reasonably possible structures by mathematical permutations of a small number of amino acids (say 4-5) as sole constituents of LRF, Dr. Guillemin will undertake the immediate synthesis of the molecules representing all permutations to study them for biological activity and physical properties; by this method the structure of hypothalamic TRF was established in less than 5 months.

B. Purification, isolation, characterization of FRF

It is still unknown if FRF exists as a true chemical entity. A new methodology is being developed based again on the simple principle

of measuring endogenous release of FSH as directly as possible, i.e. by a radioimmunoassay for rat FSH applicable to rat plasma.

With availability of an assay method for FRF, exactly the same procedure as described for LRF regarding purification, isolation, structure, etc. would be followed.

C. Synthetic antagonists to LRF

As soon as evidence has accumulated regarding even partial knowledge of the amino acid sequence of LRF, all synthetic fragments will be tested for anti-LRF activity. Once the complete structure of LRF is established, the projects efforts will turn towards the synthesis of analogs with antagonist activity to LRF.

Similar studies would be directed towards obtaining analogs of FRF if the existence of an FRF and its molecular structure can be established.

D. Evaluation of Methods

Many of the techniques to be used in this project are quite new and employ sophisticated biochemical methods.

Most of the important techniques have been described in the literature:

a) Bioassay, Gonadotropins, 1968, E. Rosenberg Ed. Geron - X Publ. New York 1968 p 313.

b) The antibodies used against LH in the bioassay, Ward et al in Gen. Comp. Endocrinol, 8, 44, 1947 and Gonadotropins, 1968, op cit p 43.

c) Purification and isolation of LRF, Guillemin, Sakiz and Ward, PSEBM 118 p 1132, 1965 and Yamashiro, Guillessen and du Vigneaud, Nature, 210, 76, 1964.

Dr. Guillemin's group is extremely well qualified to employ these advanced biochemical techniques. Their isolation and characterization of TRF is generally considered a major scientific achievement.

VI. Evaluation of Research Competence of the Investigators

Dr. Roger Guillemin at present is Associate Director, Department of Experimental Endocrinology, College de France, Paris, Professor of Physiology, Department of Physiology, and Director, Laboratories for

Neuroendocrinology, Department of Physiology, Baylor University College of Medicine, Houston, Texas. He has served on the numerous National and International Committees.

He is an acknowledged leader in the field of neuroendocrinology. He has edited four volumes in collaboration and is the author of over 200 publications in the field of endocrinology, neuroendocrinology, and mathetical analysis of bioassays.

Other senior investigators will include:

1) Thomas F. Dunn, Ph.D.

Presently, Research Associate, Department of Physiology, Baylor College of Medicine, Houston, Texas.

2) Roger C. Burgus, Ph.D.

Presently, Associate Professor of Biochemistry, Department of Physiology, Baylor College of Medicine, Houston, Texas.

3) Wylie W. Vale, Jr., Ph.D.

Presently, Assistant Professor of Physiology, Department of Physiology, Baylor College of Medicine, Houston, Texas.

4) Max St. Clair Amoss, Jr., Ph.D.

VII. Appraisal of Research Resources and Budget

The institute is a new and flexible organization, founded solidly in molecular and cellular biology and with a recognized capacity for innovation. It has established a coherent plan for expansion designed to support the entry into the field of reproductive biology and to ensure that the Institute's other activities will reinforce its proposed programs in reproductive biology.

The trustees of the Salk Institute will provide $1.5 million, and will employ grants from the Ford Foundation for $1.0 million and from the Rockefeller Foundation for $750,000 to establish basic facilities at the Institute for the field of reporductive biology. Well over half of its existing laboratory space is available for expansion into these new areas (8000 square feet).

Through its Non-Resident Fellows and its relations with such institutions as Harvard, Massachusetts Institute of Technology, California Institute of Technology, Rutgers, Cambridge (U.K.), and the

Institut Pasteur in Paris, it can effectively tap a wide range of intellectual resources in reproductive biology in a direct and practical fashion.

The Institute has developed a valuable and close relationship with the University of California, San Diego. In addition to its growing staff in the field of reproductive biology with which we will work closely, the Institute also has available the University's computer and library facilities. Most of the Institute's Fellows are professors at the University and may participate as they wish in academic programs there. There is, moreover, in each research program a large complement of postdoctoral fellows, drawn from universities both within and outside of the United States.

The work to be accomplished will require a large scale effort involving 12 full-time top level scientists, 10 technicians, and clerical personnel. Salary requests are in line with the levels of competence required. A.I.D. is requested to supply $270,550 for purchase of equipment in the first year which is essential to the research program. Dr. Guillemin's group and The Salk Institute will supply other major equipment with an approximate replacement value of $250,000.

The projected costs as set forth in the attached budget are appropriate to an effort of this magnitude and type.

VIII. Technical and Scientific Review of Proposal

Besides Dr. Guillemin's laboratory, several investigators have contributed to the now well established concept proposing the existence of hypothalamic LRF (reviews in G. W. Harris et al in the Pituitary Gland, 3 vol., Univ. Calif. Press, 1967; Schally et al, Recent Progress in Hormone Research, 24, 497, 1968; McCann et al, Ann. Rev. Physiol., 30, 414, 1968). Guillemin and Schally et al, (Endocrinology, 81, 77, 1967) have reported obtaining LRF of ovine, bovine or porcine hypothalamic origin in a high degree of purity. To this date, no one has isolated hypothalamic LRF, the molecular structure of which remains unknown. Similarly, no antagonists of LRF have been reported. Because of the minute amounts of LRF present in each hypothalamic fragment, any clinical studies with LRF or analogs will have to be performed with materials of synthetic origin -- which, of course, cannot be prepared unless the structure of the natural hormone is determined.

In the last few months, pilot studies have shown, using the new assay method and only 2 steps of further purification of the LRF con-

centrate available as starting material, that Dr. Guillemin has purified LRF preparations active in vivo in this system at 100 ng/dose. These preparations are still not homogeneous by various physicochemical tests. They show, however, no more than 5 major amino acids following 6N HCl hydrolysis. There is also evidence that enzymatic incubation destroys the biological activity; from these and other studies, the probably nature of the N-terminal amino acid of LRF is known thus decreasing considerably the number of possible permutations of other amino acids as discussed above, that would correspond to possible structure to synthesize and study for biological activity.

Probably the most significant contribution of Dr. Guillemin's group towards the goals discussed in the application, is their recent isolation of the hypothalamic hormone TRF (TSH-releasing factor), the determination of its molecular structure and its synthesis as well as that of several analogs (C.R. Acad. Sci., 268, 2116, 1969; idem, 269, 226, 1969; idem, 269, 1870, 1969; Recent Advances, Chapter 14, p. 1, Duncan's Diseases of Metabolism, vol. 2, Saunders Publ., October 1969; Endocrinology, accepted for publication, March 1970).

Outside expert review is in process. Comments of John W. Everett, Ph.D., Professor of Anatomy, Duke University School of Medicine are as follows:

"This is hardly a modest proposal, scientifically or financially. It is not only bold, but timely, well conceived and well founded in rich experience. Dr. Guillemin is widely recognized as one of a small handful of outstanding workers in this highly specialized area of investigation. I know of no one better fitted to undertake the intensive program that he outlines. Quite properly he emphasizes the recent achievement of his laboratory group in isolating the thyrotropin releasing factor (TRF) and determining its molecular structure, as well as synthesizing related compounds having TRF activity.

The question at issue, insofar as A.I.D. support is concerned, is thus whether there is a strong likelihood that the projected study will yield materials of practical value in population control. I feel that such a likelihood exists. It is pertinent that the natural TRF and its synthetic analogs are active orally. If the luteinizing hormone releaser (LRF) and its analogs can be synthesized, the development of synthetic antagonists should be feasible. As noted in the application, 'LRF antagonists may well constitute an almost ideal antifertility method' "

Pertinent comments of Dr. Charles H. Sawyer, Professor of Anatomy, University of California, Los Angeles are as follows:

"I have read Dr. Guillemin's proposal for a research grant from A.I.D. and I consider it a strong proposal of great scientific merit as a basic research program. The analysis of the structure of luteinizing hormone releasing factor (LRF) and its subsequent synthesis and application to clinical problems and population control are most worthy objectives.

Dr. Guillemin is, however, a pioneer and leading investigator in the field of "hypophysiotrophic" hormones and his efforts and proposals merit strong support. The new setting at the Salk Institute would provide a very stimulating environment for studies in molecular biology, and the Institute appears to be entering the field of population control with dedication."

The proposal was reviewed and approved by the combined review committee of the Office of Population and the Regional Bureaus on January 12, 1970 and by the Research and Institutional Grants Committee on January 22, 1970.

IX. Summary Evaluation

This proposal (determination of the structure of LRF, synthetic LRF-antagonists) is highly relevant to the problem of fertility control in LDC's.

i) Isolation of LRF and determination of its molecular structure must be accomplished before reasonable hypotheses may be advanced on which to base the synthesis of molecules with anti-LRF activity.

ii) Availability of the LRF-antagonists may well constitute an almost ideal antifertility method: antagonists to LRF should be able to inhibit activity (of LRF) at the pituitary level within a few minutes. Thus, assuming the current concepts about the pituitary origin of the luteotropic activity that maintains the early corpus luteum of pregnancy ($<$ 3 weeks) to be correct, oral administration of a dosage of anti-LRF once a month (for instance, in a simple relationship to the lunar month) should inhibit ovulation or interrupt early implantation by inhibition of the function of the early corpus luteum of pregnancy.

If this concept is correct, synthetic molecules with anti-LRF activity would be an almost ideal antifertility agent: it would be a once-a-month pill, it would likely act without peripheral effects (no biological activity of highly purified LRF has been seen in experiments other than to stimulate release of pituitary hormones, and related antagonists would logically be expected to be similarly highly specific in their loci of effect). Considerable basic research is still necessary before definitive statements can be made about these several hypotheses; we know, however, that the techniques to approach the problem are available and the Salk group is well familiar with all of them in form of biochemistry, physiology and endocrinology of reproduction.

TA/POP:JSeidel:2/5/70

Budget

June 1, 1970 - May 31, 1971

	1st Year	2nd Year	3rd Year
Salaries including Social Security benefits, etc.	$ 229,000	$ 350,000	$ 380,000
Equipment	270,550	70,000	20,000
Installation of major equipment	32,500	- -	- -
Maintenance and Outfitting Laboratory outfittings for physiological studies (constant temperature modules, cold storage facilities)	20,000	49,000	10,000
Deionization unit and installation	5,000	- -	- -
Expendables, animals, chemicals, radioisotopes, gases, radioactivity counting disposables (ca. $6,000/month)	75,000	90,000	90,000
Travel expenses, staff to other laboratories and meetings	5,000	5,000	5,000
Computer terminal $6,000, software time $8,000, hardware time $12,000	26,000	25,000	25,000
Communications, publications	2,500	2,500	2,500
Moving expenses of large equipment from Baylor to Salk Institute	15,000	- -	- -
Total Direct Costs	$ 680,550	$ 591,500	$ 532,500
Total Direct Costs	$ 1,804,550		
Total Indirect Overhead Costs (approximately 25% of total direct costs)	$ 450,000		
Grand Total Application June 1, 1970 - May 31, 1973	$ 2,254,550		

ACTION MEMORANDUM FOR THE ADMINISTRATOR

3-3-70

THRU: EXSEC

FROM: AA/TA, Joel Bernstein

Problem: Your approval is requested for the transmittal of seven research proposals to the Research Advisory Committee (RAC) for review at its 29th scheduled meeting, March 5-6, 1970.

