INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of “sectioning” the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.
INITIAL CHARACTERIZATION OF MASKED GONADOTROPIN RECEPTORS IN THE CORPUS LUTEUM OF THE Rhesus Monkey (Macaca mulatta)

The University of Arizona

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106

Ph.D. 1984
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark.

1. Glossy photographs or pages
2. Colored illustrations, paper or print
3. Photographs with dark background
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered Text follows.
14. Curling and wrinkled pages
15. Other

University Microfilms International
INITIAL CHARACTERIZATION OF MASKED GONADOTROPIN RECEPTORS IN THE CORPUS LUTEUM OF THE RHESUS MONKEY (MACACA MULATTA)

by

Douglas Robert Danforth

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHYSIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1984
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Douglas Robert Danforth entitled Initial Characterization of Masked Gonadotropin Receptors in the Corpus Luteum of the Rhesus Monkey (Macaca mulatta) and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy (Ph.D.).

Richard L. Shonfer
Date: 12/7/84

Michael Ashley
Date: 06/7/84

David Ethell
Date: 12/7/84

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Richard L. Shonfer
Dissertation Director
Date: 12/7/84
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Douglas R. Dantuff
This work is dedicated to my parents.
Their love, endless support, and quiet encouragement
are more valuable to me than they will ever know.
ACKNOWLEDGEMENTS

I would sincerely like to thank Dr. Richard L. Stouffer for his dedication, guidance, and unbelievable patience over the past four years. He has instilled in me a sense of optimism and excitement that I hope will guide me for the rest of my life. I would also like to thank my dissertation committee, including Dr. Robert J. Ryan, Bartels Professor of Cellular Biology, Mayo Clinic, for their constructive criticisms and expert advice. Thanks also go to the entire Physiology Department, especially to Matt Boegehold and Chun-su Yuan for their friendship and encouragement along the way. I am indebted to all of the members of our lab; Marty Grodin, Jessica X. Lichter, Ann Ottobre, Dr. Kathy Eyster, and Dr. Penny Graves for their cooperation and enthusiasm in making this a truly enjoyable place to work. Special thanks go to Dr. Joseph Ottobre for setting the standards of being a good friend, a fine human being, and an excellent scientist. Lastly, I thank my wife Anne. Her support and patience throughout have never ceased to amaze me.

This work was supported in part by NIH Research Grants HD-12333 and HL-07249.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>x</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. REVIEW OF THE LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>General Concepts of Hormone Action</td>
<td>3</td>
</tr>
<tr>
<td>Mechanisms of Gonadotropin Hormone Action</td>
<td>7</td>
</tr>
<tr>
<td>The Concept of Masked Receptors</td>
<td>13</td>
</tr>
<tr>
<td>The Significance of Masked Receptors</td>
<td>15</td>
</tr>
<tr>
<td>Factors Modulating Binding Site Masking/Unmasking</td>
<td>17</td>
</tr>
<tr>
<td>Membrane Fluidity</td>
<td>20</td>
</tr>
<tr>
<td>Methods of Measuring Membrane Fluidity</td>
<td>25</td>
</tr>
<tr>
<td>Effects of Membrane Fluidity on Hormone Receptor-Adenylate Cyclase Systems</td>
<td>28</td>
</tr>
<tr>
<td>3. OBJECTIVES OF THE PRESENT STUDY</td>
<td>32</td>
</tr>
<tr>
<td>4. EVIDENCE FOR TWO POPULATIONS OF MASKED GONADOTROPIN BINDING SITES IN THE CORPUS LUTEUM OF THE RHESUS MONKEY (MACACA MULATTA)</td>
<td>33</td>
</tr>
<tr>
<td>Abstract</td>
<td>33</td>
</tr>
<tr>
<td>Introduction</td>
<td>34</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>36</td>
</tr>
<tr>
<td>Source and Preparation of Luteal Tissue</td>
<td>36</td>
</tr>
<tr>
<td>Preparation of 125I-radiolabeled hLH</td>
<td>38</td>
</tr>
<tr>
<td>125I-Gonadotropin Binding Studies</td>
<td>38</td>
</tr>
<tr>
<td>Statistical Analyses</td>
<td>40</td>
</tr>
<tr>
<td>Results</td>
<td>40</td>
</tr>
<tr>
<td>Discussion</td>
<td>51</td>
</tr>
<tr>
<td>5. MODULATION OF MEMBRANE FLUIDITY IN THE PRIMATE (MACACA MULATTA) CORPUS LUTEUM: CORRELATION WITH CHANGES IN GONADOTROPIN BINDING</td>
<td>59</td>
</tr>
<tr>
<td>Abstract</td>
<td>59</td>
</tr>
<tr>
<td>Introduction</td>
<td>60</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>62</td>
</tr>
<tr>
<td>Source and Preparation of Luteal Tissue</td>
<td>62</td>
</tr>
<tr>
<td>Fluorescence Polarization Studies</td>
<td>63</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS -- Continued

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>[(^{125}\text{I})]-LH BINDING TO DETERGENT-SOLUBILIZED RECEPTORS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FROM THE PRIMATE (MACACA MULATTA) CORPUS LUTEUM:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EFFECTS OF ETHANOL EXPOSURE</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Source and Preparation of Luteal Tissue</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>(^{125}\text{I}))-hLH Binding to Luteal Particulates</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(^{125}\text{I}))-hLH Binding to Solubilized Gonadotropin Receptors</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Statistical Analyses</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>93</td>
</tr>
<tr>
<td>7.</td>
<td>SUMMARY</td>
<td>100</td>
</tr>
<tr>
<td>APPENDIX</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>LABORATORY INSTRUMENT INTERFACE SYSTEM (LIIS):</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A UNIT FOR THE ACQUISITION, TEMPORARY STORAGE, AND TRANSFER OF DATA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TO A MICROCOMPUTER</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Abstract</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>The Buffer System</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>LITERATURE CITED</td>
<td>113</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of increasing concentrations of ethanol on specific $^{125}\text{I}$-hLH binding to luteal particulates prepared from the macaque and rat corpus luteum</td>
<td>41</td>
</tr>
<tr>
<td>2.</td>
<td>Kinetic analysis of specific $^{125}\text{I}$-hLH binding to macaque luteal membranes in the presence of 0% and 8% ethanol at 25°C (2A) and 37°C (2B)</td>
<td>43</td>
</tr>
<tr>
<td>3.</td>
<td>Kinetic analysis of specific $^{125}\text{I}$-hLH binding to luteal cells prepared from the macaque corpus luteum</td>
<td>45</td>
</tr>
<tr>
<td>4.</td>
<td>Specific $^{125}\text{I}$-hLH binding to macaque luteal membranes in the presence of various concentrations of straight chain alcohols</td>
<td>46</td>
</tr>
<tr>
<td>5.</td>
<td>The effect of neuraminidase pretreatment on specific $^{125}\text{I}$-hLH binding to luteal particulates prepared from the macaque and rat corpus luteum</td>
<td>49</td>
</tr>
<tr>
<td>6.</td>
<td>Scatchard plots of specific $^{125}\text{I}$-hLH binding to macaque luteal membranes, under control conditions, in the presence of 8% ethanol, following pretreatment with neuraminidase, and following neuraminidase pretreatment plus exposure to ethanol</td>
<td>50</td>
</tr>
<tr>
<td>7.</td>
<td>Absorption and emission spectra of DPH incorporated in membranes prepared from the rat corpus luteum</td>
<td>66</td>
</tr>
<tr>
<td>8.</td>
<td>Time dependence of fluorescence intensity (8A) and fluorescence polarization (8B) of DPH incorporated into rat corpora lutea</td>
<td>68</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of temperature on fluorescence of DPH in membrane preparations of monkey and rat corpora lutea</td>
<td>70</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of ethanol on fluorescence polarization of DPH incorporated in monkey and rat luteal membranes</td>
<td>71</td>
</tr>
<tr>
<td>11.</td>
<td>Correlation of specific $^{125}\text{I}$-hLH binding with the fluorescence polarization of DPH in macaque luteal membranes</td>
<td>74</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>12.</td>
<td>Composite Scatchard plots of specific $^{125}$I-hLH binding to preparations of soluble receptors</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>and particulates of the rat corpus luteum</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Composite Scatchard plots of specific $^{125}$I-hLH binding to soluble and particulate receptors</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>from the macaque corpus luteum</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Effects of increasing concentrations of ethanol on specific $^{125}$I-hLH binding to soluble</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>receptors from the monkey corpus luteum at 4°C and 25°C</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Kinetic analysis of specific $^{125}$I-hLH binding to soluble receptor preparations of the</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>macaque corpus luteum at 4°C (15A) and 25°C (15B)</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Kinetic analysis of specific $^{125}$I-hLH binding to soluble receptor preparations of the rat</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>corpus luteum at 4°C, 25°C, and 37°C</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Schematic illustration of the Laboratory Instrument Interface System</td>
<td>106</td>
</tr>
<tr>
<td>18.</td>
<td>Block diagram of the four-channel data buffer</td>
<td>108</td>
</tr>
<tr>
<td>19.</td>
<td>Close-up drawing of the four-channel data buffer</td>
<td>109</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of ethanol and it's removal on specific $^{125}$I-hLH binding to particulate preparations of the macaque corpus luteum</td>
<td>48</td>
</tr>
<tr>
<td>2. Additive effects of ethanol and neuraminidase on specific $^{125}$I-hLH binding to particulate preparations of the macaque corpus luteum</td>
<td>52</td>
</tr>
<tr>
<td>3. Effects of ethanol and it's removal on the fluorescence polarization of DPH incorporated in macaque luteal membranes</td>
<td>73</td>
</tr>
</tbody>
</table>
ABSTRACT

This study was designed to evaluate the possible existence of masked gonadotropin binding sites in the corpus luteum of the rhesus monkey. Pretreatment of macaque luteal particulates and cells with neuraminidase increased LH binding. In vitro exposure to alcohols also enhanced LH binding to these preparations. Ethanol modulation of LH binding was a time- and temperature-dependent process. The optimal concentration of ethanol for enhancing LH uptake was inversely proportional to the incubation temperature. Longer straight-chain alcohols were more potent than ethanol in increasing LH binding. Ethanol and neuraminidase increased the number of binding sites with no affect on affinity. Moreover, the effects of ethanol and NA were additive.

Since alcohols and temperature are modulators of membrane fluidity, we examined the hypothesis that the unmasking of gonadotropin binding sites may be related to changes in the fluid state of the lipid bilayer of the luteal membrane. First, membrane fluidity was estimated from the fluorescence polarization of the membrane probe diphenylhexatriene. Conditions which resulted in enhanced gonadotropin binding (1-8% ethanol, increased temperature), increased the fluidity of luteal membranes. Moreover, changes in gonadotropin binding were highly correlated (r=-0.97) with changes in membrane fluidity under these conditions. Pretreatment of luteal particulates with neuraminidase had no apparent effect on membrane fluidity. Second, gonadotropin receptors
were removed from the luteal membrane by detergent solubilization, and
the effects of ethanol on soluble receptors were compared to those on
receptors associated with the lipid bilayer. Solubilization resulted in
the recovery of 50% more gonadotropin binding sites than are available
in particulate preparations of the corpus luteum; these sites displayed
lower affinity for gonadotropin. Moreover, conditions which increase LH
binding to luteal particulates (1-8% ethanol at 25°C) decreased LH uptake
by soluble receptors.

The data suggest that two populations of LH binding sites are
masked within the membranes of the monkey corpus luteum. The ability
of two markedly different agents, alcohol and neuraminidase, to increase
LH binding indicates that diverse mechanisms may modulate the
masking/unmasking of gonadotropin receptors in target cell membranes.
As such, changes in membrane fluidity may play an important role in this
response.
CHAPTER 1

INTRODUCTION

The interaction of peptide and protein hormones with receptors on the surface of target cells and the subsequent stimulation of adenylate cyclase are complex processes involving a number of steps and several possible regulatory loci. As such, the response of an endocrine target cell to circulating hormones may be affected by a host of different factors. The initial step in this response, the binding of hormone to the cell surface receptor, has been intensively studied over the past two decades. In the course of these studies, some investigators have observed that hormone binding can be increased above "control" levels under certain conditions. This is often due to an increase in the number of available sites, rather than to an alteration in the affinity of the receptor-hormone complex. These findings have led to the hypothesis that masked or cryptic receptors exist within the plasma membrane of certain cells.

Cryptic binding sites for the gonadotropins, i.e. luteinizing hormone (LH) and chorionic gonadotropin (CG) have been investigated in the testis, placental tissue, ovarian stroma, and corpus luteum, but conclusive evidence for the existence of these sites is lacking. Most studies on cryptic receptors have failed to critically examine the characteristics of cryptic sites. For example, it is unknown whether masked binding sites display characteristics similar to those of the
available population. Moreover, since it has not been established whether cryptic sites are functional or potentially functional receptors, the physiological relevance of masked receptors remains to be determined. A preliminary report from Cameron and Stouffer (1982c) suggests that the membrane fluidizing agent ethanol can unmask cryptic sites for LH in the corpus luteum of the rhesus monkey. In addition, changes in membrane fluidity have been proposed as a possible mechanism for the unmasking of receptors.

The present investigation was designed to carefully examine the possible existence of masked LH binding sites in the primate corpus luteum. Experiments were performed 1) to determine the conditions under which masked binding sites could be exposed, 2) to characterize gonadotropin binding to these putative sites, and 3) to investigate the possible mechanisms by which masking/unmasking of gonadotropin binding sites could occur.
CHAPTER 2

REVIEW OF THE LITERATURE

General Concepts of Hormone Action

The first step in the action of peptide and protein hormones is binding to specific sites known as receptors on the surface of target cells. The characteristics of hormone-receptor binding generally have been studied by the interaction of radioisotope-labeled hormone with intact target cells or preparations derived from these cells. These studies led to criteria for establishing that a binding site be considered a hormone receptor (Cuatrecasas, 1975; Kahn, 1976; Ryan et al., 1977). 1) There is a finite number of receptor sites on the cell surface. A target cell will usually have about $10^3$ - $10^5$ receptor sites for a given hormone (Roth, 1978). Hormone binding to these sites is termed "specific" binding, whereas nonspecific binding implies binding to other components of the system. 2) The receptor has a high affinity for the hormone. The hormone-receptor interaction is often depicted as a simple bimolecular reaction:

$$[H] + [R] \xrightleftharpoons[k_d][k_a] [HR]$$

where $[H]$ is the concentration of the hormone, $[R]$ is the concentration of the receptor, $[HR]$ is the concentration of the hormone-receptor complex, $k_a$ is the association rate constant, $k_d$ is the dissociation
rate constant, and $K$ is the equilibrium (dissociation) constant for the reaction (Kahn, 1976). Most receptors for peptide and protein hormones have dissociation constants of $10^{-8}$-$10^{-10}$M. The number of receptors and the dissociation constant(s) for the receptor are often determined by Scatchard analysis (Scatchard, 1949). The quantity of hormone bound is plotted against the ratio of bound/free, and a linear Scatchard plot indicates the presence of a single class of binding sites. The affinity constant can be estimated from the negative slope of the line, and the number of receptors is determined from the x-intercept. Linear Scatchard plots have been demonstrated for many hormones, including the gonadotropins, (Rao and Saxena, 1973; Dufau and Catt, 1976). In contrast, curvilinear plots for insulin (Kahn et al., 1972), and glucagon (Schlatz and Marinetti, 1972) binding, suggest an interaction among binding sites or the existence of two or more classes of binding sites with different affinities. 3) The receptor must be specific for the hormone, or structural analogs of the hormone. The ability of receptors on target cells to interact selectively with a specific hormone in the face of the vast excess of other factors in the extracellular milieu is the essence of the functional definition of a hormone receptor. 4) Binding of the hormone to the receptor is rapid and usually reversible. The time course of hormone-receptor association is dependent upon the temperature and concentrations of hormone and receptors. For most hormone-receptor interactions, the association rate constant is $10^4$-$10^7$ M$^{-1}$ sec$^{-1}$ (Kahn, 1976). Although early studies on hormone-receptor interactions suggested that binding of hormone to receptor is a readily reversible process, it is becoming increasingly
evident that the hormone-receptor complex may undergo a variety of
different fates. One of the events observed after hormone-receptor
binding is the clustering or aggregation of membrane receptors
(Schlessinger, 1980). In many cells the aggregation takes place over
coated pits in the membrane, and the process leads to eventual
internalization of the hormone-receptor complex. Once internalized, the
coated vesicle fuses with lysosomes and the hormone and receptor may be
degraded. In contrast, the hormone may dissociate from the receptor
intracellularly, and the receptor may be recycled to the plasma membrane
or other intracellular membranes, while the hormone is degraded or
released into the extracellular space (Gordon et al., 1980). Thus,
aggregation and internalization of hormone-receptor complexes may be a
means of terminating hormone action and/or regulating the number of
receptors on the cell membrane. 5) The interaction of hormone with
receptor should lead to the biological effect of the hormone. The
expression of the biological effect may be mediated by a variety of
membrane-associated and intracellular mechanisms. Microaggregation of
hormone-receptor complexes may be important in the chain of events
leading to the biological response. Inhibition of microaggregation with
cross-linking reagents often inhibits or abolishes the biological effect
of the hormone (Schlessinger, 1980). Phosphorylation of membrane
proteins (Czech, 1977), alteration of phospholipid metabolism
(Farese, 1983; Hirata and Axelrod, 1980), and enhanced Ca** flux across
the membrane (Rasmussen, 1970; Hinds, Larsen, and Vincenzi, 1978) are
also early events in the mechanism of action of many protein hormones.
The most thoroughly characterized mediator of hormone action however, is the stimulation of membrane-bound adenylate cyclase to form cyclic AMP (cAMP). Adenylate cyclase has been demonstrated in all nucleated cells thus far studied, and cAMP is believed to be the principle second messenger in many of these systems. Early models of the hormone receptor-adenylate cyclase system proposed a direct interaction between hormone receptors and adenylate cyclase (Robinson et al., 1967). However Rodbell and coworkers (1971) first reported that some hormone receptor and adenylate cyclase systems demonstrate a requirement for guanyl nucleotides. Their data led to the postulation and identification of a third component in the system, the nucleotide regulatory component (NRC). According to Stadel and colleagues (1978), the hormone binds to the receptor and the hormone-receptor complex then interacts with the NRC to promote the exchange of GDP for GTP. The NRC-GTP complex stimulates the catalytic unit to convert ATP into cAMP. In certain systems, the hormone receptor, NRC, and catalytic unit of adenylate cyclase may be permanently coupled (Owen and Fanger, 1976; Touraine et al., 1977). However in other systems these components are independent units within the bilayer and interact through a collision-coupling process (Tolkovsky and Levitski, 1978).