Discussion: Two of these proposals are for extension and supplemental funding of ongoing projects. The remaining five -- one in nutrition, one in economic studies, two in population and one in agriculture -- are new projects. All seven proposals were reviewed by the Agency's internal review committee, the Research and Institutional Grants Council (RIGC) on January 27-29. Its actions are reported in the attached comments on individual projects.

Following AA/PPC review, our FY 1970 OYB for non-population and population Central Research including program support were established at $8.5 million and $6.5 million respectively. The latter amount for population projects will adequately finance the two new proposals scheduled for the RAC review. For the non-population projects, the three new proposals going to the RAC may necessitate an upward adjustment of our $8.5 million OYB authority, if approved and fully implemented. We do not intend to request any OYB increase however, until our current year PASA Research projects, which were funded in July, 1969, are thoroughly reviewed. This review is now underway, with completion expected March 15, 1970. Any applicable savings will be redirected to those new proposals which the RAC approves.

If the financial requirements of the Projects approved by RAC exceed the present $8.5 million OYB, and Agency funds are available, we will request an increased OYB not to exceed the $9 million current Congressional limitation, in accordance with our agreement with AA/PPC. If Agency funds are not then available, we will follow our regular practice of implementing the projects in order of priority and feasibility within the current approved OYB level.

Agenda Items: In addition to review of the seven research proposals the RAC agenda also includes a discussion of AID research planning and evaluation on the morning of March 5, and a discussion paper entitled "An A.I.D. Nutrition Research Rationale and Program for the '70's" on the afternoon of that day.

1/ E. Relation of Dietary Caloric Intake to Protein Utilization in Man -- Massachusetts Institute of Technology. Duration, 3 years; est. total cost, $290,000; FY 70 funds required, $90,674. Milner, Langmuir, Merrill. 2/

Decision: That the project be deferred, subject to a project site visit by the subcommittee at the earliest possible moment, and that the project proposal be resubmitted to the April 29-30 meeting of the RAC. All available briefing material should be given to the subcommittee before their site visit.

F. Exchange Control, Liberalization and Development -- National Bureau of Economic Research. Duration, 2-1/2 years; est. total cost, $426,000; FY 70 funds required, $28,000. Kirk, Anderson, Clark. 3/

Decision: Approved for A.I.D. support subject to the approval of the analytical framework by a RAC subcommittee consisting of Drs. Kirk, Anderson, Tanter and one economist outside the RAC to be selected by the subcommittee.

G. Development of Inhibitors of the LH-Releasing Factor as Contraceptive Agents -- The Salk Institute. Duration, 3 years; est. total cost, $2,254,550; FY 70 funds required, $2,254,550. Merrill, Langmuir, Milner.

Decision: Approved for A.I.D. support subject to the proviso that an annual progress report be made available to the RAC.

H. Prostaglandin and Other Research for the Development of Contraceptives -- The Worcester Foundation for Experimental Biology. Duration, 3 years; est. total cost, $2,980,000; FY 70 funds required, $2,980,000. Kirk, Hagen, Linder.

Decision: To approve the full contract for A.I.D. support as proposed with the proviso that there be an annual review of the project by the RAC.

1/ Proposals listed begin with letter "E" to correspond with identification of items submitted as "Comments on Proposals to be Reviewed" attached to Action Memorandum dated March 3, 1970.

2/ RAC review subcommittee, Chairman underscored.

3/ Dr. Paul Clark of Williams College was asked to serve as a non-RAC member of this subcommittee.

I. Water Management Research in Arid and Sub-Humid Lands of the LDC's (continuation funding) -- Colorado State University/Utah State University. Duration of continuation funding, 3 years; est. total cost, $4,113,990; FY 70 funds required, $950,000. Revelle, Brady, Tanter.

Decision: Approved for A.I.D. support subject to the provisos that (1) continuation beyond one more year would be subject to review and approval by the RAC subcommittee, and (2) that the advice and guidance of the A.I.D. Office of Development Administration be obtained regarding the design and administration of the project.

J. Inheritance and Improvement of Protein Quality and Content in Maize -- Purdue University. Duration, 5 years; est. total cost, $1,053,000; FY 70 funds required, $170,860. Brady, Milner, Montgomery.

Decision: Approved for A.I.D. support with the recommendation that a RAC subcommittee consisting of Drs. Brady, Milner and Peterson work with the A.I.D. staff to develop, with Purdue and representatives of the USDA and the International Maize and Wheat Improvement Center (CIMMYT), a coordinated course of action.

K. Rural Development Analysis: Agricultural Sector Planning Models (extension) -- Michigan State University. Duration of extension, 3 months (October 1 - December 31, 1970); est. additional cost, $70,000 to be funded in FY 70. Weyl, Anderson, Tanter.

Decision: Approved for A.I.D. support.

Recommendation: That you approve the submission of these seven proposals to the RAC for review at the March 5-6 meeting.

Approved: JAH

Disapproved: _____

Date: 3/4/70

Attachments:
Summary Comments, Briefing Book

E.J.L.

TA/RUR:EJLong:mhs:3/2/70

932-0-518-0

932-0518
PN-AAF-404

52p

Application for Extension of Contract No. AID/csd 2785

Submitted to: Dr. J. Joseph Speidel
Chief, Research Division
Office of Population
Agency for International Development
Department of State
Washington, D.C. 20523

Roger Guillemin, M.D., Ph.D., Principal Investigator

The Salk Institute for Biological Studies
P.O.Box 1809
San Diego, California 92037

DEVELOPMENT OF INHIBITORS OF THE LH-RELEASING FACTOR AS CONTRACEPTIVE AGENTS.

SUBMITTED BY: The Salk Institute for Biological Studies
Post Office Box 1809
San Diego, California 92112

TYPE OF INSTITUTION: Non-Profit

DATE SUBMITTED: October 25, 1972

TITLE OF RESEARCH: DEVELOPMENT OF INHIBITORS OF THE LH-RELEASING FACTOR AS CONTRACEPTIVE AGENTS.

PRINCIPAL INVESTIGATOR: Roger Guillemin, M.D., Ph.D.
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4. SUMMARY

Our original Contract (see p. S-3) called for: "(1) isolate the luteinizing hormone (LH) and follicle stimulating hormone (FSH) gonadotropin releasing factors and establish their molecular structure; (2) to synthesize these molecules as well as a series of various analogues with luteinizing hormone releasing factor activity; (3) to synthesize analogues with anti-LRF activity as inhibitors of the secretion of LH, thus acting as anti-fertility agents; and (4) to initiate clinical studies with these substances."

As per today, October 1972, goals (1), (2), (3) of original contract have been reached. For goal (4), extensive clinical studies have been initiated with the use of synthetic LRF but not, so far, with synthetic LRF antagonists. This is because the presently available LRF-antagonists are considered to be of too low biological potency to be of clinical significance.

Extension of present Contract AID/csd 2785 is essentially to extend and reduce to practice the knowledge that the previous 3 years have allowed us to acquire.

For extension of this Contract, it is proposed -

(1) to pursue the investigations that led to isolation and characterization of LRF and have shown that substances of hypothalamic origin other than LRF may be involved in regulating secretion of gonadotropins (such as FRF and a possible natural inhibitor of the secretion of gonadotropins similar to the growth hormone inhibitor we have recently observed in hypothalamic extracts).

(2) to continue and extend the present program on a) structural configuration of LRF and related peptides; b) mechanisms of LRF binding to its receptors leading to secretion of gonadotropins, to obtain rational clues for the devising of LRF-antagonists.

(3) to extend our present series of LRF-analogues as competitive antagonists to produce more powerful inhibitors -

a) using new substituents in positions 2 and 3 of the decapeptide, in view of our current success with these compounds;
b) using new substituents in positions other than 2 and 3 in the decapeptide sequence;
c) using a different approach not used so far in the present contract, based on the concept of affinity labelling; affinity labelled compounds fix themselves on receptor sites in a semi-permanent manner which is irreversible during the biological life of the receptor protein;
d) developing chemical conjugates of LRF peptides or analogues with large biologically inert molecules such as dextran. Such conjugates-analogues should have prolonged biological life (up to several months) and would be ideal as long-range reversible inhibitors.

(4) to extend our clinical program with the Dept. of Obstetrics & Gynecology, UCSD, for testing:

a) synthetic LRF in the normal physiological menstrual cycle. The exact role of LRF in the human menstrual cycle must be clearly understood if we wish to apply adequately antagonists to LRF;
b) consider the use of LRF itself and of a long-lasting LRF as a contraceptive method as it would allow a powerful means of regulating ovulation time;
c) antagonists of LRF as anti-fertility agents in selected normal women and then in Phase II population testing.

a. Introduction and Background:

It is now well known that the hypothalamus is the ultimate controller of the secretion of adenohypophysial hormones and in particular, the gonadotropins. It is also now well established that this hypothalamic control of gonadotropin secretion is exerted by specific substances, polypeptides of hypothalamic origin. Indeed, the nature of the luteinizing hormone releasing factor (LRF) of ovine origin was elucidated as part of our efforts under present contract AID/csd 2785. There is physiological evidence that another substance (FRF for follicle stimulating hormone releasing factor) may also be involved as well as, possibly, inhibitory hypophysiotropic factors similar to the growth hormone release inhibiting factor (SRIF) recently characterized by our laboratory. The purpose of this original contract was to develop analogues antagonists of LRF as possible contraceptive agents. This had been achieved earlier in 1972 with demonstration of the anti-LRF activity of LRF analogues substituted or deleted in the -2 and -3 position. The extension of the current contract will allow us to develop other analogues antagonists of LRF with sufficient potency to be clinically significant. Also, we propose extensive human clinical studies to assess LRF per se as an inducer of ovulation in some sort of an improved rhythm method. The clinical background and physiological studies necessary for the use of LRF antagonists as well as the testing of these will also be extended.

b. Significance to A.I.D. Objectives:

The contractual research described here comes directly under the heading "Improved Means of Fertility Control" as described in A.I.D. Guidelines for Population Research Proposals. Our current and projected studies on the

use of LRF as a means of inducing ovulation in an improved rhythm method of fertility control is of obvious significance for those countries (South America, Europe) with a majority of Catholics. The development of LRF antagonists powerful enough to be used clinically should provide an ideal contraceptive agent of exquisite specificity in its locus and mode of action, in contradistinction to the ubiquitous effects of the present steroid contraceptives. Some of the research proposed in this application for extension should also lead to very long acting substances which might be administered only once a month or over an even longer period of time.

6. SCIENTIFIC PERSONNEL AND THEIR QUALIFICATIONS

All the scientific personnel involved in these studies is presently employed 100% of time and effort on the program proposed. The senior scientific staff involved is the same as has been described in earlier correspondence with A.I.D.

7. FACILITIES AND EQUIPMENT

This laboratory is adequately equipped to pursue the research proposed.

10. PROTECTION OF THE INDIVIDUAL AS A RESEARCH SUBJECT

The Salk Institute has fully complied with the spirit and the letter of this statement as evidenced by previous correspondence with A.I.D. Office of Population Research. Further evidence of our current and future compliance with A.I.D. Guidelines will be furnished, if requested.

I. SUBSTANCES OTHER THAN LRF CONTROLLING SECRETION OF GONADOTROPINS. EXISTENCE OF AN FRF. FERTILITY CONTROL IN THE MALE.

A. Chemistry

We propose to continue our present characterization of hypothalamic factors which control gonadotropin secretion. The decapeptide (luteinizing hormone, LH releasing factor, LRF) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, has already been isolated, identified and synthesized (Amoss et al., 1971; Burgus et al., 1972; Monahan et al., 1971). The natural product isolated by following its LH releasing activity has been shown also to release FSH in all the assay systems studied. The synthetic replicate has the same LH-FSH releasing ability. Nevertheless, the possibility that a separate hormone exists that is more specific for FSH release (FRF) has not been ruled out. It is possible that an FRF could be masked by the releasing activity of the LRF decapeptide in crude extract or that the extraction methods used so far have failed to extract the hypothetical FRF. Using bioassay methods specifically designed for FSH release (see section on bioassays), we propose to continue our search for an FRF.

In previous studies, ion-exchange chromatography was shown to separate two zones of LRF activity as followed by bioassay (see July 1972 report to AID); one zone corresponds to the position of LRF decapeptide ("LRF-A") and another, which behaves as a more acidic material, which we have designated "LRF-B". At this writing, it has not been established whether or not LRF-B, which also has FSH releasing activity in comparable proportions to that found for LRF-A (decapeptide), is a separate chemical entity or is a result of binding of LRF-A to another substance in the milieu. Preliminary evidence from exchange studies using [^{3}H]LRF have

indicated that if such a binding exists, the isolation of the binding substance might shed some light on the chemical nature of receptors in the pituitary cell. We will continue in attempts to characterize "LRF-B"; indeed, this material has already been further purified since the last report and biological screening is in progress. Recent observations have further confirmed the presence of more than one zone with LRF activity in purified extracts of hypothalamus. We propose to characterize the substances involved; one appears to be more potent than the decapeptide LRF in terms of LH-releasing activity. The physiological significance of such an observation is obvious--search in the hypothalamic extract for possible native inhibitors of the secretion of gonadotropins. The isolation and identification of a growth hormone-release inhibiting substance (SRIF or somatostatin) H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, has demonstrated the existence of at least one such compound of native origin. A native inhibitor of the secretion of LH and/or FSH may be a more powerful antagonist that the LRF-analogues we have designed so far.

B. Methods of Procedure - Structural chemistry of hypothalamic hormones.

1. Methods of Isolation

a) Starting materials. We have available extracts and side fractions of ca. 500,000 ovine hypothalamic fragments at various stages of purification. TRF and LRF have been removed for structural determination from 80,000 of these fragments. A batch of fresh ovine material representing 500,000 fragments is being processed as described below. We also have available for preliminary screening experiments, 3000 rat and 1000 pig lyophilized hypothalami as well as the acetone-dried powder of ca. 600 human hypothalami.

b) Extraction and purification. As part of a program on the isolation of LRF, 500,000 hypothalamic fragments have been extracted with an ethanol-chloroform solvent (Burgus and Guillemin, 1970; Amoss et al., 1971). The extract (1 kg) was subjected to manual countercurrent distribution (5 transfers) in the system 11:5:3, 0.1% aqueous acetic acid: n-butanol:pyridine. The material in the organic phase (17.2 g) was then manually distributed (5 transfers) in the system, 4:1:5 n-butanol:acetic acid:water. The material in the aqueous phase (9.2 g) was applied to an ion-exchange column of carboxymethyl cellulose (CMC) developed with an ammonium acetate gradient. In this system, LRF decapeptide, TRF, CRF and SRIF biological activities were separated, but CRF, GRF and LRF-B eluted in the same area. The LRF decapeptide from CMC is being further purified using partition chromatography on Sephadex G-25 in the system 4:1:5, n-butanol:acetic acid:water (BAW), a system which has been very effective in the isolation of TRF (Burgus and Guillemin, 1970) and LRF (Amoss et al., 1971). SRIF from this CMC column has been further purified by gel filtration and then by partition chromatography in the BAW system. The structure of SRIF was determined (Burgus et al., in preparation) using this material, of which ca. 2 mg remain for further studies.

Side fractions of these purification stages are available for studies on FRF, LRF-B, as well as other as yet unidentified factors such as pituitary hormone release inhibiting factors. If further purification is required, we have available, in addition to ion-exchange and partition systems, the other usual techniques such as zone or dextran supported high voltage electrophoresis, conventional countercurrent distribution, or (if the substance were volatile or could be derivatized) gas chromatography.

2. Characterization

All of the hypothalamic factors which have been characterized so far have been polypeptides of < 2500 molecular weight. If other hormones isolated are peptides, this laboratory is well equipped and experienced (Burgus et al., 1970; Burgus et al., 1972) in conventional sequencing techniques. Edman degradation coupled with any of the usual end-points, namely the subtractive method or direct determination of phenylthiohydantoin derivatives by gas chromatography-mass spectrometry (Burgus et al., 1972) is practiced. In addition, we have employed a sensitive method of determining Edman revealed N-termini by dansylation using ^{14}C-labelled dansyl reagent to sequence the decapeptide LRF in aliquots of 15 nanomoles (Burgus et al., 1972). We could also develop the promising new technique (Callahan et al., 1971) utilizing dipeptidyl aminopeptidase for sequencing, whereby dipeptides from enzymatic cleavage are identified by ion-exchange, paper or thin layer chromatography. If the peptide has a pyroglutamyl N-terminus as is the case with TRF and LRF, we have at our disposal an enzyme preparation (pyroglutamyl peptidase) which selectively removes the pyroglutamic acid residue (Burgus et al., 1972). In the case of another blocking group such as acetyl-, or a pyroglutamic acid linkage resistant to pyroglutamyl peptidase, we would investigate the use of enzymes such as chymotrypsin, pepsin, trypsin, to partially degrade the molecule. We also have the capability of derivatizing small peptides (up to 1000 molecular weight) for direct analysis by mass spectrometry (Bacon et al., 1969; Hakomori, 1964).

Since there is still a possibility that one or more of the hormones

may not be a polypeptide, we are sill required to consider characterization of other classes of compounds. We have equipped our laboratories for extensive characterizations of not only peptides but other classes of compounds, having experience with and at our direct disposal, in addition to the usual equipment, gas chromatograph (G.C.), melting point apparatus, a C.H.N. analyzer, various ultraviolet spectrometers, an infrared spectrometer (Beckman IR18a), a 100 MHz nmr spectrometer (Joelco) equipped with accessories, phosphorous probe, spin decoupling, a mass spectrometer (Varian Mat CH-5) equipped with direct or G. C. inlets, peak matching, and an on-line computer (Varian 620I) for data handling. In addition, we will have access to techniques within the Institute or at nearby UCSD, for measurement of circular dichroism, 220 MHz nmr, (with time averaging and Fourier transform analysis) and X-ray analysis.

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7. Callahan, P. X. et al., 1971, Fed. Proc., 30:1045.
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B. Physiology and assays

Existence of a specific hypothalamic factor controlling the release of FSH (i.e., FRF)

One of the most important questions to be answered by neuroendocrinologists involved in the hypothalamic control of reproduction is the role of the hypothalamus in controlling FSH secretion. Early reports from the laboratories of Schally and McCann claimed the separation of an FRF from LRF in the first stages of purification of porcine and ovine hypothalami using in vitro assays. Subsequently, Schally and his collaborators have stated that the decapeptide LRF is the sole mediator of FSH secretion. In recent publications from McCann it has been shown that the stimulation of various areas of the hypothalamus does result in the differential release of LH and FSH. Interpretation of several reports from the clinical literature indicate the possibility of the existence of a separate and distinct FRF. We would propose that a major effort of this laboratory over the next two years would be the establishment of the existence of such a factor, its isolation and the determination of its structure.

Previous efforts in this direction have met with little success primarily because of the lack of a physiological model or test system which would be very responsive in terms of the dynamics of FSH release. Although the chronically ovariectomized rat injected with steroids is very responsive in terms of LH release to the administration of LRF, it has been difficult to obtain large fluctuations of FSH even when very high doses of LRF are given. Based on the work of Steinberger and others

which implicates a factor released during spermiogenesis that affects FSH release in a negative feedback action (similar to the role of testosterone upon LH release), we propose to investigate several physiological models in which the entire process of spermatogenesis is impaired while at the same time leaving the cells responsible for testosterone secretion and hence the LH-gonadal axis should remain intact. Two of the models would be the adult male rat rendered cryptorchid by surgically placing the testis in the body cavity and by injections of $CdCl_2$ which destroy the cellular elements of the seminiferous tubules. Preliminary work is underway to ascertain the feasibility of using such animals in the routine study of FSH secretion.

Over one half million sheep hypothalami have been processed in this laboratory in the past two years. When the most sensitive bioassay technique for the determination of FSH control is obtained, all of the side fractions of the purification of LRF will be retested using the new system. This will include the testing of the initial extraction to ascertain the existence of an FRF in that fraction. In addition, the testing of the two residual fractions of the JN extraction process will also be undertaken. Re-extraction of the residue resulting from the alcohol-chloroform step will be made using both an acidic and an alkaline extraction. The fat soluble components are removed during the extraction of the hypothalami by the use of an ether-petroleum-ether separation. This residue, which is rich in fat soluble compounds will have to be re-extracted with a variety of partition systems in order to assess the possibility of FRF activity residing in this fraction. Preliminary work in this laboratory indicates the presence of additional

factors controlling prolactin and appears to be indicative of the presence of other biologically potent molecules, (in this residue).

a) FRF in females (ewes)

One of the difficulties encountered in the study for FSH control has been due to the fact that the assay animals involved have been primarily small laboratory rodents (rats, mice) with short duration ovarian cycles (4-5 days). We propose to study the control of release of gonadotropins in sheep with their long estrus-anestrus sequences. To this effect, extensive discussions have been conducted with Prof. J. Goding, S. S. Cameron Laboratory, State Research Farm, Werribee, Victoria, Australia, during a sojourn of J. Goding in the USA last summer and at the occasion of the First International Planned Parenthood Federation South-east Asia and Oceania Regional Medical and Scientific Congress in Sydney, Australia, August 14-18, 1972,which was attended by one of us (RG).