Cyclic AMP activates cytoplasmic protein kinases which, in turn, phosphorylate specific proteins within the cell (Walsh and Cooper, 1979). Phosphorylation of proteins may result in an increase or decrease of enzyme activity which can result in the ultimate biological effect of the hormone. The particular biological response will depend upon the particular battery of proteins that become phosphorylated.
Mechanism of Gonadotropin Hormone Action

The pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), and the placental gonadotropin, chorionic gonadotropin (CG), are glycoprotein hormones that consist of two noncovalently-linked subunits, α and β. The hormonal specificity of each hormone is determined by the β-subunit, whereas the α-subunits are essentially identical. All three gonadotropins contain sialic acid residues that occur at the end of the carbohydrate chains attached to the hormone. Sialic acids may be important for protection from hepatic inactivation; the longer half-life of CG as compared to LH is probably due to the greater amount of sialic acid present on CG. In addition, recent evidence suggests that the carbohydrate side chains are important in mediation of the hormonal signal, since deglycosylated CG binds to the gonadotropin receptor but does not stimulate cyclic AMP production (Sairam and Manjunath, 1983).

The primary target tissues for the gonadotropins are the ovaries and the testes. FSH is important for gametogenesis in both sexes by acting on somatic cells (Sertoli cells and follicle cells) which participate in germ cell development and maturation (Steinberger and Steinberger, 1975). In the male, LH stimulates testosterone production by the Leydig cells of the testis (Dufau and Catt, 1978). In the female, LH promotes estrogen production by the preovulatory follicle, ovulation of the mature follicle, and progesterone production by the corpus luteum. LH also has a trophic action to maintain the structure and function of the corpus luteum (Kaltenbach et al., 1968; Karsh et al., 1970; Nalbandov, 1970; McCraken, 1971). Chorionic gonadotropin
acts to "rescue" the corpus luteum and prolong its functional steroidogenic lifespan during early pregnancy (Knobil, 1973).

The binding of the gonadotropins, LH and CG, to receptors in the corpus luteum has been investigated in a number of species, including the rat (Lee and Ryan, 1973b; Rajaniemi et al., 1977; Bramley and Ryan, 1978a; 1978b; 1979; 1980), sheep (Diekman et al., 1978a,b), cow (Papaionannou and Gospodarowicz, 1975), monkey (Cameron and Stouffer, 1982), and human (Lee et al., 1973; Wardlaw et al., 1975; Rao et al., 1977; Halme et al., 1978; Rajaniemi et al., 1981). Although Rao (1979) suggested that the gonadotropin receptor of the bovine corpus luteum has a higher affinity for CG than LH, it is generally accepted that LH and CG bind to the same receptor with equal affinity (Lee and Ryan, 1973; Wardlaw et al., 1975; Cameron and Stouffer, 1982). The gonadotropin receptor is a high affinity, low capacity site, displaying many of the characteristics of membrane receptors found in other tissues. It has a $K_d$ of approximately $10^{-10}$M, and is specific for the gonadotropins LH and CG, displaying very little cross reactivity with FSH, prolactin, or the individual subunits of LH and CG (Lee et al., 1973; Ryan et al., 1977; Cameron and Stouffer, 1982). The hormone-receptor interaction was initially characterized as a simple bimolecular process (Catt et al., 1976). However the observations that gonadotropin hormone-receptor complexes are often internalized (Chen et al., 1977; Conn et al., 1978), and that gonadotropin receptors may be present on intracellular organelles (Mitra and Rao, 1978; Rao and Mittra, 1979), suggest that the hormone-receptor interaction is a complex process involving a number of steps which may affect the
apparent kinetics of the binding reaction. Our understanding of the gonadotropin receptor system is further complicated by the reports of specific gonadotropin binding inhibitors present in some ovarian tissues (Yang et al., 1976; Sakai et al., 1977; Kumari et al., 1980), especially after prolonged storage at -20°C.

Gonadotropin receptors of the ovary and testis can be solubilized with nonionic detergents such as Triton X-100 (Dufau et al., 1973; Dufau and Catt, 1973; Dufau et al., 1974; Lee and Ryan, 1973c; Conti et al., 1978), and Lubrol PX (Charreau, Dufau, and Catt, 1974; Dufau, Podesta, and Catt, 1975). In addition, low ionic strength buffers (Pahnke et al., 1978) and sonication (Sebokova and Kolena, 1984) have also been utilized to solubilize gonadotropin binding sites. Examination of soluble gonadotropin receptors has yielded valuable information about the physical characteristics of the receptor moiety. The gonadotropin receptor has an apparent molecular weight of 194,000 daltons (Dufau and Catt, 1976), and appears to contain phospholipid and carbohydrate moieties in addition to the major binding site protein (Dufau and Catt, 1976). Characterization of $^{125}$I-hLH binding to soluble receptors of the rat ovary (Dufau et al., 1974) and testes (Dufau and Catt, 1973) suggests that soluble receptors retain hormonal specificity, however as much as 50% of gonadotropin binding activity is lost upon solubilization (Dufau and Catt, 1973; Dufau, Charreau, and Catt, 1973). The decrease in binding activity has been attributed to a decrease in the binding capacity (Catt and Dufau, 1973; Ascoli, 1983) or affinity (Dufau and Catt, 1973) of the gonadotropin receptor.
In contrast, detergent solubilization of prolactin receptors from the luteinized rat ovary and mammary gland results in a marked increase in the number of binding sites available to interact with prolactin (Koppleman and Dufau, 1982), with no change in the affinity of the hormone-receptor interaction. Solubilization of insulin receptors from 3T3-L1 adipocytes (Deutsch, Rosen, and Rubin, 1982) and lactogenic binding sites from the rat liver (Bonifacino, Sanchez, and Paladini, 1981) also increases their binding capacity for hormone.

Recent studies have found that the number of available gonadotropin (LH-CG) receptors changes during the lifespan of the corpus luteum. In the monkey, the number of available gonadotropin binding sites increases from early to mid-luteal phase and then declines during the late luteal phase, whereas the affinity of the receptor for gonadotropin remains constant throughout the entire luteal lifespan (Cameron and Stouffer, 1982b). Comparable changes in gonadotropin binding have been reported during the development and regression of the corpus luteum of the pseudopregnant rat (Rajaniemi et al., 1977), and sheep (Diekman et al., 1978). However, the mechanisms responsible for, and the physiological significance of these receptor changes remain to be determined. The apparent change in receptor number may be due to: 1) alteration in the rates of synthesis and degradation of the receptor, 2) inhibition of the hormone-receptor interaction by the luteolytic agent, 3) changes in the number of occupied vs. unoccupied receptors, or 4) masking/unmasking of cryptic binding sites in the cell membrane. Alterations in available gonadotropin receptors may play an important role in modulating luteal sensitivity to circulating gonadotropin.
However, the loss of LH receptors during luteal regression does not appear to be an obligatory step in the initiation of spontaneous luteolysis (Cameron and Stouffer, 1982b).

Subsequent to receptor binding, the next step in the mechanism of action of gonadotropin hormones is generally believed to be the stimulation of membrane-bound adenylate cyclase. In 1966, Marsh and associates demonstrated that addition of LH to bovine luteal tissue increased cAMP levels and that exogenous cAMP mimicked the action of LH by stimulating progesterone production. They subsequently found that the increase in cAMP was due to the stimulation of adenylate cyclase rather than to an inhibition of phosphodiesterase activity (Marsh, 1970). LH stimulation of cAMP production has since been demonstrated in corpora lutea of several species (Marsh, 1975; Ryan et al., 1977) including the monkey (Eyster and Stouffer, 1982), and human (Marsh and Lemaire, 1974). As in other systems, it appears that guanine nucleotides play an important role in gonadotropin stimulation of luteal adenylate cyclase (Rodbell, 1980; Abramowitz and Birnbaumer, 1982). In addition, the presence of guanine nucleotides may influence the interaction of LH-CG with its receptors (Abramowitz et al., 1979; Rao, 1975); however this premise has been challenged recently (LaBarbera, Richert, and Ryan, 1980).

The principle site of gonadotropin regulation of progesterone production by the corpus luteum is at the conversion of cholesterol to pregnenolone (Ichi et al., 1963; Armstrong et al., 1970). LH stimulation of cAMP results in enhanced protein kinase activity (Ling and Marsh, 1977; Dufau and Catt, 1978), and protein kinases or proteins
phosphorylated by protein kinases may modulate the steroidogenic pathway by a number of mechanisms. These include: 1) increasing the production of an enzymatic cofactor, such as NADPH; 2) increasing the availability of an enzymatic substrate, such as cholesterol; 3) increasing the transport of cholesterol into the mitochondria where the first enzymes in the steroidogenic pathway are located; 4) increasing the activity of the cholesterol side-chain cleavage enzyme complex by activating or increasing the synthesis of one of the components (Marsh, 1975). LH may stimulate steroidogenesis at any or all these steps, however the exact nature of the action of LH remains to be determined.

Catt and Dufau (1973) reported evidence that maximal cAMP production and steroidogenesis occur in the rat testis when only about 1% of the available receptors are occupied with gonadotropin. Similar studies on the insulin receptor (Kono and Barham, 1971), have led to the concept of "spare" receptors existing in the plasma membrane. Catt and coworkers (1980), suggest two possible roles for spare receptors: 1) to favor the formation of hormone receptor complexes in the face of low hormone concentrations, or 2) to provide a reservoir of sites to replace those lost through receptor mediated endocytosis. However in none of the studies on spare receptors has any attempt been made to assess the functionality of the spare sites. Indeed, there is limited evidence to suggest that gonadotropin receptors located on the basolateral surface of murine luteal cells are associated with adenylate cyclase activity, while those of the microvillus border are not (Bramley and Ryan, 1978a,b, 1979, 1980). Thus, the general applicability of the concept of spare receptors to LH-CG action on the ovary is unknown.
The Concept of Masked Receptors

One of the fundamental concepts of protein hormone-receptor interaction is that the receptors are located on the cell surface. However, early investigations of hormone-receptor binding raised the possibility that receptors could exist in the plasma membrane or intracellular membranes where they were not accessible for hormone binding. One of the first investigators to suggest the presence of "masked" or "cryptic" binding sites was Cuatrecasas (1971). He noted that treatment of fat cells or fat cell membranes with various enzymes that perturbed membrane lipids led to a 3-6 fold increase in specific $^{125}$I-insulin binding. This effect was due to an increase in the number of available binding sites. The rates of association and dissociation of the hormone-receptor complex, and the equilibrium constant were similar in control and enzyme-treated cells. Only recently has this concept attracted attention, with cryptic binding sites for insulin now reported in other systems. Deutsch and colleagues (1982) noted that treatment of murine 3T3-L1 adipocytes with Triton X-100 to solubilize the receptor, resulted in 40% more insulin binding activity than was present on the surface of intact cells. This increase in binding activity was due to an increase in the number of available binding sites, and these latent sites displayed binding characteristics similar to those of the plasma membrane insulin receptor. Receptor solubilization also reveals a latent pool of insulin binding sites in rat liver and fat cells, (Cuatrecasas, 1972). Taken together, these studies strongly suggest that cryptic binding sites for insulin exist in
various tissues. However, detailed analysis of cryptic binding sites for other hormones has not been demonstrated.

There is limited evidence that cryptic binding sites for the gonadotropins (LH-CG) may be present in the gonads of several species. Early studies suggest that neuraminidase pretreatment enhances $^{125}$I-hCG binding to luteinized rat ovaries (Lee and Ryan, 1973d), and bovine corpora lutea (Rao, 1974). Recent studies on neuraminidase treatment of ovarian and testicular membranes from a variety of mammalian species (Ucer and Engel, 1981; Rajaniemi et al., 1981; Paul and Jailkharni, 1982; Muller, Ucer, and Engel, 1983), add to the concept that masked binding sites exist for the gonadotropin hormones. However, experiments were not performed in many of these studies to determine whether increases in binding were due to an unmasking of binding sites or to an alteration in binding affinity. In addition, limited characterization of the effects of neuraminidase was performed, and no attempt was made to examine the possibility that increased binding was due to an artifact of the techniques used for tissue preparation. To date, the most thorough examination of the effects of neuraminidase on gonadotropin binding has been performed by Azhar and Mennon (1981). In the bovine corpus luteum, neuraminidase from a variety of sources enhanced $^{125}$I-hCG binding and sialic acid metabolism in a dose-dependent fashion. The effect of neuraminidase was relatively insensitive to the pH of the incubation media. Moreover the increase in gonadotropin uptake was due to an increase in the number of available receptors with little or no change in affinity.
In 1982, Cameron and Stouffer reported that in vitro addition of ethanol to primate luteal membranes enhances $^{125}\text{I}-\text{hCG}$ binding in a dose-response fashion. The presence of ethanol increases gonadotropin uptake to macaque luteal tissue by as much as 80%, with a maximal effect at 8% ethanol. The increase in binding is due to an increase in the number of gonadotropin receptors, with no change in affinity. In contrast, similar concentrations of ethanol have no effect on porcine luteal membranes. Higher concentrations (20-50%) decrease gonadotropin binding in both species.

Cryptic binding sites for gonadotropin in the testis may be unmasked in vivo by exposure to homologous hormone (Hsueh et al., 1977; Huhtaniemi et al., 1981). Subcutaneous injection of ovine LH results in an acute and transient increase of rat testis LH binding sites, with no change in the affinity of the hormone-receptor complex. Similar rapid up-regulation of gonadotropin binding sites has been demonstrated in the ovine corpus luteum (Suter et al., 1980). They proposed however, that the increase in binding could be due to the insertion of secretory granule membranes (which contain gonadotropin receptors) into the plasma membrane, rather than to the unmasking of cryptic binding sites.

The Significance of Masked Receptors

The possible existence of masked binding sites in a variety of tissues, raises the questions as to the significance of these cryptic sites. Huhtaniemi and coworkers (1981) propose that cryptic sites in the rat testis, exposed by exogenous LH injections, are not coupled to adenylate cyclase, and thus do not represent functional gonadotropin receptors. In their studies, no increase in maximal cAMP production was
demonstrated, and no changes in the sensitivity of testosterone production to hCG were observed.

Depending upon a variety of conditions, a number of possibilities exist for the significance of masked binding sites. 1) Masked binding sites may serve as a reserve pool which is rapidly uncovered to alter the cell's sensitivity or maximal response to circulating hormones. Numerous studies have established a relationship between target cell responsiveness to hormones, and the number of receptors for that hormone on the cell surface. However some hormone receptor systems demonstrate the phenomenon of spare receptors, where only a fraction of the available receptors need to be occupied to achieve a maximal response. 2) Masked binding sites may be important in the phenomenon of down-regulation and desensitization (Corin and Donner, 1981), and might also be involved in rapid up-regulation by homologous hormone (Hsueh et al., 1977; Huhtaniemi et al., 1981). In addition, unmasking of receptors may allow for the rapid replacement of receptors which have been lost through receptor-mediated endocytosis (Ucer and Engel, 1981; Muller et al., 1983). 3) Cryptic binding sites may also be precursors for hormone receptors, or subunits of the receptor (Deutsch et al., 1983). Thus, masked sites may not constitute functional receptors or may display different binding characteristics than functional receptors. 4) Masked binding sites may be present on intracellular membranes in addition to the plasma membrane (Deutsch et al., 1982). Since the majority of studies on masked binding sites utilize crude membrane preparations, the location of cryptic sites is unknown. 5) Masked binding sites may represent receptor metabolites or
inactivated receptors. Internalized receptors may undergo many fates, and conditions which "unmask" binding sites may alter these processes. Although many studies have implicated the existence of cryptic binding sites, detailed characterization of these sites, and determination of their functionality has not been performed.

**Factors Modulating Binding Site Masking/Unmasking**

The receptors for most peptide and protein hormone are oligomeric glycoproteins with a molecular weight of approximately 200,000 daltons (Dufau and Catt, 1976; Kahn, 1976). The receptor protein is embedded in the bilayer of the membrane and harsh enzymatic or detergent treatment is required to extract the receptor from the bilayer. Thus, the factors or mechanisms responsible for the masking of receptors may be as complex as the membrane itself. A number of different tools have been utilized to examine cryptic binding sites in the membrane.

Many studies have altered the lipid bilayer of the membrane in order to assess the role of the surrounding lipids on receptor function. In the course of these studies, investigators have noticed that selectively increasing or decreasing the overall membrane fluidity can expose previously unavailable binding sites. Membrane fluidity relates to the ease with which membrane components can move within the membrane. In 1979, Muller and Shinitzky reported that cholesterol depletion of bone marrow cells and human erythrocytes, which increased membrane fluidity, increased the number of transferrin binding sites per cell. Cholesterol enrichment, which decreased membrane fluidity, decreased the number of binding sites for transferrin in these systems. In contrast,
decreasing membrane fluidity by cholesterol hemisuccinate treatment increases opiate (Heron et al., 1981), and serotonin (Heron et al., 1980) binding sites. Other agents which perturb membrane lipids also unmask specific hormone binding sites. Phospholipase C treatment unmasks insulin binding sites in fat cells (Cuatrecasas, 1971) and liver membranes (McCaleb and Donner, 1981). Prostaglandin I₂ increases the fluidity of mouse liver membranes, concomitant with an increase in the number of available prolactin binding sites (Dave and Knazek, 1980). A wide range of other prostaglandins examined had no effect upon prolactin binding or membrane fluidity. Incubation of primate luteal membranes with the membrane fluidizing agent ethanol, increases the number of available receptor sites for gonadotropin (Cameron and Stouffer, 1982c). Ethanol also increases the number of prolactin (Dave and Witorsch, 1983), opiate (Levine, Hess, and Morley, 1981), γ-aminobutyric (Ticku and Burch, 1980), α-adrenergic (Ciofalo, 1978) and calcium (Leskawa, 1981) binding sites in various membrane systems. It is important to note however, that treatments which are effective in unmasking receptors in some hormonal system are ineffective in others. For example, phospholipase C treatment unmasks insulin binding sites in the fat cell, but decreases gonadotropin binding to the bovine corpus luteum (Rao, 1974).

Another important constituent of cell membranes is carbohydrate attached to lipids or proteins on the external surface of the membrane. The enzyme neuraminidase, which cleaves off sialic acid residues, has been used extensively to assess the importance of membrane carbohydrates in the hormone-receptor process. Lee and Ryan demonstrated in 1973 that
the presence of 100 mg/ml neuraminidase increased specific $^{125}$I-hCG binding to luteinized ovaries of the rat by approximately 22%. Since then, a number of investigators have utilized neuraminidase to expose cryptic binding sites, as noted previously. Interestingly, Ucer and Engel (1981) reported evidence for endogenous neuraminidase activity in the neonatal rat ovary and testis. Neuraminidase activity is present by day one post-partum in the testis; however enzyme activity is not expressed until day ten in the ovary. Notably, the expression of neuraminidase activity coincides with appearance of LH-CG receptors in the gonads.

In contrast, other enzymes such as trypsin, DNase, RNAse, pronase and lipase, which affect membrane or cellular components have been ineffective in exposing cryptic sites. Therefore, the lipid and carbohydrate portions of the membrane appear to be the primary constituents involved in the process of receptor masking/unmasking. However Costlow and Hample (1982), reported that binding sites for prolactin in target cells could be unmasked with a variety of agents that markedly lower intracellular ATP levels. Although the increase in binding was not due to prevention of hormone degradation, hormone binding to internal binding sites, or to inhibition of down regulation, the exact mechanism of action remains unknown but appears to be related to energy depletion.

Masked binding sites may also be exposed by the addition of hormones which alter phospholipid metabolism. Administration of gonadotropin results in an acute and transient increase in binding sites for LH in the rat testis in vivo (Hutaniemi, 1981; Hsueh, 1977), and
stimulates phospholipid turnover in vitro (Farese, 1983). Exposure of rat liver plasma membranes to insulin stimulates phosphoinositol hydrolysis (Farese, 1983), and unmasks insulin binding sites (Corin and Donner, 1981). Recent evidence also suggests that protein hormones (Dave, Brown, and Knazek, 1982), and catecholamines (Hirata and Axelrod, 1980) increase membrane fluidity, and this may be an additional mechanism for the unmasking of binding sites by hormones.

Membrane Fluidity

The current view of the structure of biological membranes depicts a lipid bilayer within which various protein molecules are dispersed. The proteins may be loosely attached on to the surface of the membrane (extrinsic proteins) or may penetrate partially or completely through the bilayer (intrinsic or integral proteins). The fluid mosaic model which describes this system (Singer and Nicholson, 1972) states that the protein and lipid molecules are free to move within the membrane, and the relative ease of this motion has been described as the fluidity of the bilayer membrane. The term "membrane fluidity" (or its inverse, microviscosity) has been used in regard to various aspects of membrane dynamics, including: 1) motion of the hydrocarbon chain in relation to the head group of the phospholipids, 2) the lateral diffusion of lipid molecules within the bilayer, 3) rotation of lipid molecules, and 4) lateral and rotational motion of the proteins within the membrane (Robertson, 1981). Thus, fluidity is dependent upon different factors in different regions of the membrane. In the hydrocarbon core, fluidity is determined chiefly by the partial specific volume of the hydrocarbon chains. In the hydrocarbon-water
interface and hydrophilic boundary, strong intermolecular forces prevail, and fluidity is determined more by these forces than the specific volume of the constituents (Shinitzky and Henkart, 1979). There is a gradient of fluidity from the outer surface toward the center, with the interior of the membrane in a much more fluid state than the hydrophilic boundary region.

Membrane proteins and lipids are generally believed to undergo rapid rotational and translational movement within the plane of the bilayer membrane, however under normal conditions inversion of membrane proteins and "flip-flop" of phospholipids from one face to the other is extremely slow (Rousselet et al., 1976). In addition, the lipid and protein distribution between the inner and outer bilayer is asymmetrical (Rothman and Lenard, 1977). As a result, there is often a difference in fluidity between bilayer halves, and this asymmetry could have differential effects on components of the membrane located in different halves of the bilayer, such as hormone receptors and adenylate cyclase. In addition, a substantial fraction of membrane proteins are practically immobile within the membrane and may be associated with cytoskeletal components (Schlessinger et al., 1976; Jacobson et al., 1976).

As reviewed by Shinitzky and Yuli (1982), the "natural" modulators of membrane fluidity can be divided into chemical and physical factors. The four principle chemical factors which affect membrane fluidity are: 1) the cholesterol/phospholipid (C/PL) ratio, 2) the degree of unsaturation of the phospholipid acyl chains, 3) the lecithin/spingomyelin ratio, and 4) the protein/lipid ratio in the membrane.
Cholesterol is the most abundant sterol in biological membranes. In most systems, cholesterol has a rigidifying effect on lipid bilayers, achieved primarily by restricting the motion of the acyl chains relative to the head group of the phospholipid. Under normal conditions the C/PL ratio is maintained at a constant level which is optimal for cell function.

The second main determinant of membrane fluidity is the degree of unsaturation of the phospholipid acyl chains. The presence of double bonds in the hydrocarbon chain increases the specific volume of this structure, thereby increasing the fluidity of the bilayer. The degree of unsaturation is acutely modulated by intracellular metabolism, and is regarded as the main regulatory mechanism of membrane fluidity in adaptation to temperature (Cousins, 1977), metabolic disorders (Cooper, 1977), and diet. In addition, the mechanism of action of some hormones may involve phospholipid metabolism and the degree or unsaturation of the membrane lipids (Farese, 1983; Hirata and Axelrod, 1980).

The ratio of lecithin to sphingomyelin is the third main parameter of membrane fluidity. These two phospholipids constitute more than 50% of the total phospholipids present in mammalian membranes. Lecithin is highly unsaturated and thus confers fluidity, whereas sphingomyelin is highly saturated and tends to rigidify the membrane. The relative abundance of these two molecules make them very important in the regulation of overall membrane fluidity.

The effect of protein, the fourth main determinant of membrane fluidity, is essentially the same as cholesterol. As the ratio of protein to phospholipid increases, there is a net decrease in membrane
fluidity, and this effect is more prominent at low cholesterol levels. The relationship between protein and lipid can be further illustrated by the concept of vertical displacement of membrane proteins (Borochov and Shinitzky, 1976; Shinitzky and Rivnay, 1977). As membrane fluidity increases, the bulk of the membrane protein can "sink" down into the bilayer. Conversely, if membrane fluidity decreases, the bulk of the protein may become more exposed. If the bulk of a transmembrane receptor protein resides on the inner face of the bilayer and "sinks" as the fluidity increases, then the other end of the protein may become more exposed. Thus, increasing membrane fluidity may result in an increase or decrease in the exposure of membrane receptors on the outer surface of the membrane, depending upon where the bulk of the receptor protein resides.

In addition to chemical modulators, a number of physical modulators of membrane fluidity have been examined. These include temperature, pressure, pH, membrane potential, and Ca++. Except for some bacterial membranes (Quinn, 1981), most membranes of higher organisms are primarily in a liquid-crystalline state at physiological temperatures, which allows the proteins and lipids to move freely in the membrane (Cherry, 1977). As the temperature is lowered, membrane fluidity gradually decreases until, at a certain point the bilayer rapidly forms a gel-phase, in which the lipids and proteins have less molecular motion. The temperature at which this transition occurs is known as the transition temperature, and is usually 5-20°C for mammalian membranes. As a general rule, increases in pressure will lead to a decrease in membrane fluidity, while increasing pH leads to an increase
in membrane fluidity. The effects of membrane potential tend to be variable, whereas Ca$^{++}$ ions decrease the lipid fluidity (Storch et al., 1983; Rasnick and Schacter, 1982).

In addition to these "natural" modulators of membrane fluidity, there are a wide variety of other agent which have been utilized to perturb the bilayer or alter membrane fluidity. One of the most commonly investigated groups are the alcohols. In 1972, Seeman reported that the presence of n-alkanols caused an expansion of membrane surface area and a fluidization of the membrane lipids. This disordering effect has been demonstrated with a variety of membrane probes (Grisham et al., 1973; Patterson et al., 1972; Lenaz et al., 1976; Jacobson and Wobschall, 1974; Zavoico and Kutchai, 1980), and the degree of disorder is proportional to the length of the hydrocarbon chain (Zavoico and Kutchai, 1980; Lyon et al., 1981).

General and local anesthetics have also been suggested to increase membrane fluidity. Although the exact molecular mechanism of action of anesthetics is not fully understood, many studies have implicated an interaction with membrane phospholipids (Skou, 1954; Feinstein, 1964; Papahadjopoulos, 1970; Lee, 1976; Vilallunger and Phillips, 1979). Interaction with membrane proteins or competition with an endogenous ligand has also been postulated (Franks and Lieb, 1982). In addition, some anesthetics may preferentially perturb the inner or outer half of the bilayer (Houslay, Dipple, and Gordon, 1981), and thus may be useful in the elucidation of the role of membrane lipids on membrane function.
Other agents which have been demonstrated to affect membrane fluidity include; linoleic acid (Heron et al., 1981), phenylhydrazine (Rice-Evans and Hochstein, 1981), fillipin (Norman et al., 1972), alamethicin (Jones, 1980), prostaglandins (Dave and Knazek, 1980), FSH (Strulovici et al., 1981), steroid hormones (Gallay et al., 1981; Marks et al., 1982), concanavalin A (Onho et al., 1981; Cherenkevich et al., 1981), and antisickling agents (Acquaye et al., 1981).

**Methods of Measuring Membrane Fluidity**

As stated earlier, the term "membrane fluidity" has been used to describe various aspects of molecular motion in biomembranes. Examination of membrane fluidity has usually focused on the two principle components of the membrane, proteins and lipids, and a variety of techniques have been employed to examine each.

One of the most popular methods used to study protein mobility is the Fluorescence Photo-Bleaching Recovery (FPR) technique (Poo and Cone, 1974; Peters et al., 1974). Membrane proteins are "tagged" with a fluorescent probe, and a brief pulse of light is used to irreversibly bleach a well-defined region of the membrane. Measurements are then made on the rate of recovery of fluorescence in the bleached area, which presumably is due to migration of non-bleached molecules into the bleached region. This technique can be applied to a wide variety of membrane molecules, however it requires elaborate and expensive equipment, and damage to the cells due to laser bleaching and dissociation of probe-protein complexes remain potential drawbacks (Shintzky and Henkart, 1979).
The measurement of lipid fluidity can be assessed on at least three levels, the macroscopic level, submacroscopic level, and the microscopic level (Shinitzky and Yuli, 1982). At the macroscopic level, lipid domains are assessed in bulk thermodynamic terms, and fluidity is usually determined by mechanical or calorimetric means. The microscopic level deals with the movements and configurations of individual atoms or complexes at various depths within the bilayer membrane; fluidity is often assessed by the technique of nuclear magnetic resonance. The advantage of this technique is that no artificial membrane probes are utilized and the natural components of the membrane can be observed directly (Smith et al., 1978).

The most common evaluation of lipid fluidity occurs at the submacroscopic level, which provides low molecular resolution of lipid fluidity (Shinitzky and Yuli, 1982). The techniques used at this level include fluorescence polarization, which has been used extensively in a variety of artificial and natural membranes (Shinitzky and Barenholz, 1978). A fluorescent probe is inserted into the bilayer membrane or attached on to a lipid backbone. The probe is then excited with polarized monochromatic light, and the degree of depolarization is measured. If the lipid matrix is in a viscous state, the degree of rotation (and hence depolarization) of the probe will be small. However if the membrane is more fluid, the probe will be able to rotate freely and the degree of depolarization will be greater. Fluorescence polarization offers a number of advantages over other methods (Shinitzky and Barrenholz, 1978): 1) the polarized signal is sensitive and highly reproducible, 2) the technique can be applied to small quantities of
complex systems such as the biological membrane, and 3) the data are easily analyzed. In addition the technique is relatively simple and inexpensive to perform.

A wide variety of probe molecules, including dansylamide, trans-parinaric acid, and diphenylhexatriene (DPH), have been utilized in fluorescence polarization studies. DPH is the most popular because it has a number of favorable qualities as a probe of membrane fluidity (Shinitzky and Barenholz, 1978). Its fluorescence emission has a high quantum yield, and is well separated from the absorption bands of membrane proteins and lipids. It is chemically stable and easily incorporated into most membranes. It is only soluble and fluorescent in hydrophobic environments, and partitions equally well into both the gel and liquid-crystalline phases of bilayer membranes (Lentz et al., 1976). DPH has been utilized to study a wide range of biological membranes, and is therefore useful in comparing membrane properties in different species. Dansylamide is a useful probe for studying the hydrocarbon/water interface of lipids since it preferentially localizes in this region of the membrane. Trans-parinaric acid has been utilized to measure the fluidity of bovine luteal membranes (Goodsaid-Zalduondo et al., 1982).

There are several disadvantages of fluorescence polarization, however, which may influence the interpretation of fluidity measurements. 1) Probe molecules may partition preferentially into neutral membrane lipids. 2) The fluorescence signal may reflect an interaction between the probe molecules and proteins or lipoproteins within the membrane, rather than the actual fluid state or the phospholipids (Mely-Goubert
and Freedman, 1980). 3) Fluidity measurements may only reflect an average over the entire membrane, and it is the properties of the probe within the membrane that are measured, not direct measurements of the natural membrane components (Parola et al., 1981). 4) Incorporation of probe molecules into the membrane may perturb the bilayer and may affect membrane fluidity.

Despite these limitations, fluorescence polarization is one of the most popular and widely utilized techniques, and is generally considered to be a useful if not entirely accurate method for estimating membrane fluidity.

**Effect of Membrane Fluidity on Hormone Receptor-Adenylate Cyclase Systems**

One of the key features of the fluid-mosaic model is that proteins move freely within the membrane. Thus, the fluid state of the bilayer may play an important role in events such as hormone receptor interaction and the activity of enzymes associated with the membrane. As such, membrane fluidity may be a critical determinant for the successful transmission of the hormonal signal into the cell.

Alterations in membrane fluidity affect the affinity and availability of receptors for several hormones, including prolactin (Dave and Knazek, 1980), serotonin (Heron et al., 1980), and transferrin (Muller and Sninitzky, 1979). Indirect evidence also suggests that membrane fluidity may enhance receptor availability for other hormones, including the gonadotropins (Cameron and Stouffer, 1982c).

In addition, membrane fluidity may play an integral role in the modulation of enzyme function within the membrane. The specific
activity of enzymes such as Ca\textsuperscript{2+}-ATPase (Warren et al., 1977), Na\textsuperscript{+}, K\textsuperscript{+}-ATPase, (Kimelberg, 1975), and the β-galactoside transport system (Thilo et al., 1977), are affected by membrane fluidity. A number of investigators have examined the effects of membrane fluidity on adenylate cyclase activity. Agents which increase membrane fluidity enhance basal adenylate cyclase activity in rat brain membranes (Rasenick et al., 1981), turkey erythrocytes (Rimon et al., 1978), neuroblastoma cells (Stenstrom and Richelson, 1980), mouse brain membranes (Hoffman and Tabakoff, 1982), pigeon erythrocytes (Salesse et al., 1982), and rat and rabbit corpora lutea (Abramowitz and Birnbaumer, 1979). In contrast, the membrane fluidizing agent (and local anesthetic) benzyl alcohol has relatively little effect upon basal adenylate cyclase activity in the rat liver plasma membranes, however guanine nucleotide- and glucagon-stimulated activity is enhanced in the presence of this alcohol (Houslay and Gordon, 1983).

Changes in membrane fluidity may also affect hormonal stimulation of adenylate cyclase. Phenobarbital, which supposedly fluidizes only the outer half of the lipid bilayer, selectively modulates glucagon-stimulated adenylate cyclase in mouse liver plasma membranes; fluoride-stimulated adenylate cyclase activity is unaffected (Houslay et al., 1981). Ethanol stimulates both basal and hormone-stimulated adenylate cyclase activity in the rat and rabbit corpus luteum (Abramowitz and Birnbaumer, 1979). Ethanol up to a concentration of 5% enhances the adenylate cyclase response to both LH and CG. Above this concentration, ethanol depresses the adenylate cyclase response to the gonadotropins. A preliminary report by Rojas
and Asch (1984) suggests that ethanol also increases gonadotropin stimulation of adenylate cyclase activity in the human corpus luteum, but is without effect on basal activity.

In 1978 Rimon and coworkers compared the effect of altering membrane fluidity on two different hormone-receptor adenylate cyclase systems in the turkey erythrocyte. In these cells the β-adrenergic receptor is collision-coupled to adenylate cyclase (Tolkovsky and Levitzki, 1978a), whereas the adenosine receptor is permanently coupled to the enzyme (Tolkovsky and Levitzky, 1978b; Braun and Levitzky, 1979). Fluidization of the membrane with cis-vaccenic acid resulted in a greater enhancement of adrenalin-stimulated cAMP production than adenosine-stimulated cAMP production. In addition, the rate constant of the adrenalin dependent activation increased linearly with membrane fluidity, whereas the rate constant of the adenosine dependent activation was independent of membrane fluidity. They propose that modification of membrane fluidity may be a useful tool in the elucidation of the mode of receptor-cyclase coupling in other systems.

Recent reports from Carlson and colleagues (Buhr, Carlson, and Thompson, 1979; Carlson et al., 1981, Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982; Carlson, Buhr, Riley, 1984) suggest that the fluidity of the luteal membrane may change throughout the luteal lifespan. Corpora lutea undergoing spontaneous or PGF₂α-induced luteal regression have more gel-phase lipids than during the mid-luteal phase, suggesting that membranes from regressing corpora lutea are in a less fluid state. This decrease in fluidity may play an important role in luteal function, since hormone-receptor interaction and adenylate
cyclase activation are sensitive to the fluid nature of the bilayer membrane. Indeed, it is known that gonadotropin binding decreases during luteal regression (Cameron and Stouffer, 1982b), and that PGF$_2\alpha$ inhibits adenylate cyclase activity (Behrman et al., 1974). The decrease in gonadotropin binding and the uncoupling of the LH-receptor complex to adenylate cyclase may be linked to alterations in membrane fluidity. Thus, the fluid state of the luteal membrane may alter the response of the corpus luteum to circulating gonadotropins, and it is possible that decreased membrane fluidity may be a pivotal factor leading to luteal regression.
CHAPTER 3

OBJECTIVES OF THE PRESENT STUDY

The present study was designed to investigate the possible existence of masked gonadotropin binding sites in the corpus luteum of the rhesus monkey, and characterize gonadotropin binding to these putative sites. In addition, the possible mechanisms underlying the masking/unmasking of gonadotropin binding sites were examined.