Fractions of hypothalamic extracts and subsequent fractionation procedures will be injected by intracarotid catheters into well "calibrated" ewes during estrus and anestrus. Control studies will be in terms of multiple doses of the decapeptide LRF. Comparisons of the released LH and FSH assessed by specific radioimmunoassays will be the end point followed here.

b) Studies in males--Fertility control in males

The majority of the research effort towards the control of fertility has been directed toward the female because of the multiplicity of steps in this sex necessary for the completion of the reproductive process which can possibly be manipulated. The reproductive process in the male has few such steps upon which any alteration can be made

which does not lead either to permanent sterility or impairment of the production of testosterone and the involvement of secondary sex characteristics and normal male sexual behavior. One of the steps which may lead to approaches in the control of male fertility is that of spermiogenesis. Another area may be the maturation of sperm in the epididymis. Recent reports have implicated FSH as a controlling influence in the process of spermiogenesis; testosterone being a requisite for the development of sperm cells from A type spermatogonia through secondary spermatocytes and possibly the first several stages of the development of spermatid maturation appear to require the presence of FSH.

We would propose the use of several physiological constructs utilizing male rats which would impair spermatogenesis which might then allow investigations into the role of this process in controlling gonadotropin secretion. In addition to the development of a suitable assay system for FRF (see above), these models may also yield some insight into the controlling mechanisms of spermatogenesis.

1) It has been shown that treatment of adult male rats with cadmium salts results in the complete destruction of the cellular elements of the testes. However, by 90 days post-treatment there is enough androgen being secreted to maintain the seminal vesicles and the ventral prostate. Gonadotropin and androgen levels have not been reported in these animals.

2) Chronically cryptorchid rats are aspermic, although levels of gonadotropins are elevated but not to the extent of chronically castrated rats. The role of LRF and/or FRF in restoring spermatogenesis will be studied. Quantitative studies with various steroids may lead

to hypotheses concerning the role of feedback on LH and FSH release in males.

3) Recent reports have shown that males exhibit high FSH levels immediately postnatal and just prior to puberty which in rats would be approximarely 40-55 days. Studies on the dynamics of LH versus FSH release and synthesis during this period would be of value in ascertaining the role of the hypothalamus during puberty.

4) We have been able to show that female rats exposed to constant light which produces constant vaginal estrus respond to LRF by releasing LH but do not, even at very high doses, elicit a release of FSH. To our knowledge no one has studied the effect of constant light in male rats. Quantitation of plasma LH, FSH and testosterone levels, correleated with the efficacy of LRF-FRF may produce information relating higher centers of the brain to the control of gonadotropin secretion.

5) An investigation of the relationships between the anti-androgens and the hypothalamus would also be of interest in formulating hypotheses concerning the negative feedback control in the hypothalamo-hypophysial-gonadal axes.

II. RATIONALE FOR THE DESIGN OF LRF-ANTAGONISTS.

A. Structural configuration of LRF and related peptides.

With the knowledge of a native structure of known biological activity, the design of molecules as analogues of that native structure to be endowed of modified biological activity more precisely as antagonist of the parent compound, is most logically conducted with extensive knowledge of the structure/activity relationships in the parent compound. Furthermore, in any study of structure/activity relationships and receptor characterization it is necessary to have a knowledge of the conformation of the parent molecule and also to know in what manner deviation from this occurs in analogues synthesized and studied. Because of the relative simplicity of its molecule, we started this program on the conformation of TRF; some restrictions are already apparent in the configuration of the molecule and a model has been proposed. In a coordinated effort with the Department of Chemistry at the University of California at San Diego, an extensive study on the structure of LRF is proposed as part of this contract.

It is evident that if LRF exists in a preferred conformation and if this preferred conformation is the configuration seen by the receptor site, then a knowledge of that preferred conformation would be of significant assistance in predicting the biological activity of proposed LRF analogues including antagonistic properties.

Our approach combines synthesis with spectroscopic characterization (ultraviolet, circular dichroism, nuclear magnetic resonance, x-ray analysis, potentiometric titration, dipole moment measurement, etc.). Their structural assignments will then tentatively be supported by conformational

energy calculations. At this point we will enter into a little more detail on the techniques to be used before we outline the preliminary results obtained on TRF and LRF.

Three years ago Bodanszky et al.[1] raised the question whether peptide hormones possess a certain architecture: a preferred conformation. It followed from the knowledge of enzymes and other proteins with highly specific functions that their architecture was a prerequisite for the specificity in their biological activity. In the case of hormones such a requirement was far from being obvious and the concept of a hormone developing a particular conformation during the process of attachment to the receptor site was mentioned with the remark, however, that some hormones such as oxytocin, vasopressin and vasotocin had a more rigid structure because of their natural cyclic form. No such limitation can be assumed in single-chain peptide hormones and unless the more subtle forces of hydrogen bonding and side chain interactions determine their geometry, hormones like corticotropin, bradikinin, angiotensin, gastrin, glucagon, TRF, LRF and others could exist only as random coil. Since then much progress was made in instrumentation and concepts. A quick review of the techniques used and their limitations might be useful at this point.

X-Ray. X-ray analysis of some amino acids and derivatives including histidine (Bennet et al.[2]) and a derivative of proline (Karle[3]) have been reviewed[4]. A few small cyclic biological polypeptides have also been investigated (Adams et al.[5], Anderson et al.[6]) as well as a protected pentapeptide (Ueki, 1971[7]).

An objection can easily be raised in the case of these smaller, single chain peptides: no drastic conformational change can be expected

during crystallization of a highly complex, globular protein. A small, single chain peptide, however, could undergo serious changes on crystallization because of packing forces. This is why, though indispensable, any X-ray crystallographic evidence for the architecture of peptide hormones will have to be treated with certain reservations (as an example: King[8] studying the associated state(s) of glucagon showed by X-ray studies in the crystal and optical studies in solution that it had a helical character. A reinvestigation by Patel[9] by means of nmr spectroscopy at 220 MHz concluded in contradistinction to the work of Gratzer and Beaven[10] and Blanchard and King[11] that the line broadening and shifts observed at high concentration (20 mg/ml) of glucagon were assignable to an aggregate state(s).

NMR. NMR is a powerful tool because of the large number of spectral parameters it provides. Examples of the use of nmr in the study of protein structure and conformation, protein binding sites (receptor-ligand interaction) and the openings offered by probe experiments such as: enhancement of water and ligand relaxation by paramagnetic ions specifically bound to macromolecules; direct effects of macromolecules on the relaxation of solvent protons; relaxation changes of nuclei possessing a quadrupole moment (^{23}Na) on binding to macromolecules; selective changes in the spectra of ligands forming specific complexes with macromolecules; nuclear spin labelling of macromolecules especially with ^{19}F are well described and summarized by Roberts[12], Jardetzky[13], Rowe[14], Peacocke[15] and Goodman[16a]. The discussion to come on the most recent results which have appeared in the literature gives it full credit especially when coupled to the complementary spectrophotometries: ultraviolet, infrared, optical rotary dispersion and circular dichroism as well as conformational energy calculations.

On the basis of tritium exchange rates of amide protons Printz et al.[17] proposed interesting models for Angiotensin II while Grant et al.[18] in our laboratory propose in TRF a hydrogen binding in between the αN-H from histidine with the 1 position of the imidazole ring on the basis of potentiometric titration curves of more than 30 analogues and model compounds as well as the preliminary data obtained from nmr spectroscopy.

Potentiometric titrations of bovine serum albumin reported by Hormsen et al.[19] in correlation with C. D., allowed them to follow and explain the different known normal-fast transition observed in between pH 3.5 and 4.5, and neutral transition in between pH 6 and 9.

All the spectrophotometries, by their own nature, describe a very particular phenomenon that is generally related to the conformation of the peptides studied: direct correlations are however dangerous and the limitations of each one should be kept in mind.

UV absorptions of the amide bonds, for example, in poly-L-lysine hydrochloride were studied by Rosenbeck and Doty[20] and the three conformations: (pH and temp. dependent) helical, random and β conformation in aqueous solutions gave very distinct spectra shown in Fig. I.

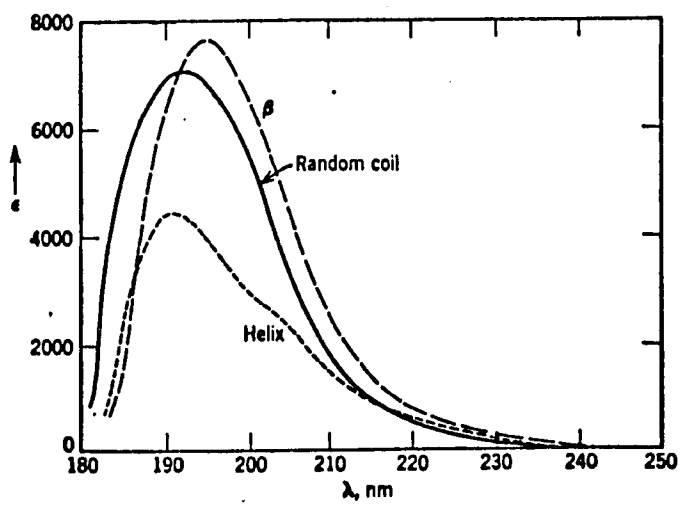


Fig. I. Absorption spectra in $\pi_1 \rightarrow \pi^*$ region for poly-L-lysine in aqueous solution: random coil, pH 6.0, 25°; helix, pH 10.8, 25°; β-form, pH 10.8, 52° (42).

The presence of other chromophores (aromatic side chains, for example) however, could undoubtedly disturb the whole picture.

Circular Dichroism. It is obvious that the examination of cyclic or linear peptides that contain various amino acid residues by circular dichroism is hampered by two fundamental problems. First, it is difficult to discern the contribution of individual chromophores to the observed Cotton effect. Second, it is often difficult to determine whether changes in the spectral patterns from solvent to solvent are related to conformational variations, specific solvation effects or a combination of both. Hence, the observation of helical structures as well as helix-coil conformational transitions in proteins is probably real, whereas some caution must be exercized in the interpretation of C. D. spectra of short chain peptides in view of the importance of end effects: (intrinsically: different contributions of the end residues to the optical properties and also different solvation).

Infrared. The IR frequencies and relative insensitivities of the amide I and II bands of polypeptides in various conformations have been reported by Goodman et al.[16b] and appear in Table I.

TABLE I

The Frequencies (cm^{-1}) and Relative Intensities of the Amide I and II Bands of Polypeptides in Various Conformations

Conformation	Designation	Amide I[a]	Amide II
Random coil (polyserine)	—	1655 (s)	1535 (s)
α-Helix	$\nu(0)$	1650 (s)	1516 (w)
(poly-γ-benzyl L-glutamate)	$\nu(\theta)$	1652 (m)	1546 (s)
Parallel-chain β	$\nu(0, 0)$	1645 (w)	1530 (s)
(α-keratin)	$\nu(\pi, 0)$	1630 (s)	1550 (m)
Antiparallel-chain β	$\nu(0, \pi)$	1685 (w)	1530 (s)
(polyglycine I)	$\nu(\pi, 0)$	1632 (s)	1540[b]
	$\nu(\pi, \pi)$	1668[b]	1550 (w)

[a] Relative intensities are given in parentheses: (s) = strong; (m) = medium; (w) = weak.

[b] Calculated value.

Once more one observes an overall effect intrinsic to the spectrophotometries.

Conformational Energy Calculations provide a test to confirm deductions obtained by other means. Various structural factors can be analyzed by using different parameters in formulating the equations for the minimum potential energy of the peptides. Contributions to the potential functions are numerous including: (a) inherent torsional potential attributable to the covalent bonding; (b) van der Waals interactions between nonbonded atoms within or directly connected to the chain backbone; (c) electrostatic interactions of polar groups in the chain backbone; (d) interactions between the respective side chains on two different amino acid units; (e) interactions between an amino acid side chain and the polymer backbone. Also, the effects of angle deformation can be considered. But for an unperturbed random coil only the first three factors are major contributors to the potential energy. On the other hand, for helical polypeptides, additional factors, such as contributions from hydrogen bonding, solvent-polymer interactions (including hydrophobic interactions), and dipole-dipole interactions between side chains and polymer backbone, should be included. Steric factors are among the most important factors in determining polypeptide conformation.

Results obtained so far and proposal

Definition of the parameters in nmr spectroscopy: The study of a tripeptide by nmr spectroscopy is a long, time-consuming process where all the parameters have to be defined if reproducible results and accurate

data are to be obtained. Our preliminary studies were to define these parameters: 1) solvents, 2) concentrations, 3) temperature, 4) pH and 5) study of exchange rates and use the best systems, that is, the most closely related to physiological conditions, in order to find the best resolving conditions leading to unequivocal assignment of each absorption to its corresponding proton.

1) Different solvents were used and are reported in Table II with their respective advantages and inconveniences.

TABLE II.

Solvent	Advantages	Inconveniences
Pyridine-d_5	N-H protons can be observed, temperature can be raised.	Extra peaks due to partially deuterated solvent mark part of the N-H region. Impossibility of varying pH.
DMSO-d_6	N-H protons can be observed, temperature can be raised.	Impossibility of varying pH.
TFA-d	Good solvent, N-H protons can be observed.	Impossibility of varying pH or to heat the sample.
D_2O	Good solvent; the HDO peak can be shielded or deshielded by changing pH or temperature. Titrations are easy.	N-H protons are not observable due to rapid exchange. Impossibility to raise the temperature above 120° in sealed tubes.
H_2O	Exchangeable hydrogens are easily observed up to pH ≈ 8.	Big peak of H_2O covers the α proton region.

On the basis of these results we decided to work in parallel in D_2O and H_2O using hexamethyl disiloxane (HMDS) as an internal reference.

2) Concentration dependance studies done in D_2O did not show any obvious difference when changing from 0.15 molar to 0.015 molar (such measurements require time averaging capabilities that we could fortunately find at UCSD). The studies were then conducted at a .15 molar concentration.

3) Spectra recorded in the different solvents above mentioned looked very similar at room temperature so that it was assumed that either one could be used. DMSO was chosen for studies up to 160°C. Our main interest was centered about the α N—C and α-CH β-CH_2 of histidine
H H

A coupling constant $J_{\alpha NH-\beta CH}$ = 8Hz corresponding to a dihedral angle of 150° according to Bystrov et al.[21] was observed, as well as two coupling constants $J_{\alpha CH-\beta CH_2'}$ = 6 Hz and $J_{\alpha CH-\beta CH_2''}$ = 8 Hz corresponding to two dihedral angles of 30° and 140° respectively according to Karplus et al[22]. It turned out that these couplings are neither influenced by temperature 0°-160°, nor by changing the pH from 1 to 8 or / solvents. This is our first evidence of some rigidity of the TRF molecule. The fact that the Pro-NH_2 moiety appears to be in the trans form only is another interesting feature.

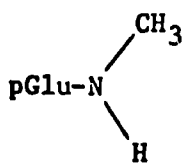
4) pH titration in D O of the C_2-H, C_4-H of the imidazole ring as well as the α-CH of histidine gave an average value of 6.2 for the pK_a of the imidazole ring; it is in agreement with potentiometric studies.

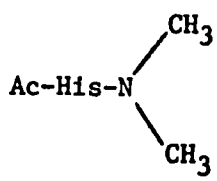
pH titration of the different NH amide of the TRF molecule (4 of them) from pH 1 to 8 (pH at which NH exchange too rapidly with water to be observable) did not show any significant variation of their chemical shifts

indicating that they are probably not involved in a hydrogen bonding (we stay, however, very open-minded since contradictory reports have appeared in the literature on that subject).

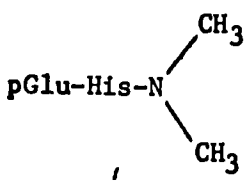
5) Preliminary data show no difference in the exchange rate, which is very rapid, of the different exchangeable protons. Some more controlled experiments have, however, to be conducted.

The following model compounds were synthesized so that a careful study of the chemical shifts of each proton could be performed.

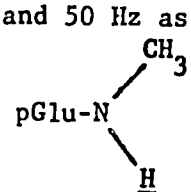


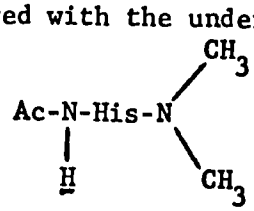
, 

, Ac-Pro-NH_2, pGlu-Pro-NH_2, Ac-His-Pro-NH_2.



has not yet been synthesized.

We do not want to enter here into too many tedious details, but one striking observation can be made: the α-N<u>H</u> of histidine is deshielded by as much as 100 Hz and 50 Hz as compared with the underlined hydrogen in both compounds, 

and 

respectively, indicating the possibility of a strong anisotropic effect of the aromatic imidazole moiety: This restricts considerably the number of possible conformations. The Overhauser effect (successfully used in our laboratory on model compounds) not yet used in the peptide field will most probably be of great help to confirm our hypotheses.

Energy calculations are carried on at the present time in collaboration with Dr. M. Goodman (UCSD) using the data aforementioned for a first approximation.

Circular dichroism (C. D.)

Preliminary data were obtained on TRF and the three first model compounds aforementioned. To summarize, we can say: 1) that the C. D. spectrum of TRF is not the sum of those of the 3 model compounds; 2) that the C. D. spectrum of TRF is highly dependent on pH giving rise to two definite conformations at low and high pH with a flexible form around pH 6.5 (pK_a of histidine). These apparently contradictory data in between C. D. and nmr can best be explained by the fact that the time scale of nmr spectroscopy as compared with that of C. D. is much slower giving an average picture. We, therefore, want to remain very open-minded until more data are obtained.

NMR of LRF

We have now available gram quantities of synthetic LRF as well as of LRF-analogues for these studies. We are at the stage of accumulating data in order to be able to assign unequivocally each proton to each peak. The series of nonapeptides des-1 amino acid-LRF, shorter peptides from the N- and C-termini have already been run.

Many model compounds will have to be synthesized, but we do not expect any problem since the synthesis of N-methyl amides as described in the first part of this report seems to work adequately.

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B. Mechanism of action of hypothalamic releasing factors at the cellular and subcellular level.

Our attempts to date to examine the steps in the secretion of gonadotropins have been limited to exploratory ones into the feasibility of such studies. The discovery of the structure and subsequent synthesis of a factor which inhibits the release of a pituitary hormone (i.e., SRIF) and our desire to develop compounds which block gonadotropin secretion has prompted us to consider the biochemical mechanisms involved in secretion and to investigate not only the site at which the compounds work but also the mechanism by which the peptides either enhance or block the secretion rate of a particular hormone. Using established double antibody, gel electrophoretic and cell fractionation techniques we intend to monitor the progress of nascent hormone molecules through the secretory apparatus of the pituitary cell and to ascertain how and at what level the hypothalamic regulatory peptides exert their effects. This will be done in classical double label, pulse-chase experiments, being careful to document yields and losses in all subsequent cell fractionations (1).

It has been possible to deduce from the kinetics of interaction that the LRF antagonists already synthesized act competitively at the LRF receptor (2). The site of action of SRIF has not been ascertained, however, a parallel inhibiting factor in the LRF series working via a receptor distinct from the defined LRF receptor (3) would be of obvious practical as well as academic interest in our studies. Therefore, we feel our efforts in studying the cellular mechanism by which secretion rates may be enhanced or reduced will function to open avenues of work in the control of secretion of the gonadotropins as yet unapproached in our investigations.

It has been well established that c-AMP acts as a secondary (intracellular) messenger for several mammalian hormones, while other hormones (steroid) effect their responses more directly by being transferred from cytoplasm to nucleus attached to a receptor. This established involvement of c-AMP in some hormonal systems gives rise to the possibility of a role for c-AMP in the action of hypothalamic releasing factors. The effects of such releasing factors on their pituitary target cells are not easily studied, since pure population of target cells must necessarily be obtained for these studies to be done. In particular, the action of thyrotropin releasing factor (TRF) on the thyrotrophs of the pituitary is best clarified if pure clones of thyrotrophs can be isolated and maintained in tissue culture. Such isolation and maintenance is the major thrust of this research. Several means of selecting for thyrotroph growth in mouse pituitaries exist; two of these methods being thyroidectomy by radiation or PTU treatments. Once the inhibitory influence of the thyroid is removed, the cells producing thyroid stimulating hormone (TSH) within the pituitary become hyperplastic and eventually neoplastic. Clones of such primary tumor lines would be quite suitable for c-AMP and adenyl cyclase studies, if they continued to secrete elevated levels of TSH in response to incubation with TRF.

Along these same lines, tumors of luteinizing hormone (LH)-producing cells are being utilized in order to obtain pure populations which are responsive to luteinizing hormone releasing factor, LRF. Studies on c-AMP levels and adenyl cyclase activities during stimulated secretion of LH could thus be performed. The effects of steroids, prostaglandins and various drugs on the c-AMP levels (and adenyl cyclase activity) will

similarly be looked for in these pituitary cells after incubation with LRF.

Thus this part of the project proposes to resolve the question of whether or not c-AMP is acting as a "secondary" messenger for hypothalamic releasing factors within their target pituitary cells. This study will be carried out by cloning pure lines of target cells and checking c-AMP levels and adenyl cyclase activity in them before and after stimulation by releasing factors. In the event that the answer to the c-AMP riddle demonstrates the non-invovlement of this "ubiquitous" messenger in the action of the TSH and LH-releasing factors, the study will provide cell lines and systems for our future physiological studies.

Our in vitro techniques, and culture methods in concert with a number of thyrotropin and gonadotropic pituitary tumor cell lines which we have in culture, provide a superb system for the complete study of pituitary regulatory mechanisms. In particular, the interrelationship with peripheral factors and hypothalamic factors is of interest. In the absence of in vivo stimuli, pituitary cells in culture often lose their capacity to synthesize and secrete hormones. It is our intent to study this phenomenon and, if possible, reverse the capacity of the cells by studying both the induction and repression of synthesis and secretion of the pituitary glycoproteins. We have already shown that the releasing factors do stimulate prolonged but finite secretory abilities.