Experiments were performed 1) to identify and characterize the conditions under which masked gonadotropin binding sites can be exposed in the corpus luteum of the rhesus monkey, 2) to characterize gonadotropin binding to these putative sites with respect to species specificity, tissue preparation, affinity for LH, and kinetics of the gonadotropin-receptor interaction, and 3) to examine the possible role of membrane fluidity in the masking/unmasking of cryptic sites, a) by estimating the fluidity of luteal membranes under conditions which expose masked sites and correlating these changes in fluidity with changes in gonadotropin binding, and b) by removing the gonadotropin binding site from the lipid bilayer of the luteal membrane and examining gonadotropin binding to solubilized sites under conditions which expose cryptic receptors in luteal particulates.
CHAPTER 4

EVIDENCE FOR TWO POPULATIONS OF MASKED GONADOTROPIN BINDING SITES IN THE CORPUS LUTEUM OF THE Rhesus Monkey (MACACA MULATTA)

Abstract

To evaluate the possible existence of masked gonadotropin receptors in the corpus luteum, we characterized the effects of alcohols and neuraminidase on $^{125}$I-hLH binding to in vitro preparations of luteal tissue from the rhesus monkey and pseudopregnant rat. The presence of 1-8% (v/v) ethanol enhanced specific LH binding to macaque luteal particulates under steady-state conditions (25°C, 20h incubation), with a maximal effect at 8% ethanol (166% of control uptake; p<0.05). However, 1-8% ethanol had no effect on LH binding to rat luteal tissue. Higher concentrations of ethanol (20%) decreased LH binding relative to control in both species. Ethanol modulation of LH binding to macaque luteal particulates and dispersed cells was a time- and temperature-dependent process. At 4°C and 25°C, ethanol increased LH uptake at all times during 32h incubation. However at 37°C, ethanol increased LH uptake at 30 min., binding peaked at 2h and then returned to control levels within 20h. The optimal concentration of ethanol for enhancing LH uptake was inversely related to the incubation temperature. The increase in LH binding to macaque luteal particulates in the presence of ethanol was reversible; binding returned to control levels if ethanol was removed prior to the addition of labeled LH. Longer straight-chain alcohols
(butanol, pentanol, and octanol) were progressively more potent than ethanol in enhancing LH binding to macaque luteal particulates and dispersed luteal cells. Pretreatment of luteal particulates from either the rat or monkey with neuraminidase increased LH uptake, with a maximal effect (160% of control) at 1 mg/ml enzyme. Scatchard analysis revealed that both ethanol and neuraminidase increased \( \text{p}(0.05) \) the number of LH binding sites without altering the affinity for gonadotropin. Moreover, the effects of ethanol and neuraminidase were additive, i.e., increased LH binding during combination of the two treatments approximated the sum of the individual effects.

The data suggest that two distinct populations of LH binding sites are masked within the membranes of the monkey corpus luteum. The ability of two markedly different agents, alcohol and neuraminidase, to increase LH binding indicates that diverse mechanisms may modulate the masking/unmasking of gonadotropin receptors in target cell membranes. Finally, the inability of ethanol to enhance LH binding in the rat suggests species differences in the receptor population or milieu of luteal membranes.

**Introduction**

The population of receptors for peptide/protein hormones on the surface of target cells is in a dynamic state. Receptors are continuously synthesized and inserted into the plasma membrane, and are lost via internalization, endocytosis, and lysosomal degradation (Gorden et al., 1980; Schlessinger, 1980; Posner et al., 1981). The factors which regulate the number of available receptors at a given time are
poorly understood. In many systems, exposure to elevated levels of hormone leads to a loss of receptors on the cell surface, a process known as down-regulation (Dufau et al., 1978; Dufau and Catt, 1978; Catt et al., 1979). However, the presence of hormone can also lead to up-regulation of receptors (Hsueh, Dufau, and Catt, 1977; Huhtaniemi, Martikainen, and Tikkala, 1978; Chan and Davies, 1979; Suter et al., 1980) within minutes of treatment. The rapidity of up-regulation, plus evidence that certain in vitro treatments of luteal preparations increases the number of available binding sites for a hormone (Rao, 1974; Azhar and Mennon, 1981; Rajaniemi et al., 1981; Cameron and Stouffer, 1982c), has led to the hypothesis that masked or cryptic receptors exist within the membranes of target cells which are unable to interact with circulating gonadotropin.

Investigators have proposed that gonadal tissues (Rao, 1974; Azhar and Mennon, 1981; Rajaniemi et al., 1981; Ucer and Engel, 1981; Berman and Sairam, 1982; Cameron and Stouffer, 1982c; Paul and Jailkhani, 1982; Muller, Ucer, and Engel, 1983) such as the corpus luteum (Rao, 1974; Azhar and Mennon, 1981, Rajaniemi et al., 1981; Cameron and Stouffer, 1982c) contain masked binding sites for the gonadotropins, LH/CG. Azhar and Menon (1981) observed that treatment of luteal membranes with neuraminidase, an enzyme that removes cell-surface sialic acid, increased specific $^{125}$I-hCG binding. Moreover, a preliminary report by Cameron and Stouffer (1982c) indicated that in vitro exposure to ethanol increased the ability of macaque luteal particulates to bind hLH. However, conclusive evidence for the existence of masked gonadotropin binding sites and detailed
characterization of these sites with respect to available receptors is lacking.

Consequently, we undertook a series of experiments to examine in detail the possible presence of masked binding sites for LH/CG in the corpus luteum of the rhesus monkey and the psuedopregnant rat. In this paper we report on conditions which expose binding sites in vitro and selected characteristics of gonadotropin binding to exposed sites.

Materials and Methods

Source and Preparation of Luteal Tissue

We removed the corpus luteum from adult female rhesus monkeys on days 21-24 of the menstrual cycle by methods described previously (Cameron and Stouffer, 1982a). Preliminary studies by Cameron and Stouffer (1982a) suggested that corpora lutea removed at this time contained masked gonadotropin binding sites.

For some experiments, we induced multiple ovulations in monkeys by using a regimen of human menopausal gonadotropins (hMG, Pergonal, Serono Laboratories Inc., Randolph, MA) followed by hCG (APL, Ayerst Laboratories, New York, NY). Monkeys were injected im with 18 I.U. hMG twice per day for 8-10 days; then 1000 I.U. hCG was injected to promote ovulation and luteinization of the developed follicles. Typically, 3-5 corpora lutea formed on each ovary. The corpora lutea were removed 6-7 days after hCG injection and were used primarily for cell studies which required more tissue than could be obtained from a normal cycling monkey.
The freshly excised corpus luteum was freed from extraneous tissue, weighed, and minced into small pieces. Crude homogenates were prepared according to the procedures of Cameron and Stouffer (1982a). Briefly, we homogenized the minced tissue in 0.05M Tris-HCl buffer (pH 7.4, containing 5 mM MgCl2, 0.15M NaN3, and 8% sucrose) with a hand-held glass (Dounce) homogenizer, and then filtered the homogenate through fine Japanese silk to remove large debris. For studies on particulates, we centrifuged the homogenate at 20000g for 15 minutes, and suspended the resulting pellet in buffer at a concentration of 2.5 mg tissue equivalents/ml.

We prepared collagenase-dispersed luteal cells according to the methods of Stouffer and colleagues (1976). The minced tissue was incubated in Ham’s F-10 nutrient media containing 2% bovine serum albumin (BSA) and 0.2% collagenase (Worthington, CLS) at an initial concentration of 75 mg tissue/ml. Incubations were carried out at 37°C in capped polyethylene tubes containing an atmosphere of 95% O2-5% CO2 with gentle shaking. The cells dispersed by the collagenase treatment were removed every 10 minutes, washed, and placed in ice-cold nutrient media. Isolated cells were collected by centrifugation (160g) and resuspended in nutrient media containing 2% BSA at 4°C, at a final concentration of approximately 5x10^4 cells/0.25ml.

Prepubertal rats (20-22 days old, Sprague Dawley, Animal Resources Breeding Colony) were injected with 50 I.U. pregnant mares serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO) sc, followed 56h later by 50 I.U. hCG (APL) to induce multiple ovulations and corpora lutea (Birnbaumer et al., 1976). The rats were decapitated
and their ovaries were removed 8-10 days after PMSG injection. We prepared particulates using the same methods described for the macaque corpus luteum.

In general, experiments were performed on fresh tissue. Due to the large amount of tissue obtained from the pseudopregnant rat and superovulated monkey, we performed some experiments on tissue frozen at -70°C prior to use. Freezing and storing the tissue in this manner did not alter gonadotropin binding or inhibit the unmasking of binding sites.

Preparation of ¹²⁵I-radiolabeled hLH

We radiolabeled purified hLH (NIH-I-1, National Pituitary Agency) with ¹²⁵I by the lactoperoxidase method (Cameron and Stouffer, 1982a). Human LH (5 μg) was incubated with 200 ng lactoperoxidase (B grade, Calbiochem, La Jolla, CA), and 0.5 mCi Na⁻¹²⁵I in the presence of 3x200 ng H₂O₂ added at 5 minute intervals. We separated labeled hormone from free iodine by filtration through a Biogel P-60 column equilibrated with 0.05M phosphate buffered saline (PBS) containing 2.0% bovine serum albumin. The specific activity (Cameron and Stouffer, 1982a) of the radiolabeled LH was 68.53 +/- 2.87 uCi/ug. Radiolabeled LH was stored at 4°C and was used within 3 weeks of iodination.

¹²⁵I-Gonadotropin Binding Studies

We used the general techniques of Cameron and Stouffer (1982a) to assess gonadotropin binding to preparations of luteal tissue. Homogenates and particulates of luteal tissue, or dispersed luteal cells were incubated with various concentrations of ¹²⁵I-hLH for up to 32h in
a gyrotory shaker bath. Incubations of homogenates and particulates were performed in Tris-HCl buffer in a final volume of 0.25ml. Intact cells were incubated with Ham's F-10 nutrient media in polyethylene tubes at a final concentration of 0.25ml. After incubation, 1 ml ice-cold Tris-HCl buffer was added to each sample, and the $^{125}$I-hLH bound to the luteal tissue was separated from free hormone by filtration through Metritcel membrane filters (pore size=0.45μm) presoaked in buffer containing 2% bovine serum albumin (BSA). We rinsed the incubation tubes with 2 ml buffer and the filters with an additional 5 ml buffer. The amount of radioactivity remaining on the filter was determined in a Searle 1197 automatic gamma counter. Non-specific binding of $^{125}$I-hLH was assessed by co-incubating samples with a 100-fold excess of unlabeled gonadotropin (Ayerst APL). Specific binding was calculated by subtracting non-specific uptake from the total amount of $^{125}$I-hLH bound. Steady-state $^{125}$I-hLH binding was analyzed by the method of Scatchard (1949) to determine the maximum binding capacity (Bm) of the tissue and the affinity (equilibrium dissociation constant, Kd) of the binding sites for LH. Data were analyzed and calculated on a Zenith Z-100 microcomputer interfaced with the gamma counter via a four-channel data buffer system (see Appendix).

Luteal particulates and cells were incubated with 0-20% alcohol (vol/vol) at 4C, 25C, and/or 37C, and the effects on gonadotropin binding were assessed. For the experiments with neuraminidase (NA), luteal preparations were preincubated with 0-2.0 mg NA/ml for 30 minutes at 37C. The tissue was then washed twice with 1 ml ice-cold buffer to
remove the NA, and resuspended at the appropriate concentration for the binding studies.

Statistical analyses

Differences among levels of gonadotropin binding during exposure to 0-20% ethanol and following pretreatment with 0-2.0 mg neuraminidase/ml were determined by a two-way analysis of variance for a randomized complete block design. Individual corpora lutea were treated as blocks in these analyses. To examine the possible interaction between neuraminidase and ethanol in one of these analyses, the treatment sum of squares was partitioned. Following demonstration of significance (p<0.05) with an F test, comparisons were made between pairs of means using the Least Significant Difference Test (Snedcor and Cochran, 1967). Student's t-tests were used to examine possible changes in numbers and affinities of LH binding sites due to ethanol exposure and neuraminidase treatment.

Results

The effects of ethanol on $^{125}\text{I}-\text{hLH}$ binding to luteal particulates prepared from the monkey and the pseudopregnant rat are depicted in Figure 1. In these studies, gonadotropin binding was assessed following incubation at conditions (25°C for 20h) which result in steady-state uptake to luteal particulates (Cameron and Stouffer, 1982a). As the concentration of ethanol in the incubation medium increased from 1-8%, specific LH binding to macaque luteal membranes was enhanced, with a maximal effect at 8% ethanol (p<0.05). In contrast, the presence of 1-8% ethanol had no effect on LH binding to rat luteal
Figure 1. Effect of increasing concentrations of ethanol on specific
$^{125}$I-hLH binding to luteal particulates prepared from the macaque and
rat corpus luteum. Luteal membranes (2.5 mg tissue equivalents) were
incubated with 3 ng $^{125}$I-hLH and 0-20% ethanol for 20h at 25°C. Each bar
represents the mean +/- SEM of four experiments. Note different scale
for monkey versus rat.
membranes. Higher concentrations of ethanol (20%), decreased (p<0.05) gonadotropin binding to luteal particulates prepared from both the monkey and the rat to a similar extent. When similar experiments were performed at 4C, the dose of ethanol which maximally increased LH uptake to macaque luteal membranes was the highest concentration examined, i.e. 20% (data not shown). In contrast, experiments performed at 37C for 20h revealed no increase in LH binding to monkey luteal membranes at any concentration of ethanol tested.

To further investigate the importance of temperature on the effects of ethanol, we examined the kinetics of LH binding to macaque luteal particulates at 25C and 37C in the presence and absence of 8% ethanol (Figure 2). Under control conditions at 25C, LH binding reached steady-state levels within 6h (Figure 2A). In the presence of 8% ethanol, binding at 25C was greater than control within 30 minutes, and remained elevated throughout the entire incubation. At 37C (Figure 2B), binding under control conditions increased rapidly and reached steady-state levels within 2h. In the presence of 8% ethanol, binding at 37C was greater than control within 30 minutes, peaked at 2h, and then declined to control levels within 20-32h. Thus, the effect of ethanol on LH binding to macaque luteal membranes was both a time- and temperature-dependent process. Subsequent experiments performed at 37C following a 2h incubation revealed a biphasic dose-response with a maximum increase in LH binding observed in the presence of 4-6% ethanol (data not shown).

To determine whether ethanol increased $^{125}$I-hLH binding to intact cells as well as particulate preparations of macaque corpora
Figure 2. Kinetic analyses of specific $^{125}$I-hLH binding to macaque luteal membranes in the presence of 0% (●) and 8% (O) ethanol at 25°C (Fig. 2A) and 37°C (Fig. 2B). Aliquots of luteal particulates (2.5 mg) were incubated with 3 ng $^{125}$I-hLH and 0% or 8% ethanol for 30 minutes to 32h.
lutea, suspensions of dispersed luteal cells were incubated in the presence and absence of ethanol, and LH binding was assessed. Concentrations of ethanol used were those shown to maximally enhance LH binding to particulate preparations. The kinetics of LH binding to macaque luteal cells in the presence and absence of ethanol are depicted in Figure 3. Under control conditions, LH binding to intact cells at 4°C was minimal throughout the entire incubation (3B). The presence of 20% ethanol at 4°C progressively increased gonadotropin binding throughout the incubation. At 37°C (Figure 3B), binding to intact cells reached steady-state levels within 2h, and remained elevated for up to 20h. The presence of ethanol (4%) at this temperature increased LH uptake above control levels within 30 minutes, binding peaked at 2h, and then declined to approach control levels by 20h. The patterns of LH uptake in the presence and absence of ethanol were similar in both intact cells and luteal particulates at all three incubation temperatures, i.e., 4, 25 (not shown), and 37°C.

Exposure to other straight-chain alcohols, in addition to ethanol, increased LH binding to macaque luteal membranes (Figure 4). Butanol, pentanol, and octanol increased LH uptake in a biphasic manner similar to that observed with ethanol. The concentrations of alcohols required to increase LH uptake were inversely proportional to the length of the hydrocarbon chain of the alcohol. The relatively smaller increase in LH uptake elicited by octanol may be related to the limited characterization of the dose-response relationship with this alcohol. Butanol (0.75%) and pentanol (0.25%) also increased LH binding to dispersed luteal cells to a similar extent as 6% ethanol (data not
Figure 3. Kinetic analysis of specific $^{125}$I-hLH binding to luteal cells prepared from the macaque corpus luteum. Each tube contained 50,000 cells, 5 ng $^{125}$I-hLH, in the presence (○) or absence (●) of ethanol. Incubations were carried out at 4°C (Fig. 3A), or 37°C (Fig. 3B) for time periods of 30 minutes to 20h.
Figure 4. Specific $^{125}$I-hLH binding to macaque luteal membranes in the presence of various concentrations of straight chain alcohols. Luteal particulates (2.5 mg) were incubated with 3 ng $^{125}$I-hLH in the presence of 0.01-0.1% octanol, 0.1-1.5% pentanol, 0.25-2.5% butanol, or 1.0-20% ethanol for 20h at 25°C. Binding is expressed as % of control (no alcohol treatment). Note log scale on abscissa. Each point represents the mean of at least 3 experimental values.
Cell viability, as determined by Trypan Blue exclusion was maintained in the presence of butanol and pentanol (97%±2.2% and 101%±6.1% of control, respectively), whereas ethanol decreased cell viability to 72%±4.5%.

The ability of ethanol to increase LH binding to macaque luteal membranes was a reversible phenomenon (Table 1). When luteal membranes were incubated with 8% ethanol for 20h at 25°C, and then the alcohol was removed prior to the addition of labeled gonadotropin, no increase in LH binding was observed (p>0.05). However, exposure to 20% ethanol prior to the addition of labeled gonadotropin markedly decreased LH uptake relative to control (p<0.05). Thus, the increase in LH binding observed in the presence of 8% ethanol is readily reversed when the ethanol is removed. However, the loss of LH uptake observed at higher ethanol concentrations appears to be a permanent phenomenon.

The effect of neuraminidase (NA) pretreatment of monkey and rat luteal particulates on ¹²⁵I-hLH binding is depicted in Figure 5. Neuraminidase pretreatment increased specific LH binding to luteal particulates from both species in a dose-dependent manner, with a maximal effect at 1-2 mg NA/ml. Pretreatment with trypsin (2 mg/ml; 30 min at 37°C) markedly decreased LH uptake by macaque luteal membranes, whereas DNAse (100μg/ml) had no effect on gonadotropin binding (data not shown).

Scatchard analysis of ¹²⁵I-hLH binding to macaque luteal particulates in the presence and absence of ethanol or neuraminidase yielded linear plots (Figure 6). Exposure of macaque luteal membranes to 8% ethanol increased the maximum binding capacity for LH from 4.84
Table 1. Effects of ethanol and its removal on specific $^{125}$I-hLH binding to particulate preparations of the macaque corpus luteum.