The use of cultured hormone secreting pituitary tumors in parallel to normal cell cultures is anticipated as these homogeneous cell types can be constantly maintained and intracellular events more simply examined. In particular, the role of peripheral factors (steroids and/or thyroxine) in the induction of intracellular regulatory proteins can be ascertained in such tumor cells using double isotope labelling

and co-electrophoretic acrylamide gel techniques. Assay of levels of induced or repressed synthesis and/or secretion of pituitary hormones can be carried out by either established bio- or radioimmunoassay methods as well as the previously mentioned electrophoretic systems.

The study of the involvement of peripheral and neural factors in the regulation of both synthesis and secretion of the pituitary hormones will be further clarified by attempts to examine steps in the secretory processes within the pituitary cells. The system of choice for this study is the pituitary tumor cell cultures, although we have been able to do some experiments with normal cells. We feel that the steps in assembly of glycoproteins, the stepwise addition of sugar residues, the interchange of secretory membrane structures can all be examined using established, tested techniques of cell fractionation and synthesis of nascent proteins. This program of study on secretory mechanisms requires only that we learn to stabilize the secretory process and responsivity of the various pituitary cells to the peripheral and neural influences. To date, no apparent attempts have been made in this direction, although it is known that cells in culture do require not only the influence of ligand type hormones but also macromolecular serum factors to maintain the functional integrity. Our initial program will be to experiment with cultures of established normal or neoplastic cell lines to determine under what conditions peripheral and serum factors might stabilize pituitary cell functions. Subsequent to pituitary stabilization, the project of cell secretion can be examined using established techniques. Although long-term and partially dependent upon improving culture conditions, we feel this study will complement and benefit from the structure/activity relationships, characterization of

pituitary receptors and study of induction and repression phenomena in pituitary cells.

C. Isolation of LRF-Receptors on Pituitary Cell Membranes

Efforts to obtain and use "isolated receptors" in any biological system have not been very successful to date. This is undoubtedly due to the fact that the receptors are incorporated into large multi-macromolecular structures such as cell membranes. The physiochemical interrelations between the receptor molecules and the adjoining molecules are essential for the characteristics of the receptor in situ. Separation of the receptor and its surronding membranes will, as a rule, change the shape and/or charge distribution of the receptor macromolecule compared to its in situ properties. Recently, however, the liver cell membrane receptor for insulin has been separated from its membrane and its molecular properties preliminarily characterized. The use of an ingenious and intriguing technique for the assay of the insulin binding activity in an aggregated, non-soluble form may be of significant importance in the success of the isolation technique.

We propose to use the same technology to characterize the LRF receptor for cells of normal and/or neoplastic origin. Such an accomplishment would allow us to further assess the role of the pituitary receptors in the action of the releasing factors, as well as aid us by simplifying the complicated interaction of the receptor with labelled releasing factor. This simplification is necessary for the complete interpretation of the physiochemical kinetic data which we have at present (on the TRF receptor) and anticipate obtaining in the future with respect to the LRF receptor. Our present techniques of receptor assay will serve as a very adequate basis for such a project. In

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addition, the development and use of active site affinity labels as described below would allow us an even more convenient means of isolating the LRF receptor.

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III. EXTENSION OF THE PRESENT SERIES OF LRF-ANALOGUES AS COMPETITIVE ANTAGONISTS OF LRF.

A. New substituents in LRF.

A large number of LRF analogues have been synthesized in our laboratory and thoroughly tested in several biological systems. These results have given us an appreciation of the structural requirements for LRF activity and have allowed us to infer the factors (binding, intrinsic activity, rate of metabolism, access to the active site, etc.) which limit the activity of each analogue. We propose that this program be extended and expanded.

Our laboratory has developed several excellent procedures for testing the biological activities of releasing factor analogues:

1) An *in vitro* method for assaying all factors which influence the rate of secretion of all anterior pituitary hormones. We have reported on the functional validation of this method using primary cultures of enzymatically dispersed rat anterior pituitary cells and have had extensive experience with this procedure over the last year. This method is proving to be our most efficient, reliable and sensitive assay for releasing factors and their analogues. Using this procedure, multiple treatments can be compared against each other simultaneously; it is now possible to carry out truly quantitative bioassays and to test directly for competitive antagonism of releasing factors. We are now using this method extensively on fragments of human pituitary obtained at time of surgery for hypophysectomy. The end point is the measurement of the various pituitary hormones by specific radioimmunoassay for human gonadotropins.

The reaults of in vitro assays of various LRF analogues have been in good agreement with in vivo assays on the same substances. Using this technique, the relative binding affinity, intrinsic activity and ability to antagonize the natural product of each releasing factor analogue can be inferred.

2) An assay in the castrated estrogen/progesterone pretreated rat which is used to test analogues for LH and FSH releasing activity in vivo.

3) The relative binding affinity of each LRF analogue can be determined directly by competitive binding assays which test the ability of each analogue to compete with tritiated LRF for binding to the hypothalamic releasing factor receptor on a variety of anterior pituitary cell types. This procedure has been used to determine the binding affinities of over 40 TRF analogues and there has been a good correlation between the relative binding affinities determined by this method and the specific biological activities estimated with the in vivo and in vitro TRF assays.

With the aid of the methods described above, we can now determine the various biological properties associated with each analogue in order to gain insight of the topography of the various releasing factor receptors in the pituitary gland. We have already found three classes of LRF analogues, pure agonists, partial agonists and antagonists. The assignment of structural analogues to those classes allows an appreciation of the parts of the molecule important for binding or for intrinsic activity. This appreciation will be indispensible to the designing of better LRF antagonists. We have already deduced that the presence of the [histidine^2] residue in LRF is critical for the intrinsic

activity of LRF; whereas virtually all parts of the LRF molecule must be present in order to maintain full affinity for the LRF receptor. We further know that the [Trp^3] residue also contributes to the efficacy of the LRF molecule and, in fact, that analogues with modifications to the [Trp^3] position are also antagonists although with lower potency than the two histidine modified LRF analogues that we recently reported to antagonize LRF. We are synthesizing many LRF analogues with modifications at the 2 and 3 positions, trying to establish the minimum changes which will compromise the efficacy of LRF hoping that high binding affinities will be maintained. Another approach which we will pursue is to find a means to enhance the potency of LRF and incorporate those modifications into a known antagonist of LRF (i.e., des-His^2-LRF) in hopes of producing a more potent antagonist. The feasibility of finding "super-potent" releasing factor analogues was demonstrated by our observation that [Me^{τ}-His^2]TRF is 8 times more potent than TRF. If the conformation of LRF as it interacts with the LRF receptor can be established (see below) then analogues can be planned which would "lock" the molecule into that conformation and might thereby result in a more potent LRF. The importance of the conformation of LRF to its biological activity has been recently emphasized by the comparative activity of [L-Ala^6]LRF (1%) and [D-Ala^6] (> 50%). Most of the LRF analogues that have been synthesized up to this time have involved substitution of one amino acid in LRF by another conventional amino acid. In some cases, very informative analogues with definite changes in certain functional groups can be prepared. For example, [Cys^4]LRF, [Phe^5]LRF and [Cit^8]LRF. In most situations, the alterations will be more profound such as [Met^2]LRF, [Phe^2]LRF, [$Ileu^2$]LRF, [Lys^2]LRF and [Asp^2]LRF. We intend in the future

to prepare analogues of LRF which do not contain naturally occurring amino acids such as [F-His^2]LRF, [B-1-Methyl-3-indolyl-alanine^3]LRF, [Thienyl-Ala^2]LRF, [2-thiazolealanine^2]LRF, [1,2,4-triazole-3-alanine^2]LRF, in which the size and shape of the natural amino acid can be maintained while the electronic properties of a given region can be altered in a predicted manner. The converse might also be exploited in which functional groups will be maintained while size and shape will be altered.

It is possible that some of the analogues that are being synthesized will have properties which will prove of practical significance such as having a longer biological half-life. Analogues will be prepared that we have reason to speculate might bind to plasma proteins thereby providing a large inert reserve of LRF which could be in equilibrium with the free LRF available to be biologically active. For example, substance such as thyroxine might be incorporated into LRF and result in the binding of LRF to thyroxine binding plasma proteins. We have recently discovered that the TRF activity of N-formyl-Pro-His-Pro-NH_2 is resistant to the plasma enzyme that destroys the biological activity of TRF. In view of the reports that LRF also is inactivated by plasma, modifications that would render LRF resistant to plasma inactivation are of interest. A plasma resistant LRF analogue might be further modified to retard its loss through the kidneys (in addition to the approach mentioned above, see section on analogues bound to macromolecules).

Thus, it appears that we have reliable means to reliably and efficiently characterize the biological activities of analogues which would affect the secretion of the gonadotropins. Our past experience with LRF and TRF analogues has given us an appreciation of the requirements of various parts of those molecules for their biological activities.

We feel confident that we already have the means and experience to find more potent antagonists of LRF than the original molecules obtained so far.

B. The Chemical Synthesis of Specific Affinity Labelling LRF Analogues.

Chemically, the concept of utilizing affinity labelling of the receptor(s) of LRF involves the preparation of peptide analogues of this hormone which have several properties: the affinity labeller must be able to react with the active site so that it becomes covalently (and therefore, permanently, i.e., through the biological life of the receptor) bound to the site; it must not react either intramolecularly or intermolecularly with itself (e.g., it must not polymerize or cyclize upon itself); it must be an analogue which retains a high degree of specific biological activity.

All of these problems must be faced when planning the chemical synthesis of analogues of LRF. The reactive nature of the amino acid residues of LRF virtually rules out the possibility of using one reactive group while maintaining the remainder of the molecule intact. For example, if one amino acid residue (such as tyrosine) were activated to the extent that it could react to form a covalent bond at the active site, then it is very probable that the same activated residue would react intramolecularly with another residue in the molecule (such as histidine). Therefore, it is necessary to make appropriate amino acid substitutions in the peptide prior to activating the affinity labeller. Our current and past work on substitution analogues of LRF indicates that modifications of the hormone are possible which will yield a material which conforms to these requirements.

The first approach (currently being investigated under present contract) utilizes the preparation of an analogue of LRF which contains

the reactive bromo-acetyl function. In this analogue, the pyroglutamyl residue is replaced with a bromo-acetyl group which react as an alkylating reagent. In principle, this group should irreversibly alkylate a nucleophile at the active site. Unfortunately, side reactions involving the molecule itself are also possible (histidine and tyrosine could be alkylated by this reagent, leading to cyclization or polymerization). Even though this side reaction is possible, we are continuing our preliminary efforts in its preparation.

The second approach involves the preparation of LRF analogues in which the tyrosine residue is replaced with a functional group which can be activated as an affinity labeller. Such a compound is available in the form of p-amino-phenylalanine. p-aminophenylalanine is reacted with nitrosobenzene to form p-phenylazophenylalanine. The p-phenylazo group will protect the p-amino function during synthesis and cleavage and can be easily removed by hydrogenation. This will yield [-p-amino-Phe^5]LRF, which can be diazotized with nitrous acid to yield the corresponding diazonium salt. In this case, it will be necessary to make a further substitution in the two-position due to the ease with which aromatic diazonium salts couple with the imidazole of histidine. Fortunately, the internal coupling problem can be alleviated by substitution of histidine with phenylalanine or isoleucine ([Phe^2]LRF has 4% biological potency and [Ileu^2]LRF has 1% potency). Thus either diazotized [Phe^2, p-amino-Phe^5]LRF or [Ileu^2, p-amino-Phe^5]LRF ought to yield an efficient and internally stable affinity labeller.