<table>
<thead>
<tr>
<th>Ethanol (vol/vol)</th>
<th>Specific $^{125}$I-hLH binding, (cpm/2.5mg tissue equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol present a</td>
</tr>
<tr>
<td>0%</td>
<td>10,241 ± 360</td>
</tr>
<tr>
<td>8%</td>
<td>18,786 ± 647</td>
</tr>
<tr>
<td>20%</td>
<td>6,263 ± 499</td>
</tr>
</tbody>
</table>

a) Luteal particulates (2.5 mg) were incubated with 0, 8, or 20% ethanol in the presence of 3ng $^{125}$I-hLH for 20h at 25C.

b) Luteal particulates (2.5mg) were incubated with 0, 8, or 20% ethanol for 20h at 25C, washed twice, and then incubated with 3ng $^{125}$I-hLH for 20h at 25C. Values are the mean ± SEM of 3 observations.
Figure 5. The effect of neuraminidase pretreatment on specific $^{125}$I-hLH binding to luteal particulates prepared from the macaque and rat corpus luteum. Aliquots of luteal membranes (2.5 mg) were incubated with 0-2.0 mg neuraminidase/ml for 30 minutes at 37°C, washed twice with ice-cold buffer, and then incubated with 3 ng $^{125}$I-hLH for 20h at 25°C. Binding is expressed as % of control (no neuraminidase pretreatment). Each bar represents the mean +/- SEM of at least 2 experiments.
Figure 6. Scatchard plots of specific $^{125}$I-hLH binding to macaque luteal membranes. Luteal particulates (2.5 mg) were incubated under control conditions (no treatment; ○), in the presence of 8% ethanol (●), following pretreatment with 1mg neuraminidase/ml (■), and following neuraminidase pretreatment plus exposure to ethanol (▲). Incubations were carried out in the presence of 1-25 ng $^{125}$I-hLH for 20h at 25°C to achieve steady-state conditions. Each point is the mean of 3 observations in this representative experiment.
+/- 0.38 to 8.83 +/- 0.49 fmol/mg (p<0.05, n=4 experiments), whereas the affinity of the binding site for LH was unchanged (Kd = 3.97 +/- 0.71 x10^-10 M vs. 3.13 +/- 0.46 x10^-10 M; p>0.05). Pretreatment of macaque luteal membranes with 1 mg/ml neuraminidase also increased the maximum binding capacity of the tissue for LH from 5.94 +/- 1.52 to 7.97 +/- 1.04 fmol/mg (p<0.05, n=3), without affecting affinity (Kd=6.10 +/- 1.85 x10^-10 M for control vs 7.31 +/- 1.84 x10^-10 M; p>0.05).

Figure 6 also depicts the results of a typical experiment directly comparing the effects of ethanol exposure, neuraminidase (NA) pretreatment, and the combination of ethanol exposure and NA pretreatment on gonadotropin uptake by macaque luteal membranes. The combination of NA pretreatment and ethanol exposure resulted in a further increase in binding capacity (Bm) above that seen with either NA or ethanol alone; again there was no effect on Kd. Table 2 summarizes the results of 5 experiments comparing the effects of ethanol and NA on gonadotropin binding to macaque luteal membranes. Both NA and ethanol increased ^125I-hLH binding to luteal particulates, and the combination of NA pretreatment plus ethanol exposure further increased LH uptake above the levels seen with either treatment alone. Similar results were observed with intact cells. Statistical analysis revealed no interaction between NA pretreatment and ethanol exposure indicating that the effects of these two agents were additive.

Discussion

The data suggest that two distinct populations of LH binding sites are masked within the membranes of the macaque corpus luteum; one
Table 2. Additive effects of ethanol and neuraminidase on specific 125I-hLH binding to particulate preparations of the macaque corpus luteum.

<table>
<thead>
<tr>
<th>Tissue Treatment</th>
<th>Specific 125I-hLH binding (% control uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Neuraminidase (NA) pretreatment, 1mg/ml</td>
<td>153 ± 11.6</td>
</tr>
<tr>
<td>Ethanol (EtOH) exposure, 8% v/v</td>
<td>171 ± 13.5</td>
</tr>
<tr>
<td>NA + EtOH</td>
<td>214 ± 14.3</td>
</tr>
</tbody>
</table>

Luteal particulates were incubated for 20h at 25C

Values are the mean ± SEM, n=5 experiments
population that is exposed upon addition of alcohol and a second population that is unmasked by treatment with neuraminidase. The ability of two markedly different agents to increase LH binding indicates that diverse mechanisms may modulate the masking/unmasking of gonadotropin receptors in target cell membranes.

These studies confirm and expand on the report of Cameron and Stouffer (1982c) that ethanol increases LH binding in particulate preparations of the macaque corpus luteum. Our current experiments clearly suggest that the effect of ethanol is not a methodologic artifact of tissue preparation, since ethanol increased LH uptake by intact cells and particulates of the macaque corpus luteum in a similar fashion. In addition, ethanol did not increase LH uptake by rat luteal particulates even though these are prepared in an identical manner as macaque membranes. The studies with intact cells also suggest that the location of the masked binding sites may be in the plasma membrane, but a possible intracellular location cannot be ruled out.

The effects of alcohol on LH binding were sensitive to the temperature and duration of the incubation, as well as to the concentration and type of alcohol. At 37°C ethanol only transiently increased LH binding. This may be due to metabolism of ethanol by alcohol dehydrogenases in the corpus luteum; these enzymes are more active at 37°C than at lower temperatures (Li, 1977). Alternatively, the presence of ethanol at 37°C may damage the membrane or binding site, and may solubilize the receptor from the bilayer (Bhalla and Reichert, 1974). At lower temperatures, a persistent response was observed, however higher concentrations of ethanol were required to maximally enhance LH
uptake. In addition, the concentration of alcohol which effectively
increased LH binding was a function of the length of the carbon chain of
the alcohol. These characteristics suggest that the increase in LH
binding may be related to the ability of alcohols to change the fluidity
of the cellular membrane (Goldstein and Chin, 1981a; Goldstein and Chin,
1981b; Sauerheber, Esgate, and Kuhn, 1982). Alcohols are membrane
fluidizers in many systems with the longer straight-chain alcohols more
potent agents (Zavoico and Kutchai, 1980, Lyon et al., 1981). Recently,
Dave and Witorsch (1983) reported that certain straight chain alcohols,
including ethanol, increased the number of prolactin binding sites and
the fluidity of mouse hepatic membranes. Other agents which are
reportedly effective in altering membrane fluidity have also been shown
to increase hormone binding in certain tissues (Muller and Shinitzky,
1979; Dave and Knazek, 1980; Dave, Brown, and Knazek, 1982; Neufield and
Cobro, 1982). Finally, the ability of ethanol to increase LH binding
was acutely sensitive to the temperature of the incubation. Temperature
also has marked effects on membrane fluidity (Quinn, 1981), e.g., as the
temperature is lowered membrane fluidity decreases. We hypothesize that
at lower temperatures, higher concentrations of ethanol are required to
overcome membrane order and elicit a maximal increase in gonadotropin
uptake.

The explanation for the inability of ethanol to unmask
gonadotropin binding sites in the rat corpus luteum, as opposed to the
monkey corpus luteum, is unknown. Cameron and Stouffer (1982c) reported
a similar lack of effect of ethanol on $^{125}$I-hLH binding to porcine
luteal membranes. This may be related to different physical and
chemical properties of these luteal membranes as compared to the monkey, such that ethanol may be ineffective in altering the membrane enough to expose masked sites. Although monkeys were anesthetized during luteectomy and rats were sacrificed by decapitation, it is unlikely that the effects of ethanol are due to the presence of anesthetics, since enhanced LH binding in response to ethanol was not observed in rats anesthetized prior to sacrifice (Danforth and Stouffer, unpublished data). Alternatively, this disparity may be due to a difference in physiologic state between the rat corpus luteum of pseudopregnancy and the macaque corpus luteum of the menstrual cycle. Finally, the rat corpus luteum may not contain a population of masked binding sites that can be exposed by ethanol.

There are several reports that neuraminidase increases gonadotropin binding to target tissues, including the corpus luteum of the cow (Azhar and Mennon, 1981), and the human (Rajaniemi et al., 1981). In the current study, neuraminidase increased LH binding to both macaque and rat corpora lutea; this effect was due to an increase in the number of LH binding sites rather than to any change in affinity. The data indicate that there may be masked gonadotropin binding sites in both species which can be exposed by neuraminidase. These findings also suggest that sialic acids are important in the interaction of gonadotropin with its binding site in the membrane. Carbohydrate moieties on the receptor or on neighboring phospholipids may sterically modulate the availability of LH/CG binding sites. Alternatively, removal of sialic acids in the vicinity of the gonadotropin receptor may
alter the charge in that region of the membrane which might affect hormone-receptor interaction (Kohn et al., 1982).

Ethanol and neuraminidase unmasked two populations of gonadotropin binding sites in the luteal membrane which were indistinguishable from available receptors in terms of affinity. However, the question remains whether these binding sites constitute functional gonadotropin receptors, i.e., that they can they stimulate adenylate cyclase or increase steroid production (Niswender et al., 1980). Huhtaniemi and colleagues (1978) suggest that in the rat, cryptic gonadotropin binding sites which are exposed by exogenous LH injections are not coupled to adenylate cyclase and thus do not constitute functional gonadotropin receptors. In their studies, no increase in maximal cAMP production was detected and no major changes in the sensitivity of testosterone production to hCG was observed. It is possible that masked binding sites represent a form of receptor precursor or receptor metabolite (Deutsch et al., 1983) that have retained the capacity to bind gonadotropin but are not capable of transmitting that signal into a biological response. In contrast, Abramowitz and Birnbaumer (1979) reported that incubation of rat and rabbit luteal membranes with 1-10% ethanol increased gonadotropin stimulated adenylate cyclase activity up to 7-fold. In addition, a preliminary report by Rojas and Asch (1984) suggests that ethanol increases gonadotropin-stimulated adenylate cyclase activity in the human corpus luteum as well. Although a direct effect of ethanol on the adenylate cyclase enzyme or on the coupling of receptor with enzyme cannot be ruled out, these studies suggest that ethanol unMASKS a
population of LH receptors which are capable of stimulating adenylate cyclase.

There is some evidence that certain hormone binding sites may be unmasked in vivo. Ucer and Engel (1981) reported that endogenous neuraminidase activity is present by day one post partum in the rat testis, however enzyme activity is not expressed until day 10 in the ovary. Importantly, the expression of neuraminidase activity coincides with the appearance of gonadotropin receptors in the gonads. In addition, binding sites in several tissues can be unmasked following exposure to homologous hormone (Hsueh, Dufau, and Catt, 1977; Huhtaniemi, Martikainen, and Tikkala, 1978; Chan and Davies, 1979; Suter et al., 1980). Other endogenous substances such as prostaglandins (Dave and Knazek, 1980) and certain phospholipases (Cuatrecasas, 1971; Dave, Knazek, and Liu, 1981) also unmask hormone binding sites in target tissues, possibly by changing the fluidity of cellular membranes. Thus, target tissues may possess several different mechanisms for altering the number of available binding sites for a hormone.

The importance of masked receptors in the regulation of corpus luteum function remains to be elucidated. The masking or unmasking of gonadotropin binding sites at different stages of the luteal lifespan may alter the sensitivity of the corpus luteum to the low levels of circulating gonadotropin that are present during the luteal phase of the menstrual cycle. Indeed, the changes in the number of available gonadotropin binding sites that occur during the luteal phase of the menstrual cycle (Cameron and Stouffer, 1982b) may be related in part to the masking/unmasking of gonadotropin receptors. Changes in available
receptors may also be important in regulating the response of the corpus luteum to hCG during early pregnancy (Ottobre, Ottobre, and Stouffer, 1984), and as such could involve the masking/unmasking of gonadotropin receptors.

In summary, the corpus luteum of the rhesus monkey and the psuedopregnant rat contain a population of masked gonadotropin binding sites which can be exposed by \textit{in vitro} treatment with the enzyme neuraminidase. In addition, the macaque corpus luteum contains a second population of masked sites which can be exposed upon addition of alcohol to the membranes. Whereas the affinities of masked and available gonadotropin binding sites are similar, the functionality and importance of masked sites to corpus luteum regulation remains to be determined.
CHAPTER 5

MODULATION OF MEMBRANE FLUIDITY IN THE PRIMATE (MACACA MULATTA) CORPUS LUTEUM: CORRELATION WITH CHANGES IN GONADOTROPIN BINDING

Abstract

Addition of alcohols to particulate or cellular preparations of the monkey corpus luteum unmasks gonadotropin binding sites via a temperature-sensitive process. Since alcohols and temperature are known modulators of membrane fluidity, we measured the fluidity of luteal membranes and determined whether the effects of ethanol and temperature on gonadotropin binding correlated with changes in the fluid state of the membrane. The fluidity of membranes from the macaque and rat corpus luteum was estimated from the fluorescence polarization of the lipophilic membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). The absorption and emission spectra of DPH incorporated into luteal membranes were typical of those in other systems. Fluorescence intensity increased rapidly during the first 60 min of incubation and reached steady-state conditions within 3h. In contrast, polarization was constant within minutes and was insensitive to pH, ionic strength, tissue concentration or DPH levels over the ranges tested. Fluorescence polarization was acutely sensitive to the temperature of the assay medium; polarization decreased as temperature increased from 4-50°C and no phase transitions were observed. Addition of 4-20% and 8-20% ethanol to monkey and rat membranes, respectively, decreased (p<0.05)
polarization relative to control. However, ethanol was less effective on rat membranes, such that 20% ethanol was required to elicit a similar change in polarization as 8% ethanol in macaque membranes. The decrease in polarization was reversed to control levels when ethanol was removed from the incubation medium. Changes in fluorescence polarization of DPH-labeled macaque membranes elicited by ethanol and temperature correlated significantly ($r=-0.97$) with changes in specific $^{125}$I-hLH binding. In contrast, pretreatment of luteal membranes from the monkey and the rat with neuraminidase, which unmasks another population of LH binding sites in both species, did not alter polarization.

We conclude that the fluorescence polarization of DPH is a useful tool for estimating membrane fluidity in the corpus luteum. Furthermore, changes in membrane fluidity may play an important role in the masking/unmasking of alcohol-sensitive (but not neuraminidase-sensitive) gonadotropin binding sites in the macaque corpus luteum. Finally, the lesser effects of ethanol in the rat suggest important species differences in the receptor milieu and composition of luteal membranes.

**Introduction**

The plasma membrane is an important locus for many aspects of cellular function; ion transport, enzyme activity, and hormone-receptor interaction are vital processes associated with the membrane. The fluid state of the plasma membrane (Cherry, 1976; Quinn, 1981), that is the ease with which lipid and protein molecules move about, may play a role in the regulation of many membrane processes. For example, aliphatic
alcohols are potent membrane fluidizers (Zavoico and Kutchai, 1980; Goldstein and Chin, 1981a,b; Lyon et al., 1981; Sauerheber, Esgate, and Kuhn, 1982) and reportedly modulate the number of available receptors for hormones, including prolactin (Dave and Witorsch, 1983), opioids (Levine, Hess, and Morley, 1981; Charness, Gorden, and Diamond, 1983), and chemotactic peptides (Liao, and Freer, 1980). Likewise, other agents which influence membrane fluidity such as cholesterol (Heron et al., 1980), and prostaglandins (Dave and Knazek, 1980) modulate the availability of hormone binding sites in test systems.

This laboratory recently reported that in vitro exposure to ethanol increased the number of available LH binding sites in particulate and cellular preparations of the monkey corpus luteum (Cameron and Stouffer, 1982c; Danforth and Stouffer, 1983), but not in luteal tissue from the rat (Danforth and Stouffer, 1983) or pig (Cameron and Stouffer, 1982c). Pretreatment of ovarian membranes from the monkey (Danforth and Stouffer, 1983), rat (Muller, Ucer, and Engel, 1983), and several other species (Muller, Ucer, and Engel, 1983; Rao, 1974; Azhar, and Mennon, 1981; Rajaniemi et al., 1981; Berman and Sairam, 1982) with neuraminidase to remove cell surface sialic acid also unmasks gonadotropin binding sites. The effects of neuraminidase treatment on membrane fluidity have not been reported. Indeed there have been few investigations of membrane fluidity in ovarian tissues (Buhr, Carlson, and Thompson, 1979; Carlson et al., 1981; Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982; Carlson, Buhr, and Riley, 1984) and the relationship between the fluid state and gonadotropin binding to membranes remains undetermined.
In the current study, we have characterized the fluidity of membranes prepared from the monkey and rat corpus luteum via the fluorescence polarization of the membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH). In addition, we have examined the effects of ethanol and neuraminidase treatment on fluorescence polarization in order to clarify the relationship between membrane fluidity and the availability of LH receptors in the corpus luteum.

**Materials and Methods**

**Source and Preparation of Luteal Tissue**

Adult female rhesus monkeys were housed in the Division of Animal Resources at the University of Arizona as described previously (Cameron and Stouffer, 1982a). We induced multiple ovulations in monkeys by using a regimen of human menopausal gonadotropin (HMG, Pergonal, Serono Laboratories Inc., Randolph, MA) followed by human chorionic gonadotropin, hCG (APL, Ayerst Laboratories Inc., New York, NY). Starting at menses, monkeys were injected intramuscularly with 18 or 37 I.U. of HMG, twice each day for 8-10 days to stimulate follicular growth. Monkeys were then injected with 1000 I.U. hCG to promote ovulation of the developed follicles. Using this regimen of gonadotropins (Danforth and Stouffer, 1984), approximately 3-5 corpora lutea were formed on each ovary. We removed the ovaries 6-7 days after hCG injection.

The freshly excised corpora lutea were weighed and minced. The minced tissue was homogenized in 0.05M Tris-HCl buffer, pH 7.4 with a glass homogenizer (Wheaton; Cameron and Stouffer, 1982a). We filtered
the homogenate through fine Japanese silk to remove tissue chunks and large debris, and then centrifuged it at 20000g for 15 minutes. The supernatant was discarded and the pellet was resuspended at a final concentration of 10 mg tissue equivalents / ml.

Prepubertal female rats (20-22 days old, Sprague Dawley, Animal Resources breeding colony) were superovulated with a subcutaneous injection of 50 I.U. pregnant mares serum gonadotropin (PMSG) followed 56 hours later by 50 I.U. hCG (APL). The rats were decapitated and their ovaries were removed 8-10 days after PMSG injection. We prepared particulates using the same methods as described for macaque corpora lutea.