A third approach involves the preparation of LRF analogues in which the histidine residue has been activated as a diazonium salt. With this substitution, it will be necessary to protect the five-position (tyrosine)

from cross coupling with diazotized histidine. This can be accomplished by substituting phenylalanine ([Phe^2]LRF has ≥ 50% potency) or alanine ([Ala^2]LRF has 2% potency) for tyrosine. The high activity of [Phe^2]- and [Ala^2]LRF makes this approach seem more promising than the two approaches cited above, expecially since our best inhibitors involve substitutions at the 2-position. Working in collaboration with Drs. Cohen and Kirk at NIH, we expect to receive sufficient quantities of 4-phenylazo-L-histidine for incorporation into LRF in the near future. Conversion of [4-phenylazo-histidyl^2]LRF will be accomplished by hydrogen reduction as in the case of the [p-amino-Phe^5]LRF analogues. Again, diazotization should yield the appropriate diazonium peptide.

The above synthetic approaches are based on sound chemical techniques and will hopefully lead to new hormonal analogues which may be used to block the active site and may provide a means for the isolation of such a site. The latter could yield extensive knowledge on the physical and topological properties of the receptor. Certainly, such knowledge could be of inestimable value in designing appropriate inhibitors of the hypothalamic releasing factors.

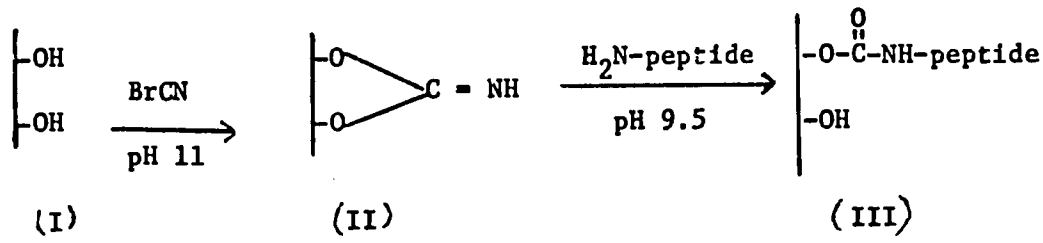
Practically, such an inhibitor of LRF would have a biological life of several days and possibly weeks and could thus represent or lead to a powerful contraceptive agent with prolonged activity following single administration.

C. <u>LRF or LRF antagonists linked to large molecular weight polymers</u>.

We have recently observed that a TRF analogue linked through the C-terminal end to dextran T-2000 (mw 2,000,000) has TSH releasing activity. A similar derivative of LRF or LRF antagonist might yield very long acting (dextran-LRF would not be excreted) LRF or LRF antagonist. These

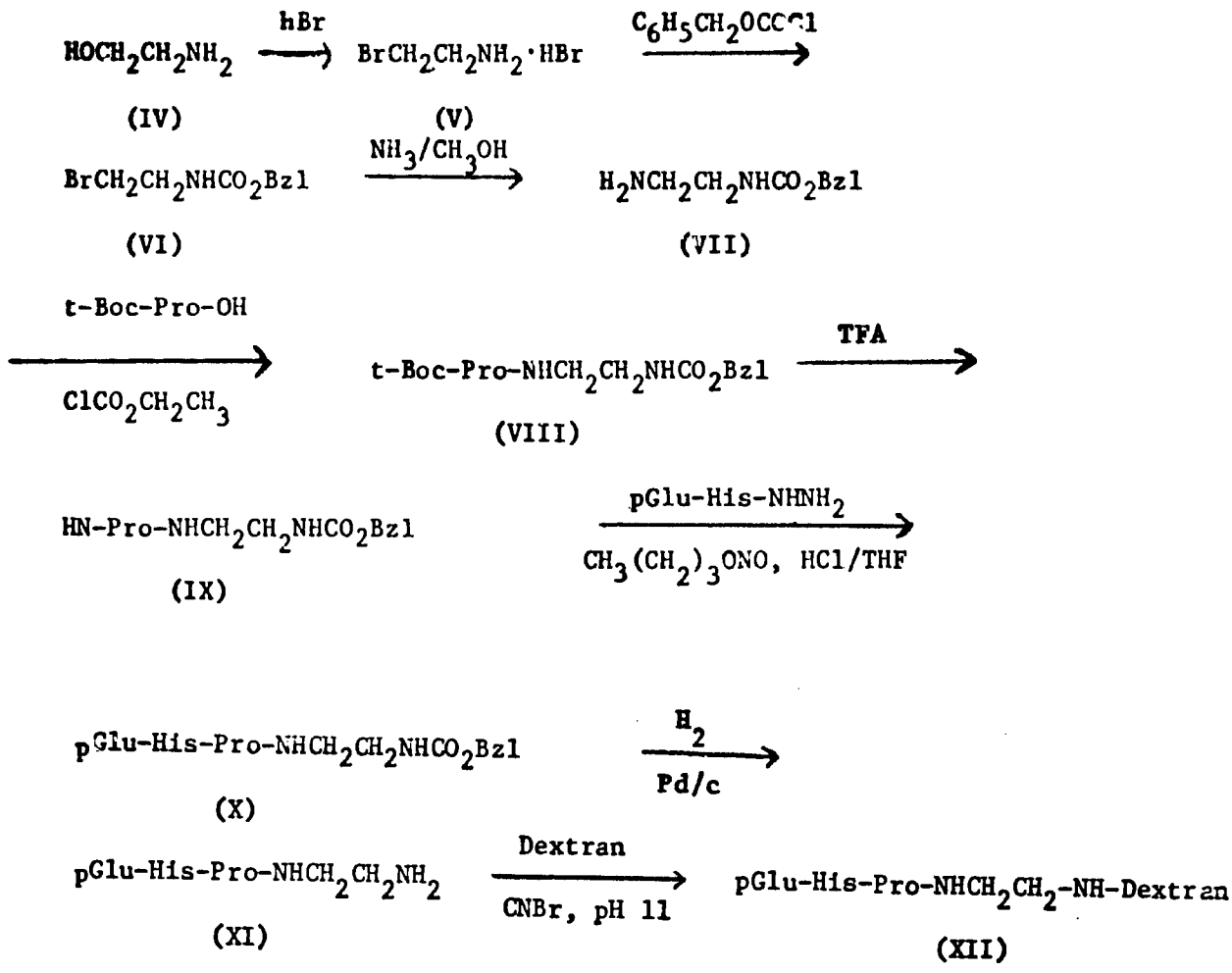
substances will also be useful in determining the primary site of interaction of LRF as being at the plasma membrane. Furthermore, they will be of use as antigens for immunization.

We have chosen dextran T-2000, which has a molecular weight of approximately 2,000,000 and is essentially linear, as the first candidate of large molecules for the conjugation to LRF.



Dextran (I), when activated with cyanogen bromide in water over a period of 6 minutes at pH 11, will form an iminocarbonate (II) which can conjugate with any peptide that has a free amino group to give product III. But before we can embark on such an endeavor with LRF, it is logical for us to test our experimental procedure with a relatively simple tripeptide analogue of TRF to see if it is feasible to attach it to dextran and to study its resulting biological activity.

The TRF analogue that we have in mind is pGlu-His-Pro-NH-CH_2-CH_2-NH_2 since TRF itself does not possess a free amino group. This molecule is an N-(β-aminoethyl)-amide of TRF. We know from our series of synthetic TRF analogues that replacing one of the amide hydrogens on the carboxyl end of the molecule with an ethyl or β-hydroxyethyl group still renders at least 10% biological activity to the molecule. Therefore, pGlu-His-Pro-NH-CH_2-CH_2-NH_2 should have some biological activity if the free amino group is attached to dextran.



The scheme that we propose to synthesize this TRF conjugate as a model for studies with LRF, is to react aminoethanol (IV) with concentrated hydrobromic acid, yielding β-bromoethylamine hydrobromide (V). The amino moiety of V is then protected with a carbobenzoxy group to give VI and the bromine is replaced by a primary amino grouping with methanol saturated with ammonia. The resulting β-carbobenzoxyaminoethylamine (VII) is coupled to t-BOC-proline through a mixed anhydride method using ethyl chloroformate to yield VIII. After deprotection of the BOC-group with trifluoroacetic acid, IX is combined with pGlu-His-$NHNH_2$ through the azide method to give X. Hydrogenation of X with palladium on charcoal would yield XI which can then be coupled to the activated dextran to form the conjugate XII for biological studies.

If this experimental procedure is successful in conjugating a TRF analogue to dextran, we plan to extend this process to the LRF peptides. Luteinizing hormone releasing factor itself does not bond to activated dextran even though it possesses a free amino group on the arginine residue. This is because the guanidino grouping is very basie (pK_a 12.48) and hence, it is completely protonated in the medium (pH 9.5) in which the coupling to dextran is performed. As a result, we have to synthesize some LRF analogues that possess a free amino group besides the guanidino grouping.

The logical position to attach this free amino group is at the carboxyl end of the LRF molecule because we have found that the pyroglutamic acid terminus is required for biological activity whereas deleting the glycine residue at the other terminus still renders 10% biological activity to the molecule.

As a logical extension of the dextran coupling work done on the TRF analogues, we can couple HN-Pro-$NHCH_2CH_2NHCO_2Bzl$ to the octapeptide, pGlu-His(im-Bzl)-Trp-Ser(O-Bzl)-Tyr(O-Bzl)-Gly-Leu-Arg(NO_2)-$NHNH_2$, via the azide method to give pGlu-His(im-Bzl)-Trp-Ser(O-Bzl)-Tyr(O-Bzl)-Gly-Leu-Arg(NO_2)-Pro-$NHCH_2CH_2NHCO_2Bzl$. Hydrogenation of the product with palladium will give pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$NHCH_2CH_2NH_2$ which can then be attached to activated dextran. Synthesis of the protected octapeptide can be accomplished by the usual solid-phase methodology. When the synthesis is completed, the protected peptide is cleaved from the resin by hydrazine in dimethylformamide thus yielding the desired hydrazide. Alternatively, the protected octapeptide can be cleaved as the free acid, pGlu-His(im-Bzl)-Trp-Ser(O-Bzl)-Tyr(O-Bzl)-Gly-Leu-Arg(NO_2)-OH, with base in dioxane which can then be coupled to

HN-Pro-$NHCH_2CH_2NHCO_2Bzl$ via cyclohexyl carbodiimide and 1-hydroxybenzotriazote to give the same protected LRF analogue.

Another interesting LRF peptide that can be bonded to dextran is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NHCH_2CH_2NH_2$. This compound contains the same sequence of amino acids as LRF and can be synthesized in the following manner. Starting with the chloromethyl resin, a series of protected amino acids corresponding to the sequence of LRF is coupled to the polymer. After the coupling is finished, the protected peptide pGlu-His(O-Bzl)-Trp-Ser(O-Bzl)-Tyr(O-Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-resin, can be cleaved with hydrazine and the resulting product joined to $H_2NCH_2CH_2$-$NHCO_2$-Bzl by the azide method. Hydrogenation of the product will give the desired compound for coupling to dextran. On the other hand, the protected peptide can be cleaved from the resin as the free acid which in turn can be coupled to $H_2NCH_2CH_2NHCO_2Bzl$ by the mixed anhydride method. Hydrogenation of the resulting compound will give the same product for bonding to dextran.