**Fluorescence Polarization Studies**

A stock solution of 1mM 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich Chemical Company, Milwaukee, WI) in tetrahydrofuran was diluted 500-fold with 0.05M phosphate buffered saline, pH 7.4, and was stirred vigorously for 1 hour at 25°C (Shinitzky and Barenholz, 1978). We added 1 ml of the resulting suspension of DPH (2μM) to 0.5 ml of luteal membranes (10 mg/ml) and 0.5 ml of ethanol solution or buffer. The tubes were incubated in the dark for at least 3 hours. We examined various parameters of the assay system to determine the optimal conditions for measuring membrane fluidity. The system was characterized with respect to pH, ionic strength, tissue concentration, and DPH concentration. In addition, the effects of temperature on fluorescence polarization were examined. Membranes were either cooled to 4°C and then polarization measurements were taken every 2°C up to 50°C,
or first heated to 50°C and fluorescence polarization measured as the membranes cooled to 10°C.

Steady-state fluorescence polarization was performed according to the methods of Shinitzky and Barenholz (1978). Polarization measurements were performed with a Perkin-Elmer model MPF-2A fluorescence spectrophotometer fitted with a polarizer accessory. Samples were excited by continuous exposure to polarized monochromatic light (365 nm), and the emission intensities were read at 430 nm through polarizers oriented parallel and perpendicular to the plane of the excitation beam. We used a 390 nm cutoff filter in the emission path to remove scattered light from the tissue, which comprised less than 10% of the fluorescence in our system. Fluorescence polarization was calculated from the equation

\[ P = \frac{I_{vv} - I_{vh}(C)}{I_{vv} + I_{vh}(C)} \]

where \( I_{vv} \) and \( I_{vh} \) are the emission intensities measured through a polarizer oriented parallel and perpendicular to the plane of the excitation light, respectively. \( C \) is a correction factor which compensates for the unequal transmission of the components of the polarized light which arises from the dispersion of the beam as it passes through the emission monochromator grating (Vorbeck et al., 1982). A decrease in polarization of the probe corresponds to increased
movement in the lipid bilayer and can be equated to an increase in the fluidity or disorder of the lipid bilayer of the cell membranes.

Luteal particulates were incubated with DPH in the presence of 0-20% ethanol for at least three hours before fluorescence polarization measurements were taken. For the experiments with neuraminidase (NA, Sigma type V, Sigma Laboratories, St. Louis, MO), aliquots of luteal particulates (10 mg/ml) were incubated with 0-2.0 mg NA/ml for 30 minutes at 37°C. One ml ice-cold buffer was then added and the tubes were centrifuged at 5000g for 5 minutes. The supernatant was discarded and the membranes were washed again with buffer. The pellet was then resuspended in buffer at a final concentration of 10 mg/ml.

Differences among levels of fluorescence polarization during exposure to 0-20% ethanol and following pretreatment with 0-2.0 mg neuraminidase/ml were determined by a two-way analysis of variance for a randomized complete block design. Individual experiments were treated as blocks in these analyses. Following demonstration of significance (p<0.05) with an F test, comparisons were made between pairs of means using the Least Significant Difference Test (Snedecor and Cochran, 1967). Student's t-tests were used to examine possible differences in fluorescence polarization values between the monkey and the rat in response to ethanol treatment.

**Results**

The absorption and emission spectra of DPH-labeled rat luteal particulates are depicted in Figure 7. The absorption spectrum is
Figure 7. Absorption and emission spectra of DPH incorporated in membranes prepared from the rat corpus luteum. Luteal membranes (5.0 mg/ml) were incubated with DPH (1μM) for 3h at 25°C before fluorescence measurements were taken. Relative fluorescence is expressed in arbitrary units.
centered at approximately 365 nm and is well separated from the emission spectrum centered at 430 nm. These spectra are typical of those reported for DPH in other membrane systems (Shinitzky and Barenholz, 1974; Billard et al., 1983; Cranney et al., 1983). Figure 8 depicts the fluorescence intensity (8A) and fluorescence polarization (8B) of DPH as a function of time after addition to rat luteal membranes. Fluorescence intensity increased rapidly during the first 60 minutes of incubation indicating a progressive increase in DPH incorporation into the membrane. Fluorescence reached steady-state conditions within 2-3 hours of incubation. Fluorescence polarization appeared to decrease during the first 30 minutes of the incubation, and then remained constant for the remainder of the experiment. The initial decrease in polarization is likely an artifact due to the rapid increase in fluorescence intensity and our inability to measure $I_{vv}$ and $I_{vh}$ simultaneously with our instrumentation.

We assessed the effect of various parameters of the assay on steady-state polarization to determine the optimal conditions for subsequent experiments. Altering the amount of membranes in the system (0.01-25 mg tissue equivalents / ml) or changing the concentration of DPH in the incubation (0.01-100μM) had no effect on polarization. In addition, fluorescence polarization was insensitive to the pH (5.0-8.5) or ionic strength (0-500 mM phosphate-buffered saline) of the incubation. We used the following assay conditions for all subsequent studies; 1μM DPH incubated for at least 3 hours with 2.5 mg tissue equivalents /ml, in 50 mM phosphate buffered saline at pH 7.4.
Figure 8. Time dependence of fluorescence polarization of DPH incorporated into rat corpora lutea. Fluorescence intensity (A) and fluorescence polarization (B) were measured at time points of 4 minutes to 3.5 hours. Luteal membranes (2.5 mg/ml) were incubated with DPH (1μM) at 25°C for the various times indicated.
Due to the large amount of tissue obtained from the superovulated monkey and the pseudopregnant rat, some of the membranes were frozen at -70°C until used in an experiment. Previous studies from this laboratory indicated that freezing and thawing of luteal membranes had no deleterious effects on $^{125}$I-hLH binding or adenylate cyclase activity. Likewise, fluorescence polarization values obtained at 25°C from fresh (P=.232 +/- .007), vs frozen (P=.230 +/- .007) membranes were not different (P>0.1, n=3).

Fluorescence polarization was acutely dependent on the temperature of the assay medium. DPH-labeled membranes of both monkey and rat corpora lutea exhibited a decrease in polarization as temperature increased (Figure 9). Arrhenius plots of these temperature profiles were linear and no phase transitions were observed over the scanned temperature range. Under control conditions at 25°C, the fluorescence polarization of DPH was similar in both monkey (P=.225 +/- .010) and rat (P=.224 +/- .004) corpora lutea (p>0.1, n=4).

The effect of ethanol on the polarization of DPH incorporated into monkey and rat luteal membranes is depicted in Figure 10. As the concentration of ethanol in the incubation increased, fluorescence polarization decreased in both rat and monkey membranes. Addition of 4-20% ethanol to monkey luteal membranes, and 8-20% ethanol to rat luteal membranes significantly decreased polarization values relative to control (p<0.05, n=4). Ethanol was less effective on rat membranes, such that 20% ethanol was required to elicit a similar change in polarization as 8% ethanol did in the macaque membranes.
Figure 9. Effect of temperature on fluorescence of DPH in membrane preparations of monkey and rat corpora lutea. Luteal membranes from the monkey and the rat were incubated with DPH (1μM) for 3h at 25°C. Each value represents the mean of 3 observations.
Figure 10. Effect of ethanol on fluorescence polarization of DPH incorporated in monkey and rat luteal membranes. Luteal membranes (2.5 mg/ml) were incubated with DPH (1μM) and ethanol (0-20%) for 3h at 25C. Polarization is expressed as the difference between the value observed at the various alcohol concentrations and in the absence of ethanol. (mean +/- SEM, n=4).
The decrease in polarization upon addition of ethanol was reversed when ethanol was removed from the incubation media (Table 3). When we incubated monkey luteal particulates with 8% and 20% ethanol, the polarization of DPH incorporated in the membranes decreased (p<0.05) relative to control (0% ethanol). However, when the membranes were exposed to ethanol for 3h, and then the ethanol was removed prior to the addition of DPH, no change in the fluorescence polarization was observed (p>0.1).

Pretreatment of macaque luteal membranes with 0.01-2.0 mg neuraminidase / ml had no effect on the polarization of DPH-labeled membranes (p>0.1, data not shown), compared to untreated controls (P=0.247±0.017). The combination of 8% ethanol plus neuraminidase (P=0.216±0.013) decreased fluorescence polarization values to a similar extent as ethanol alone (P=0.218±0.015).

To determine if a relationship exists between the fluid state of the luteal membrane and the level of gonadotropin binding, we plotted (Figure 11) the polarization values obtained in the present study versus gonadotropin binding data obtained from a parallel series of experiments reported earlier (Danforth and Stouffer, 1984). As temperature decreased from 37°C to 4°C, membrane fluidity and gonadotropin binding both declined. As the concentration of ethanol in the incubation increased up to 8% ethanol, both membrane fluidity and LH uptake were enhanced. The level of gonadotropin binding (expressed as the percent of specific binding observed following 20h incubation at 25°C in the absence of ethanol, B₀) under these incubation conditions was highly
Table 3. Effects of ethanol and its removal on the fluorescence polarization of DPH incorporated in macaque luteal membranes.

<table>
<thead>
<tr>
<th>ETHANOL (vol/vol)</th>
<th>FLUORESCENCE POLARIZATION</th>
<th>Ethanol present&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ethanol removed&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td></td>
<td>0.280 +/- 0.008</td>
<td>0.280 +/- 0.008</td>
</tr>
<tr>
<td>8%</td>
<td></td>
<td>0.256 +/- 0.005*</td>
<td>0.286 +/- 0.003</td>
</tr>
<tr>
<td>20%</td>
<td></td>
<td>0.226 +/- 0.003*</td>
<td>0.275 +/- 0.004</td>
</tr>
</tbody>
</table>

<sup>a</sup> Membranes were incubated with DPH in the presence or absence of ethanol.

<sup>b</sup> Membranes were incubated w/wo ethanol for 3h, washed twice, and then incubated with DPH for 3h.

* Values are significantly different from control (p<0.05)
Each value is the mean +/- SEM of 3 observations.
Figure 11. Correlation of specific $^{125}$I-hLH binding with the fluorescence polarization of DPH in macaque luteal membranes. Binding is expressed as the percent of LH uptake at 25°C in the absence of ethanol ($B_0$). Fluorescence polarization and steady-state gonadotropin binding were measured at 4°C, 25°C, and 37°C, and in the presence of 0%, 1%, 4%, and 8% Ethanol. Each value represents the mean of at least 3 experiments.
correlated with the fluorescence polarization of DPH-labeled luteal membranes (r=0.979, p<0.01, n=16).

**Discussion**

This study constitutes the first characterization of membrane fluidity in the primate corpus luteum. The data suggest that the fluid state of the lipid bilayer modulates the interaction of gonadotropin with specific binding sites in the luteal membrane and as such, may play an important role in the masking/unmasking of gonadotropin binding sites within the primate corpus luteum. In addition, there may be important species differences in the receptor population or membrane composition of the rat versus the macaque corpus luteum which could affect luteal function.

The initial characterization experiments indicate that the DPH incorporated in luteal membranes responds as predicted from other membrane systems. Fluorescence intensity increased as more probe molecules became incorporated into the membrane, whereas fluorescence polarization generally remained constant over this period. Fluorescence polarization was independent of the probe concentration, tissue concentration, and duration of the incubation. The fluorescence polarization of DPH was sensitive to the effects of ethanol and temperature which are reported to be modulators of membrane fluidity (Shinitzky and Barenholz, 1978). Thus, the fluorescence polarization of DPH appears to be a useful tool for measuring membrane fluidity in the corpus luteum. Fluorescent probes such as DPH and trans-parinaric acid have been used by Carlson and coworkers (1984) to monitor membrane
fluidity in the rat corpus luteum. It is important to note that polarization values obtained with DPH reflect an overall average of the fluidity of the phospholipid bilayer, and may not reflect changes within localized regions of the membrane. In addition, we are estimating in these initial studies, the fluidity of all of the various membranes in a particulate preparation of the corpus luteum. Therefore, selective perturbations of individual components such as the plasma membrane might not be detected in this system.

Ethanol was highly effective in increasing the fluidity of the primate luteal membrane. These data are in agreement with many other systems (Zavoico and Kutchai, 1980; Goldstein and Chin, 1981a,b; Lyon et al., 1981; Sauerheber, Esgate, and Kuhn, 1982). In addition, the increase in membrane fluidity in the presence of ethanol was highly correlated with the increase in gonadotropin binding observed under these conditions. Other investigators have shown that ethanol increases the number of binding sites for hormones including prolactin (Dave and Witorsch, 1983), opioids (Levine, Hess, and Morley, 1981; Charness, Gorden, and Diamond, 1983), and chemotactic peptides (Liao, and Freer, 1980). The data suggest that ethanol unmasks gonadotropin binding sites in the macaque corpus luteum by increasing the fluidity of the luteal membrane. An increase in the fluid state of the lipid bilayer could lead to increased exposure of integral proteins within the membrane. Alternatively, ethanol may have a direct effect on the gonadotropin binding site, or on the lipid annulus immediately surrounding the protein. Since the effect of 8% and 20% ethanol on membrane fluidity was a reversible phenomenon, it appears that ethanol at these
concentrations does not permanently alter the region of the membrane monitored by DPH. The unmasking of gonadotropin binding sites in the macaque corpus luteum by 8% ethanol was also reversed when the ethanol was removed from the membrane (Danforth and Stouffer, 1984). However, the decrease in LH uptake observed in the presence of 20% ethanol was irreversible (Danforth and Stouffer, 1984), suggesting that at higher concentrations, ethanol caused a permanent alteration in the interaction of gonadotropin with its binding site without causing major damage to the lipid bilayer. These high concentrations of ethanol may be denaturing membrane proteins or actually solubilizing the receptor proteins from the membrane (Bhalla and Reichert, 1974).

Ethanol was less effective in increasing the fluidity of rat luteal membranes as compared to membranes prepared from the monkey corpus luteum. In addition, ethanol was ineffective in unmasking gonadotropin binding sites in the rat corpus luteum (Danforth and Stouffer, 1984). The different responses to ethanol, in terms of membrane fluidity and gonadotropin binding may reflect important species differences in the receptor population (Cameron and Stouffer, 1981) or membrane composition (Carlson et al., 1981; Goodsaid-Zalduondo et al., 1982) between the macaque and the rat corpus luteum. Chin and Goldstein (1984) reported that mouse brain synaptosomal membranes enriched in cholesterol were more resistant to the disordering effect of ethanol than control membranes. Thus, the rat luteal membrane may contain a higher cholesterol content thereby decreasing its sensitivity to the disordering effects of ethanol. There may also be other species differences in the composition or receptor population of the macaque vs
the rat luteal membrane. Finally, the corpus luteum of the pseudopregnant rat may be at a different stage in its functional lifespan than the corpus luteum of the superovulated monkey. If the different response to ethanol is due to a physiological rather than species difference, this would suggest that membrane fluidity changes during the functional lifespan of the corpus luteum.

Alterations of membrane fluidity may play an important role in regulating the function and lifespan of the corpus luteum. Carlson and colleagues have examined membrane fluidity of the rat (Buhr, Carlson, and Thompson, 1979; Carlson et al., 1981; Carlson, Buhr, and Riley, 1984), and bovine (Carlson et al., 1982; Goodsaid-Zalduondo et al., 1982), corpus luteum. They demonstrated that membranes prepared from regressing tissues contained more gel-phase lipid (Buhr, Carlson, and Thompson, 1979), had higher transition temperatures (Carlson et al., 1982), and were less fluid (Carlson, Buhr, and Riley, 1984) than membranes prepared from mid-luteal phase corpora lutea. Such changes in membrane fluidity could alter the response of the luteal cell to circulating hormones by increasing or decreasing the number of available binding sites for gonadotropin, by altering enzyme activity within the corpus luteum, or by affecting the coupling of receptor with adenylate cyclase. Indeed, both receptor number (Cameron and Stouffer, 1982b), and adenylate cyclase activity (Eyster, Ottobre, and Stouffer, 1984c) diminish around the onset of luteolysis, at a time when membrane fluidity may be decreasing in these tissues. However, there have been no direct studies relating hormone-receptor interaction or adenylate cyclase activity to changes in membrane fluidity during the lifespan of
the corpus luteum. Moreover, the factors responsible for the changes in membrane fluidity throughout the luteal phase have not been reported. Prostaglandins, which have marked effects on various aspects of luteal function, including gonadotropin binding (Cameron and Stouffer, 1982b; Sotrel et al., 1981), adenylate cyclase activity (Behrman, Ng, Orczyk, 1974), and steroid production (Stouffer, Nixon, and Hodgen, 1979; Ellinwood, Nett, and Niswender, 1978), have also been reported to increase membrane fluidity in some tissues (Dave and Knazek, 1980). Thus, some of the luteolytic actions of prostaglandins may be mediated through changes in membrane fluidity.

Whereas ethanol was highly effective in increasing membrane fluidity in the macaque corpus luteum, neuraminidase, which also unmasks gonadotropin binding sites in this species (Danforth and Stouffer, 1984), had no apparent effect on membrane fluidity. These data agree with the hypothesis that ethanol and neuraminidase act on the luteal membrane by different mechanisms, and support the idea that two different populations of masked gonadotropin binding sites exist within the primate corpus luteum (Danforth and Stouffer, 1984); one that is exposed by increasing the fluidity of the membrane, and another population which is unmasked by removal of sialic acid residues. However, it is not known whether these sites exist in the plasma membrane or in other intracellular membranes.

In summary, the fluorescence polarization of DPH appears to be a sensitive tool to examine the fluid state of luteal membranes prepared from the rhesus monkey and pseudopregnant rat. In addition, membrane fluidity may be an important modulator of the interaction of
gonadotropin with its binding site in the luteal membrane, and possibly in the masking/unmasking of gonadotropin binding sites within the primate corpus luteum.


CHAPTER 6

[\textsuperscript{125}\textit{I}]LH BINDING TO DETERGENT-SOLUBILIZED RECEPTORS FROM THE PRIMATE (MACACA MULATTA) CORPUS LUTEUM: EFFECTS OF ETHANOL EXPOSURE

Abstract

\textit{In vitro} exposure to alcohols unmasks gonadotropin binding sites in the corpus luteum of the rhesus monkey, possibly by altering the fluidity of the lipid bilayer of the luteal membrane. To examine this hypothesis, we solubilized gonadotropin receptors from the luteal membrane and compared the effects of ethanol on soluble receptors to those on receptors associated with the lipid bilayer. In the monkey, solubilization with 1\% Triton X-100 for 30 min at 4\textdegree C, followed by precipitation of the hormone-receptor complex with polyethylene glycol, resulted in the apparent recovery of 50\% more gonadotropin binding sites than are available in particulate preparations (p<0.05); these sites displayed lower affinity for labelled gonadotropin (p<0.05). Solubilized gonadotropin binding sites from rat luteal particulates also displayed reduced affinity as compared to particulates (p<0.05), however no increase in the binding capacity of the tissue was observed. At equilibrium, gonadotropin binding to soluble receptors from the macaque corpus luteum was 50\% higher at 25\textdegree C than at 4\textdegree C. In contrast, binding at 25 and 4\textdegree C was similar in receptors solubilized from the rat corpus luteum. Conditions which increase gonadotropin binding to macaque luteal membranes, 1-8\% ethanol at 25\textdegree C, decreased LH uptake by soluble
gonadotropin receptors. In contrast, at 4°C the presence of 1-20% ethanol markedly increased LH uptake by soluble receptors; binding approached steady-state levels present at 25°C.

The data suggest that gonadotropin binding sites of the rat and monkey corpus luteum can be effectively solubilized with Triton X-100. Indeed, solubilization results in an apparent increase in the number of available binding sites in the macaque corpus luteum. In addition, the differences in affinity and responses to ethanol between particulate and soluble receptors suggests that the interaction of the gonadotropin receptor with the lipid environment of the luteal membrane appears to be an important factor in modulating the availability and affinity of gonadotropin receptors.

**Introduction**

Cameron and Stouffer (1981, 1982a,b) have recently characterized the interaction of the gonadotropins, LH and CG, with particulate preparations of the macaque corpus luteum. Their studies indicated that the monkey corpus luteum contains a single class of binding sites which display specificity and equally high affinity for primate gonadotropins of pituitary (LH) and placental (CG) origin. In addition, in vitro studies in this laboratory (Cameron and Stouffer, 1982c; Danforth and Stouffer, 1984) suggest that gonadotropin binding sites exist within the macaque luteal membrane which are unable to interact with circulating hormone. One population of masked or cryptic sites can be exposed by pretreatment of luteal membranes/cells with the enzyme neuraminidase, and a second population is unmasked by addition of alcohol. We hypothesize that the unmasking of gonadotropin binding sites by alcohols
may be related to their ability to change the fluidity of the lipid bilayer of the luteal membrane (Danforth and Stouffer, 1984; Danforth, Wells, and Stouffer, 1984). Extraction of the gonadotropin receptor from the luteal membrane might be a useful approach to examine this hypothesis since the effects of ethanol on soluble receptors could be compared to those on receptors associated with the lipid bilayer.

Gonadotropin receptors in the rat ovary and testis can be solubilized by extraction with nonionic detergents such as Lubrol PX (Charreau, Dufau, and Catt, 1974; Dufau, Podesta, and Catt, 1975) or Triton X-100 (Dufau et al., 1973; Dufau and Catt, 1973; Dufau et al., 1974; Conti et al., 1978). These binding sites retain hormone specificity and have been used to gain valuable information on the physical characteristics of the receptor moiety (Dufau and Catt, 1976). However, solubilization often results in a loss of as much as 50% of gonadotropin binding activity (Dufau and Catt, 1973; Dufau, Charreau, and Catt, 1973), which may be due to a decrease in affinity (Dufau and Catt, 1973) or in the number of binding sites (Catt and Dufau, 1973; Ascoli, 1983) in solubilized preparations. Solubilization of gonadotropin receptors from the primate corpus luteum has not been reported.

In the present study we have examined the characteristics of gonadotropin binding to soluble receptors of the macaque and rat corpus luteum. In addition, we have investigated the effect of ethanol on LH binding to soluble receptors in a further attempt to elucidate the mechanism of action of ethanol in the unmasking of gonadotropin receptors in the primate corpus luteum.
Materials and Methods

Source and Preparation of Luteal Tissue

Corpora lutea were obtained from adult female rhesus monkeys on days 21-24 of the menstrual cycle, approximately 10 days after the midcycle LH surge (Cameron and Stouffer, 1982a). For some experiments, we induced multiple ovulations in monkeys by using a regimen of human menopausal gonadotropins (hMG, Pergonal, Serono Laboratories Inc., Randolph, MA) followed by hCG (APL, Ayerst Laboratories, New York, NY). Monkeys were injected im with 37 I.U. hMG twice per day for 8-10 days; then 1000 I.U. hCG was injected to promote ovulation and luteinization of the developed follicles. Typically, 3-5 corpora lutea formed on each ovary. The corpora lutea were removed 6-7 days after hCG injection.

Prepubertal rats (20-22 days old, Sprague Dawley, Animal Resources Breeding Colony) were injected with 50 I.U. pregnant mares serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO) sc, followed 56h later by 50 I.U. hCG (APL) to induce multiple ovulations and corpora lutea (Birnbaumer et al., 1976). The rats were decapitated and their ovaries were removed 8-10 days after PMSG injection.

In general, experiments were performed on fresh tissue. Due to the large amount of tissue obtained from the pseudopregnant rat and superovulated monkey, we performed some experiments on tissue frozen at -70C prior to use. Freezing and storing the tissue in this manner did not alter gonadotropin binding.
125I-hLH Binding to Luteal Particulates.

The freshly excised corpus luteum (or luteinized ovary) was freed from extraneous tissue, weighed, and minced into small pieces. Luteal particulates were prepared according to the procedures of Cameron and Stouffer (1982a). Briefly, we homogenized the minced tissue in 0.05M Tris-HCl buffer (pH 7.4, containing 5 mM MgCl2, 0.15M NaN3, and 8% sucrose) with a hand-held glass (Dounce) homogenizer, and then filtered it through fine Japanese silk to remove large debris. The homogenate was centrifuged at 20000g for 15 minutes and the resulting pellet was used for gonadotropin binding studies.

Purified hLH (NIH-I-1, National Pituitary Agency) was radiolabeled with 125I by the lactoperoxidase method (Cameron and Stouffer, 1982a). Luteal particulates were incubated with various concentrations of 125I-hLH for 20h in a gyrotory shaker bath. Incubations were carried out in Tris-HCl buffer in a final volume of 0.7ml. After incubation, 1 ml ice-cold Tris-HCl buffer was added to each sample, and the 125I-hLH bound to the luteal tissue was separated from free hormone by filtration through Metricel membrane filters (pore size=0.45μm) presoaked in buffer containing 2% bovine serum albumin (BSA). We rinsed the incubation tubes with 2 ml buffer and the filters with an additional 5 ml buffer. The amount of radioactivity remaining on the filter was determined in a Searle 1197 automatic gamma counter. Non-specific binding of 125I-hLH was assessed by coincubating samples with a 100-fold excess of unlabeled gonadotropin (Ayerst APL). Specific binding was calculated by subtracting non-specific uptake from the total amount of 125I-hLH bound. Steady-state 125I-hLH binding was analyzed by
the method of Scatchard (1949) to determine the maximum binding capacity (Bm) of the tissue and the affinity (equilibrium dissociation constant, Kd) of the binding sites for LH.

\[ ^{125}\text{I}}\text{-hLH Binding to Solubilized Gonadotropin Receptors } \]

We followed the general procedures of Dufau and colleagues (1973) to assess gonadotropin binding to solubilized binding sites. Luteal particulates were resuspended in the presence of 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 4°C. After dilution to 0.05% Triton with 0.05M Tris-HCl buffer, the solution was centrifuged at 50,000 x g for 2h to remove unsolubilized debris. The resulting supernatant was used for gonadotropin binding studies. Under these conditions less than 10% of gonadotropin binding activity remained in the precipitate. In initial experiments, we determined that these conditions (solubilization with 1% Triton followed by dilution to 0.05% Triton) were optimal for recovery of gonadotropin binding activity.

Soluble receptor preparations (2.5 mg tissue equivalents/ml) were incubated with various concentrations of \(^{125}\text{I}}\text{-hLH for up to 32h. Incubations were performed in Tris-HCl buffer in a final volume of 0.7 ml. Receptor-bound gonadotropin was separated from free hormone by polyethylene glycol (PEG, Sigma) precipitation (Dufau, Charreau, and Catt, 1973). After incubation with labeled gonadotropin, 1 mg bovine gamma globulins (0.2 ml of a 5 mg/ml solution) was added to the assay tubes, followed by 0.5 ml of 30% PEG. The tubes were incubated at 4°C for 10 min before centrifugation at 1500g for 10 min. The supernatant was decanted and 0.9 ml of 0.05% Triton was added to the pellet. After
10 min, 0.5 ml 30% PEG was added and the tubes were incubated for 10 minutes prior to centrifugation at 1500g for 10 min. The supernatant was discarded and the amount of radioactivity remaining in the pellet was determined.

Statistical Analyses

Student's t-tests were used to examine possible changes in numbers and affinities of LH binding sites in particulate vs soluble gonadotropin receptor preparations. Differences among levels of gonadotropin binding during exposure to 0-20% ethanol were determined by a two-way analysis of variance for a randomized complete block design. Individual corpora lutea were treated as blocks in these analyses. Following demonstration of significance (p<0.05) with an F test, comparisons were made between pairs of means using the Least Significant Difference Test (Snedcor and Cochran, 1967).

Results

Scatchard analyses of 125I-hLH binding to preparations of soluble receptors and particulates from the rat corpus luteum yielded linear plots (Figure 12). The maximum binding capacity was similar in soluble (Bm=55.5 ± 9.0 fmol/mg equivalent tissue weight) and particulate (53.2 ± 10.3 fmol/mg, p>0.05, n=3) preparations. However, the apparent affinity of the hormone-receptor interaction was lower for soluble receptors (Kd=13.8 ± 3.0 x 10^{-10}M) as compared to particulates (Kd=5.7 ± 1.1 x 10^{-10}M, p<0.05).

In contrast, solubilization of gonadotropin receptors from the macaque corpus luteum increased the number of available binding sites
RAT

- PARTICULATE RECEPTOR
  \[ K_d = 5.7 \pm 1.1 \times 10^{-10} \text{M} \]
  \[ B_m = 53.2 \pm 10.3 \text{ fmol/mg} \]

- SOLUBLE RECEPTOR
  \[ K_d = 13.8 \pm 3.0 \times 10^{-10} \text{M} \]
  \[ B_m = 55.5 \pm 9.0 \text{ fmol/mg} \]

Figure 12. Composite Scatchard plots of specific ¹²⁵I-hLH binding to preparations of soluble receptors and particulates of the rat corpus luteum. Soluble and particulate preparations (2.5 mg tissue equivalents/ml) were incubated with 0.5-25 ng ¹²⁵I-hLH for 20h at 25°C to achieve steady-state conditions. Each point represents the mean of 3 experiments.
above that observed in particulates (Figure 13). The maximum binding capacity of the solubilized receptors was $8.2 \pm 1.4 \text{ fmol/mg}$ vs $5.6 \pm 1.0 \text{ fmol/mg}$ for particulates ($p<0.05$, $n=6$). In addition, the apparent affinity of the soluble receptor-hormone interaction was lower than that for particulates ($K_d=8.0 \pm 1.8 \times 10^{-10} \text{ M}$ vs $2.8 \pm 0.3 \times 10^{-10} \text{ M}$, respectively, $p<0.05$).

The effect of ethanol on $^{125}$I-hLH binding to soluble receptors prepared from the macaque corpus luteum is depicted in Figure 14. At $25^\circ\text{C}$, the presence of 1-20% ethanol decreased LH uptake relative to control levels, with a maximal effect at 20% ethanol. Lower levels of ethanol (0.1-0.5%) had no effect on LH binding. In contrast, at $4^\circ\text{C}$, the presence of 1-20% ethanol markedly increased gonadotropin binding relative to control, with a maximal effect at 12% ethanol ($p<0.05$, $n=3$). Importantly, under control conditions the level of gonadotropin binding at $25^\circ\text{C}$ was higher than at $4^\circ\text{C}$. The presence of ethanol at $4^\circ\text{C}$ increased LH uptake to levels present at $25^\circ\text{C}$ under control conditions. The presence of 1-20% ethanol at $4^\circ\text{C}$ also increased LH binding to soluble receptors prepared from the rat corpus luteum (data not shown).

To investigate further the importance of temperature on the effect of ethanol, we examined the kinetics of LH binding to soluble receptors of the macaque corpus luteum at $25^\circ\text{C}$ and $4^\circ\text{C}$ in the presence and absence of ethanol (Figure 15). Under control conditions at $4^\circ\text{C}$, LH binding reached steady-state levels within 20h (Figure 15A). In the presence of 20% ethanol binding at $4^\circ\text{C}$ was greater than control within 30 minutes and remained elevated throughout the entire incubation. At $25^\circ\text{C}$ (Figure 15B), binding under control conditions increased rapidly and
Figure 13. Composite Scatchard plots of specific $^{125}$I-hLH binding to soluble and particulate receptors from the macaque corpus luteum. Incubations were carried out as described in Figure 12. Each point represents the mean of 6 experiments.
Figure 14. Effects of increasing concentrations of ethanol on specific $^{125}$I-hLH binding to soluble receptors from the monkey corpus luteum at 4C and 25C. Soluble receptor preparations (2.5 mg tissue equivalents/ml) were incubated with 3 ng $^{125}$I-hLH and 0-20% ethanol for 20h. Binding is expressed as % of control uptake (0% ethanol). Each bar represents the mean ± SEM of 3 experiments.
Figure 15. Kinetic analysis of specific $^{125}\text{I}$-hLH binding to soluble receptor preparations of the macaque corpus luteum at 4°C (Figure 15A) and 25°C (Figure 15B). Soluble receptors (2.5 mg tissue equivalents/ml) were incubated with 3 ng $^{125}\text{I}$-hLH and 0, 8, or 20% ethanol for time periods of 30 minutes to 20h. Each point represents the mean of 3 observations.
reached steady-state levels within 8h. In the presence of 8% ethanol, binding was depressed below control within 30 minutes and continued to decline throughout the remainder of the experiment. At 37°C (data not shown), LH uptake increased during the first 30 minutes and then steadily declined throughout the remainder of the incubation, such that by 20h binding at 37°C was approximately 50% of that present at 4°C.

The kinetics of gonadotropin binding to soluble receptors prepared from the rat corpus luteum are depicted in Figure 16. Gonadotropin uptake was rapid at 25°C, and reached steady-state levels within 4h. At 4°C, uptake proceeded more slowly; steady-state conditions were reached at approximately 20h. At 37°C, LH binding increased during the first 30 minutes but remained below the levels seen at 25°C and 4°C throughout the incubation. Interestingly, the maximal level of gonadotropin binding at 25°C and 4°C was similar, which is in contrast to the effect observed in the monkey where steady-state binding at 4°C was less than 50% of that at 25°C.

**Discussion**

These studies indicate that gonadotropin binding sites in the corpus luteum of the rhesus monkey can be effectively solubilized with the nonionic detergent Triton X-100. Solubilization results in the apparent recovery of more gonadotropin binding sites than are available in particulate preparations of the macaque corpus luteum; these solubilized sites display a lower affinity for labelled gonadotropin. In addition, the presence of 1-8% ethanol at 25°C, a condition which markedly increased gonadotropin binding to macaque luteal particulates
Figure 16. Kinetic analysis of specific $^{125}$I-hLH binding to soluble receptor preparations of the rat corpus luteum at 4C, 25C, and 37C. Soluble receptors (2.5 mg tissue equivalents/ml) were incubated with 3 ng $^{125}$I-hLH for time periods of 30 minutes to 20h. Each point represents the mean of 3 observations.
(Cameron and Stouffer, 1982c; Danforth and Stouffer, 1984), decreased LH uptake by soluble gonadotropin receptors. The data suggest the interaction of the gonadotropin receptor with the lipid environment of the luteal membrane may modulate the availability and affinity of gonadotropin with its receptor in the corpus luteum.

Solubilization of gonadotropin binding sites in the macaque corpus luteum results in a single class of binding sites with lower apparent affinity for LH than membrane-bound receptors. A similar decrease in affinity has been observed in detergent-solubilized receptors of the rat ovary (Dufau et al., 1974; present study), and testes (Dufau and Catt, 1973), but not in receptors solubilized from the bovine corpus luteum by sonication (Sebokova and Kolena, 1984). The decrease in affinity may be due to removal of the receptor from the membrane environment, thus altering the structure or conformation of the protein (Dufau, Charreau, and Catt, 1973). Interaction of the receptor protein with detergent molecules may also affect the affinity of the hormone-receptor interaction, however water-soluble receptors of the luteinized rat ovary also display decreased affinity as compared to particulate receptors (Wimalasena and Dufau, 1982). The apparent reduction in affinity could also be due to increased degradation of gonadotropin (Lee and Ryan, 1973) by solubilized enzyme systems which would be more apparent at lower levels of bound gonadotropin.

Interestingly, solubilization of gonadotropin binding sites from the macaque corpus luteum results in an apparent increase in the total number of binding sites available to bind gonadotropin. This is in contrast with several reports that solubilization decreases the binding
activity of gonadotropin receptors by as much as 50% (Catt and Dufau, 1973; Dufau and Catt, 1973; Dufau, Charreau, and Catt, 1973; Ascoli, 1983). However, estimation of the maximum binding capacity and affinity of soluble receptors were not performed in many of these earlier studies, and the decrease in gonadotropin binding activity could be due to the decreased affinity of the soluble receptor preparation. Indeed, in the present study, the levels of gonadotropin binding at a given hormone concentration were often lower for soluble receptors as compared to particulates, due to the pronounced decrease in affinity.

The apparent increase in gonadotropin binding sites after solubilization suggest that extra gonadotropin receptors exist in the macaque luteal membrane that are unavailable to interact with circulating gonadotropin. Indeed, previous studies from our laboratory (Cameron and Stouffer, 1982c; Danforth and Stouffer, 1984) indicate that masked gonadotropin binding sites are present in the luteal membrane of the rhesus monkey. These sites can be exposed by in vitro treatment of luteal particulates with alcohols. A second population of gonadotropin binding sites in the corpus luteum of both the rat and monkey can be exposed by preincubation of luteal membranes with the enzyme neuraminidase, which removes cell membrane sialic acids. Since solubilization does not increase the number of binding sites in the rat corpus luteum, we hypothesize that in the monkey, the apparent increase in gonadotropin binding sites after solubilization is due to the exposure of "alcohol sensitive sites" (Danforth and Stouffer, 1984) in the luteal membrane.
The decrease in LH uptake by solubilized receptors in the presence of ethanol at 25°C is in contrast to the effects seen in particulates and whole cells under these conditions (Danforth and Stouffer, 1984). Incubation of luteal particulates with 1-8% ethanol unmasks a population of gonadotropin binding sites in the macaque corpus luteum which are indistinguishable in terms of affinity from available receptors (Danforth and Stouffer, 1984). The unmasking of gonadotropin binding sites in the macaque corpus luteum may be due to changes in the fluidity of the luteal membrane (Danforth and Stouffer, 1984; Danforth, Wells, and Stouffer, 1984). The inability of ethanol to increase LH binding to receptors solubilized from the membrane is consistent with this hypothesis.