The practical significance of an LRF-dextran complex would be considerable, allowing as it would, prolonged biological activity of LRF for several days. The same reasoning and interest apply to the possible LRF antagonist-dextran complex, yielding an LRF antagonist with antagonistic biological activity lasting for several days.

CLINICAL STUDIES

The initial phase of investigations of synthetic LRF in normal human subjects was started in January 1972 under the direction of Dr. S.S.C. Yen, Professor and Chairman, Department of Obstetrics and Gynecology, University of California, San Diego, School of Medicine. To date, approximately 400 separate investigations have been performed with normal subjects, who indeed represent the population which should eventually be administered LRF or LRF-antagonists. Data obtained concerning the dose-response relationship, variability, reproductibility and characteristics of pituitary gonadotropin responsiveness under a variety of physiological situations (in both male and female subjects) have provided the essential framework in the formulation of future studies and the contemplated clinical application of LRF-analogues as antagonists. We are now prepared to extend this program to a Phase II investigation. Concentrated efforts will be directed to the following projects:

Physiological and Pharmacological Studies on the Effects of Synthetic LRF in the Normal Menstrual Cycle.

1) Quantitation of LRF in biologic fluids. The development of a sensitive radioimmunoassay for LRF has been initiated and a satisfactory dynamic study of the assay system has recently been accomplished. Application of this assay method in the assessment of metabolish, biologic half-life, distribution and secretory pattern of endogenous LRF as well as the disposition of exogenous LRF would constitute a critical step in the interpretation of clinical data.

2) The effects of LRF-induced acute gonadotropin release mimicking the midcycle surge observed in the ovulatory cycle surge will be studied during different phases of the cycle. The pituitary responsiveness (circulating gonadotropin pattern) and the ovarian responsiveness (circulating ovarian steroid pattern) will be monitored during the experimental as well as subsequent spontaneous cycles. The dose and route of LRF administration as related to the quantitative and qualitative response in gonadotropin and gonadal steroids will be assessed.

3) When the maximal sensitivity of pituitary gonadotropin and ovarian responsiveness during the mesntrual cycle are defined, immediate application in the investigation of appropriately "timed ovulation" will be made with the aim to improve the reliability of the rhythm method of contraception.

Clinical Studies of LRF-Antagonists as Anti-Fertility Aspects.

Subjects with previous surgical sterilization and normal women with documented ovulatory cycles will be selected for this study. The effect(s) of single and repeated administration of LRF-analogues during different phases of the cycle will be assessed. The duration of action and its reversibility of different types of LRF analogues (short acting and long acting) will be extensively explored. These physiological and pharmacological events will be monitored by daily gonadotropin and gonadal steroid determinations. Assessment will be made concerning the immediate and long term effect(s) of LRF-analogues on pituitary and ovarian functions with the aim to develop a new and effective form of anti-fertility agent.

In a second phase of this proposal, true Phase II studies are contemplated with the most powerful of the LRF-analogues antagonists that will have been developed in the laboratory. These Phase II studies will be undertaken on a large-scale (through our Obstetrics and Gynecology outpatient and open clinic) after the studies described above have been statisfactorily completed.

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A.I.D. CONTRACT RENEWAL

PROPOSED BUDGET
for PERIOD June 1, 1973 - May 31, 1975.

	June 1/73- May 31/74	June 1/74 - May 31/75	TOTAL
* Salaries:			
Professional (12)	$171,260	$180,665	$351,925
Non-Professional (17)	136,600	144,105	280,705
* Fringe Benefits: 3/			
Professional	37,920	47,944	85,864
Non-Professional	21,462	22,655	44,117
Travel & Transportation	5,000	5,000	10,000
Subsistence Allowance - per diem	2,500	2,500	5,000
Equipment	10,000	10,000	20,000
Materials & Supplies:			
Expendables: (animals, chemicals, radioisotopes, gases, radioactivity counting disposables, glassware,) Central Services: (glassware washing, animal care, photo lab, lab support shop, biochemical lab). (calculated at ca. $15,000/mo)	180,000	180,000	360,000
Computer terminal & hardware	12,000	12,000	24,000
Other Direct Costs:			
Maintenance contracts on equipment (presently in lab and new)	9,250	9,250	18,500
Publications & Communications	5,000	5,000	10,000
Consultants (@ $100/day)	2,000	2,000	4,000
	592,992	621,119	1,214,111
Clinical Studies (as a subcontract to UCSD, Dept. Obst. & Gyn. - see attachment)			420,330
TOTAL DIRECT COSTS			$ 1,634,441

Overhead to be negotiated in keeping with current Institute contracts with federal agencies.

(October 25, 1972)

* See attached for breakdown.

THE SALK INSTITUTE

PROPOSED BUDGET FOR CLINICAL PROGRAM

for Period June 1, 1973 - May 31, 1975

	1st Year	2nd Year	TOTAL
Salaries:			
Registered Nurses:			
(Susan Donley, R.N. and	8,000	8,440	16,440
1 to be named)	8,000	8,440	16,440
Research Associate, Ph.D. (to be named)	18,000	18,990	36,990
Laboratory Technicians:			
(Nancy Czekala (B.S.)	11,000	11,605	22,605
(to be named)	9,000	9,495	18,495
Principal Director (20% time) S.S.C. Yen, M.D.	24,000	25,320	49,320
Fringe Benefits:	11,700	12,340	24,040
Travel & Publication Costs	5,000	5,000	10,000
Equipment:			
Mark II Beta Counter (Nuclear-Chicago)	20,000		
Centrifuge	4,000		
Gamma Counter	17,000		
Micromedic	15,000		56,000
Consumable Supplies:	60,000	60,000	120,000
Research Subjects and Patient Costs	25,000	25,000	50,000
TOTAL DIRECT COSTS	$ 235,700	$ 184,630	$ 420,330

Overhead to be negotiated in keeping with current Institute contracts with federal agencies.

(October 25, 1972)

CLINICAL PROGRAM - Budget Justification:

Due to the anticipated increase in clinical and laboratory activities, additional personnel and equipment are needed.

We have in our laboratory one Gamma and one Beta counter and they are fully occupied for the present working load. It is anticipated that the clinical investigation will be doubled, therefore, additional counters, (one of each) is required.

Since the hospital is located in San Diego and our research laboratory is located at the La Jolla campus, a centrifuge should be provided at the hospital where all the samples are being collected. (We do not have one there at the present time). Separation of serum from blood shortly after the experiment would be highly desirable.

Two Registered Nurses will be needed for obtaining blood samples. All our studies of this nature are sampled at 5 to 15 minute intervals in order to define the dynamics of response to LRF and LRF analogues. In the past, Research Fellows have been completely occupied by these procedures. With two Registered Nurses we could free up the M.D. for analysis of data.

A person at the Research Associate level with a Ph.D. degree in steroid chemistry will be needed for running and supervising the portion of radioimmunoassay of gonadal steroids. We have one such individual (Dr. Siler) now in charge of the Radioimmunoassay Laboratory for protein hormones.

A.I.D. Contract Renewal
No. AID/csd 2785
Dr. Roger Guillemin

THE SALK INSTITUTE

PROPOSED PERSONNEL BUDGET

	June 1, 1973 - May 31, 1974					June 1, 1974 - May 31, 1975			
	Present Annual Rate	Proposed Annual Rate	Man Months	Salary Expense	2/ Benefits	Proposed Annual Rate 3/	Man Months	Salary Expense	2/ Benefits
*Biochemist Roger Burgus, Ph.D. Senior Research Assoc.	24,000	25,300	12	25,300	6,325	26,690	12	26,690	6,672
*Biochemist Michael Monahan, Ph.D. Research Associate	13,650	14,400	12	14,400	3,600	15,190	12	15,190	3,800
*Physiologist Wylie Vale, Ph.D. Senior Research Assoc.	18,000	19,000	12	19,000	4,750	20,045	12	20,045	5,011
*Physiologist Max Amoss, Ph.D. Research Associate	17,000	17,950	12	17,950	4,660	18,930	12	18,930	4,730
*Molecular Biologist Geoffrey Grant, Ph.D. Research Associate	13,800	14,560	12	14,560	3,640	15,360	12	15,360	3,840
*Mass Spectrocopist Nicholas Ling, Ph.D. Research Associate	13,650	14,400	12	14,400	3,600	15,190	12	15,190	3,800
*FMR Spectrocopist Jean Rivier, Ph.D. Research Associate	13,650	14,400	12	14,400	3,600	15,190	12	15,190	3,800
*Programmer Ann Pitzer	13,500	14,250	12	14,250	2,140	15,035	12	15,035	8,269
3 Postdoctoral Fellows	1/	10,000	36	30,000	5,500	10,550	36	31,650	7,912
Postdoctoral Fellow Catherine Rivier, Ph.D.	6,600	7,000	12	7,000	105	7,385	12	7,385	110
TOTAL (Professional)				171,260	37,920			180,665	47,944
15 Technicians, etc.		8,000	180	120,000	18,000	8,440	180	126,600	19,000
*1 Admin. Assist.	9,250	9,750	12	9,750	2,437	10,280	12	10,280	2,570
1 Secretary	6,300	6,850	12	6,850	1,025	7,225	12	7,225	1,085
TOTAL (Non-professional)			204	136,600	21,462		204	144,105	22,655
GRAND TOTAL				$307,860	$59,382			$324,770	$70,599

(Explanation of footnotes on next page)

THE SALK INSTITUTE

PROPOSED PERSONNEL BUDGET

FOOTNOTES TO PAGE 1

1/ The Salk Institute now recruiting candidates.

2/ Fringe benefits included in these costs are:

(a) Social Security - Rate as provided by law, currently 5.85 percent up to $10,800.

(b) Group Health Insurance - Current cost is $16.50 monthly for single coverage and $52.05 monthly including dependent coverage. Full cost is paid by the Institute.

(c) Group Life Insurance - The Institute furnishes group life insurance equal to three times annual salary. The current premium, borne entirely by the Institute, is $.55 per thousand dollars of coverage per month.

(d) Disability TIAA Insurance - For persons covered in the Institute Retirement Program, the Institute provides a total disability insurance through TIAA after one year's employment. The current premium is $5.83 per month per covered employee.

(e) TIAA/CREF Retirement - The cost of the annuity provided through TIAA/CREF for eligible employees is 15 percent of gross salary. The entire cost is born by the Institute except for certain employees who contribute 50 percent of this cost. Positions for which the Institute pays full retirement costs are indicated with an asterisk. (*)

(f) Unemployment Insurance - Premium paid entirely by the Institute at the rate of 3.9 percent on base of first $4,200 earned per person.

3/ Salary increases have been computed at 5.5 percent per year in accordance with present governmental directives.