In contrast, at 4°C ethanol markedly increased LH binding to both particulate (Danforth and Stouffer, 1984), and soluble receptors of the macaque corpus luteum. The increase in binding under these conditions cannot be explained solely by an increase in membrane fluidity, as solubilized receptors have been removed from the overall membrane environment. Upon solubilization however, many membrane proteins are still associated with a small amount of membrane lipid (Dufau et al., 1973), and these may have important effects on protein function. Ethanol may be affecting the annular lipids (Jost et al., 1973) or the interaction of these lipids with the protein (Lenaz, 1975; Lee, 1976; Lenaz, 1979), thereby influencing hormone-receptor interaction. Alternatively, ethanol may have a direct effect on the receptor protein itself (Baker et al., 1975; Schoenborn, 1968), possibly decreasing the transition temperature of the protein (King and White, 1976). At 4°C
gonadotropin receptors may be below their transition temperature and in a conformational state that inhibits the interaction with LH. The presence of ethanol may decrease the transition temperature of the receptor allowing it to bind LH more effectively at this temperature.

The kinetics of gonadotropin binding to soluble receptors is markedly different than to particulate receptors. In macaque luteal particulates LH binding is inversely proportional to the temperature of the incubation, such that the rate and maximal level of LH uptake is greater at 37°C than at 25 or 4°C (Danforth and Stouffer, 1984). In contrast, LH binding to soluble receptors is not strictly a function of temperature; binding is greater at 25°C than at 4°C and 37°C. The lower levels of LH uptake seen at 37°C may be due to greater degradation of receptors and may be a consequence of enzyme degradation (Dufau, Charreau, and Catt, 1973). Since ethanol never increased LH uptake to levels greater than those seen under steady-state conditions at 25°C, ethanol was ineffective at unmasking gonadotropin binding sites in soluble receptor preparations.

In summary, extraction with Triton X-100 is an effective method for solubilizing gonadotropin binding sites from the corpus luteum of the rhesus monkey and the psuedopregnant rat, and these binding sites display a lower apparent affinity for gonadotropin than membrane-bound receptors. In addition, solubilization results in an apparent increase in the total number of binding sites present in the monkey but not the rat corpus luteum. The different responses to ethanol, and the differences in affinity between soluble and particulate receptors suggests that the interaction of the gonadotropin receptor with the
lipid environment of the luteal membrane may be an important factor in modulating the availability and affinity of gonadotropin receptor.
CHAPTER 7

SUMMARY

The data presented in this dissertation expand our understanding of hormone-receptor interaction in the corpus luteum, and provide evidence that masked gonadotropin binding sites exist within luteal membranes. These studies suggest that the corpus luteum of the rhesus monkey and the pseudopregnant rat contains a population of masked gonadotropin binding sites which can be exposed \textit{in vitro} with the enzyme neuraminidase. In addition, the corpus luteum of the monkey, but not the rat, contains a second population of masked sites which can be exposed upon addition of alcohol to the membranes. The different response to ethanol may reflect important species differences in the receptor population or membrane composition of the macaque versus the rat corpus luteum. The interaction of the gonadotropin receptor with the lipid environment of the luteal membrane and/or changes in overall membrane fluidity may play an important role in the masking/unmasking of gonadotropin binding sites in the macaque corpus luteum.

The importance of masked receptors in the regulation of luteal function remains to be elucidated. The masking or unmasking of gonadotropin binding sites at different stages in the luteal lifespan may alter the sensitivity of the corpus luteum to the low levels of circulating gonadotropin that are present during the luteal phase of the menstrual cycle. Indeed, the changes in the number of available
gonadotropin binding sites that occur during the luteal phase of the menstrual cycle may be related in part to the unmasking/unmasking of gonadotropin receptors. Changes in available receptors may also be important in regulating the response of the corpus luteum to hCG during early pregnancy, and as such could involve the masking/unmasking of gonadotropin receptors.

There are several lines of evidence suggesting that changes in membrane fluidity may be important in the interaction of gonadotropin with its receptor in the corpus luteum. 1) Alcohols, which are well-known modulators of membrane fluidity, unmask a population of gonadotropin binding sites in the corpus luteum of the rhesus monkey. The longer straight-chain alcohols, which are more potent membrane fluidizers, are more potent at enhancing LH binding. 2) The effect of alcohols on LH binding is sensitive to the temperature of the system, and temperature also affects membrane fluidity. 3) The level of gonadotropin binding in the presence of alcohol and at various temperatures is highly correlated with changes in membrane fluidity under these conditions. 4) The ability of ethanol to increase LH uptake by the macaque corpus luteum at 25°C is lost if the receptor is removed from the lipid bilayer of the luteal membrane. Taken together, these data suggest that hormone-receptor interaction in the corpus luteum may be modulated by changes in the lipid bilayer of luteal membranes.

Alterations of membrane fluidity may play an important role in regulating the function and lifespan of the corpus luteum. There is evidence that the fluid state of the luteal membrane changes during the luteal lifespan. Changes in membrane fluidity could alter the response
of the luteal cell to circulating hormones by increasing or decreasing
the number of available receptors for gonadotropin, by altering enzyme
activity within the corpus luteum, or by affecting the coupling of
receptor with adenylate cyclase.
APPENDIX

LABORATORY INSTRUMENT INTERFACE SYSTEM (LIIS); A UNIT FOR THE ACQUISITION, TEMPORARY STORAGE, AND TRANSFER OF DATA TO A MICROCOMPUTER

Abstract

A simple method of interfacing clinical and research laboratory equipment with a microcomputer is described. A four-channel buffer system has been constructed which stores data generated from laboratory instruments and then transmits the data directly to a microcomputer. The system is highly flexible with respect to the type of laboratory equipment and model of computer that can be interfaced, and it allows for virtually automatic data acquisition and analysis.

Introduction

The affordability and increased computing power of the current generation of microcomputers has resulted in their utilization in many clinical and research laboratories. Personal and small business computers are well suited for calculation and analysis of data obtained from many types of biological and biochemical experiments. However, data from laboratory equipment often must be entered into the computer by time-consuming and repetitive manual input at the keyboard. To circumvent this problem the computer can be connected directly to the laboratory equipment. Indeed, many laboratory instruments are marketed with built-in microcomputers for data reduction; however it becomes costly and inefficient to have a separate computer for each laboratory.
instrument. Alternatively, a central computer can be connected directly to several laboratory instruments. However, this necessitates the continuous operation of the computer which prevents other use during data acquisition unless the computer is equipped with expensive and sometimes complicated multitasking capabilities. It is also possible to record the data on paper (or magnetic) tape, which can later be fed into the computer by means of a paper tape reader or tape recorder (Angelis and Chang, 1976). However, these devices tend to be slow and inefficient, and often require several extra pieces of hardware.

To eliminate these drawbacks and to make greater use of the speed and flexibility of the microcomputer, we have developed a simple and relatively inexpensive laboratory instrument interface system (LIIS). This device stores (in volatile memory) the raw data generated by the laboratory equipment and then on demand outputs the data directly to the laboratory computer for subsequent data reduction and analysis. The transfer of data is fast, efficient, and extremely reliable, and allows for the calculation of large experimental data sets with very little manual input. The buffer can be interfaced to four different laboratory instruments and can store up to 15000 characters from each piece of equipment. This system allows for virtually automatic data acquisition and analysis with a minimum of human error, and still leaves the laboratory computer free for other tasks during data acquisition.
The Buffer System

LIIS is schematically illustrated in Figure 17. The data are transmitted from laboratory equipment to the buffer over Electronic Industry Association (EIA) four-wire cable at a rate of 300 baud. All interfaces between laboratory equipment, buffer, and computer are standard RS-232C format. Each input channel of the buffer may be configured as Data Terminal or Data Communication equipment. Virtually every microcomputer available today has at least one RS-232C port, and many different types of laboratory instruments are equipped with this interface. Laboratory instruments usually provide ASCII (American Standard Code for Information Exchange) RS-232C (bit serial) data. Since the buffer is only a first in, first out storage device, it performs no manipulation or formatting of the data. All data are sent to the computer exactly as received, and all processing of the data is performed by the host computer. Thus, the system is highly flexible with respect to the type of laboratory equipment and computer system that can be interfaced with the buffer. Instruments located up to 300 feet from the laboratory computer can be connected using this protocol. Utilization of low capacitance cable can extend this range to 500 feet. Since the buffer stores the data in volatile memory, voltage spikes or power surges may destroy stored data. Thus, it is beneficial to utilize an external surge suppressor to eliminate voltage spikes. Moreover, a battery backup system might be useful if data are stored in the buffer from extended periods of time.

The system is designed around an STD Bus microcomputer system using three Applied Microtechnology Inc. products: an ST4102 single
Figure 17. Schematic illustration of the Laboratory Instrument Interface System. Laboratory equipment may be any type that provides an RS-232C interface and may be located up to 300 feet from the computer.
board computer (Z80 based CPU), 2 ST4302 dual serial boards, and 1 ST4203 64KB dynamic memory board (Figure 18). The STD bus was chosen for its compactness and functional modularity. The buffer is equipped with four separate channels for input, and thus can be connected to four different laboratory instruments. In the present configuration channels three and four share the same memory allotment but are selected to operate at 110 and 300 baud respectively by an internal switch. Each channel is designed with 20 K of memory and operates independently of the other channels. In addition, separate sets of data from one instrument can be stored on the same channel. Each start/stop event causes the LIIS to delimit the current block of data. The buffer can transmit data to the computer and receive data from laboratory instruments simultaneously. The program loop responsible for inputting data to a buffer space takes less than 5 milliseconds. It is therefore possible for the 4 channels to be serviced in one character time at baud rates up to 1200. The communication between the host computer and the buffer is interleaved with the input task, and requires about 1 millisecond. The status of each channel (transmit, receive, channel full, etc.) is indicated by two light-emitting diodes. The activity of each channel is initiated or terminated by a separate toggle switch (Figure 19). Any time a group of data is transmitted to the host, the available space in that channel increases by the size of the group.

Data are transmitted from the buffer to the computer one character at a time. The computer must then echo this character to the buffer to receive the next character. A bad echo from the computer
Figure 18. Block diagram of the four-channel data buffer. The unit utilizes a switching power supply which provides +5 volts and ±12 volts. The system is cooled by an IMC Model PWS2107FL internal fan.
Figure 19. Close-up drawing of the four-channel data buffer. Each channel is controlled by a separate toggle switch and the status is indicated by the two light-emitting diodes above the switch. The end warning lamp flashes when the channel is approximately 85% full. The device is compact and measures 6"H x 10"W x 11"L.
signals the buffer to re-send the previous character. Although this extensive error-checking scheme slows the transfer of data, it essentially eliminates errors due to data transmission. The host and LIIS communicate at 9600 baud although this is software selectable.

The buffer is capable of transmitting as frequently as it polls an input for data, therefore the data rate is controlled primarily by the host program. The program utilized to download data from the buffer, print the data, and store it on disk is written in Basic and occupies less than 2 K of random access memory (RAM). If desired, the speed of data transmission can be greatly increased by using a compiled basic program or an assembly language routine to download from the buffer. As such, a large experiment (100-300 samples) can be downloaded from the buffer and calculated in less than 5 minutes. In addition, errors due to manual input of the data are completely eliminated.

Currently in our laboratory, the data buffer is connected to a Beckman LS 1800 liquid scintillation counter, a Searle 1197 gamma counter, and an Isocap 300 liquid scintillation counter in an adjacent laboratory. The laboratory computer is a Zenith Z-100 color computer with 128K RAM and two 320 K 5 1/4 inch disk drives. We have developed programs to utilize data received from the buffer for the calculation of radioimmunoassay (Rodbard, 1971), hormone-receptor binding parameters (Munck, 1976), adenylate cyclase activity (Birnbaumer et al., 1976), and the plotting of calculated data.

The buffer device was constructed by Michael Bosnos at the University Instrument Shop, University of Arizona at a cost of approximately $2000. All specifications on the buffer system and the
programs utilized to access the data are available from the authors. Hardware enhancements can include: large memory, more input channels, remotely-set baud rate and channel control, battery backup, and multiple output channels. Using a technique known as bank switching, 60 K of memory can be assigned to each channel. Changes such as these require small modifications to the software. STD bus memory costs about $400 per 64K and serial channels about $150 each. Adding more of each is not possible in this unit because there are no space provisions in the chassis. A larger device with more channels or memory can be built for the additional cost of the circuit boards as the software is written to allow easy modification.

Remote baud rate setting would require the inclusion of a command dialogue with the host. Currently the LIIS unit initializes itself upon power-up. More switch selectable baud rates and data protocols would require a digital I/O circuit board at a cost of about $350. Software changes for this modification are small.

The control program currently occupies less than 2K of memory space. The single board computer has space for 4K of EPROM. The software was developed on a CP/M based system using the Microsoft M80 assembler package. The program is basically one loop which handles one data channel at a time where parameters of each are defined in a "file control block". This allows easy modification to any feature of that channel or its memory allotment as well as the number of channels. It is further possible to cause a channel to screen and format the data but
this would detract from the universality of the unit and of course require specific programming for each unit or channel.

Summary

There are several methods available to automate data acquisition from laboratory instruments. Direct connection of computer to laboratory equipment is a reliable means of data transfer but weds computer to instrument and allows no access to the computer during data acquisition. Off-line data reduction by means of a paper tape reader or tape recorder is slow and inefficient, and often requires several extra pieces of equipment. We have developed a buffer system which temporarily stores data generated from laboratory equipment, and then outputs the data to a laboratory computer. During data acquisition the computer is free to perform other tasks such as word processing or analyzing data from other experiments. Transfer of data from buffer to computer is fast and efficient and essentially eliminates human error in data analysis. The system is highly flexible in that any laboratory instrument equipped with a standard RS-232C interface can be connected to the buffer, and all data formatting and analysis is performed by the computer. This system provides an excellent means for interfacing existing laboratory computers with equipment, and offers an alternative to purchasing expensive laboratory equipment that has data reduction capabilities enslaved in the unit.
LITERATURE CITED


Bhalla VK, Reichert LE 1974 Interaction of an ethanol soluble factor with with human FSH and LH. J Biol Chem 249:7996

113


Bramley TA, Ryan RJ 1978a Interactions of gonadotropins with corpus luteum membranes I. Properties and distributions of some marker enzyme activities after subcellular fractionation of the superovulated rat ovary. Endocrinology 103:778

Bramley TA, Ryan RJ 1978b Interactions of gonadotropins with corpus luteum membranes II. The identification of two distinct surface membrane fractions from superovulated rat ovaries. Endocrinology 103:796


Bramley TA, Ryan RJ 1980 Interactions of gonadotropins with corpus luteum membranes VIII. The different properties of rat luteal cell light and heavy membranes cannot be explained by fractionation of inside-out and outside-out plasma-membrane vesicles. Mol Cell Endocrinol 19:21


Cameron JL, Stouffer RL 1982a Gonadotropin receptors of the primate corpus luteum I. Characterization of 125I-labelled human luteinizing hormone and human chorionic gonadotropin binding to luteal membranes from the rhesus monkey. Endocrinology 110:2059
Cameron JL, Stouffer RL 1982b Gonadotropin receptors of the primate corpus luteum II. Changes in available luteinizing hormone and chorionic gonadotropin binding sites in macaque luteal membranes during the nonfertile menstrual cycle. Endocrinology 110:2068


Carlson JC, Buhr MM, Riley JCM 1984 Alterations in the cellular membranes of regressing rat corpora lutea. Endocrinology 114(2):521


Chan V, Davies TF, Regulatory inhibition of LH and prolactin receptors in the Leydig cell. 61st Annual Meeting of The Endocrine Society, Anaheim CA, 1979 (Abstract 510)


Chen TT, Abel JH, McClellan MC, Sawyer HR, Diekman MA, Niswender GD 1977 Localization of gonadotropic hormones in lysosomes of ovine luteal cells. Cytobiologie 14:412

Cherry RJ 1976 Protein and lipid mobility in biological and model membranes. Biological Membranes 3:47

Chin JH, Goldstein DB 1984 Biomembranes enriched in cholesterol are resistant to the disordering effects of ethanol. Fed Proceed 43(3):2188


Cousins AR 1977 Adaptation of biological membranes to temperature. Biochim Biophys Acta 470:395

Cranney M, Cundall RB, Jones GR, Richards JT, Thomas EW 1983 Fluorescence lifetime and quenching studies on some interesting diphenylhexatriene membrane probes. Biochim Biophys Acta 735:418


Cuatrecasas P 1972 Isolation of the insulin receptor of liver and fat cell membranes. Proc Natl Acad Sci 69(2):318


Danforth DR, Stouffer RL 1983 Ethanol and neuraminidase treatment unmask gonadotropin receptors in the primate corpus luteum. Endocrine Soc Abstr # 810.

Danforth DR, Stouffer RL 1984 Evidence for two populations of masked gonadotropin binding sites in the corpus luteum of the rhesus monkey (Macaca mulatta). Endocrinology, in preparation.


Dave JR, Knazek RA 1980 Prostaglandin I₂ modifies both prolactin binding capacity and fluidity of mouse liver membranes. Proc Natl Acad Sci USA 77:6597

Dave JR, Knazek RA, Liu SC 1981 Arachidonic acid, bradykinin, and phospholipase A₂ modify both prolactin binding capacity and fluidity of mouse hepatic membranes. Biochim Biophys Res Comm 103(2):727


Deutsch PJ, Wan CF, Rosen OM, Rubin CS 1983 Latent insulin receptors and possible precursors in 3T3-L1 adipocytes. Proc Natl Acad Sci USA 80:133

Diekman MA, O'Callaghan P, Nett TM, Niswender GD 1978a Validation of methods and quantification of luteal receptors for LH throughout the estrous cycle and early pregnancy in ewes. Biol Reprod 19:999


York, p 135


Dufau ML, Catt KJ 1978 Gonadotropin receptors and regulation of steroidogenesis in the testis and ovary. Vitam Horm 36:462


Eyster KM, Stouffer RL 1982 Demonstration of gonadotropin-responsive adenylly cyclase activity in the primate (macaque) corpus luteum. Endocrine Soc Abstr 290


Feinstein MB 1964 Relation of local anesthetics with phospholipids. J Gen Physiol 48:357


Hare F, Lussan C 1977 Variations in microviscosity induced by different rotational behavior of fluorescent probes in some aliphatic environments. Biochim Biophys Acta 467:262


Heron D, Shinitzky M, Hershkowitz M, Samuel D 1980 Lipid fluidity modulates the binding of serotonin to mouse brain membranes. Proc Natl Acad Sci USA 77(12):463


Houslay MD, Dipple I, Gordon LM 1981 Phenobarbital selectively modulates the glucagon-stimulated activity of adenylate cyclase by depressing the lipid phase separation occurring in the outer half of the bilayer of liver plasma membranes. Biochem J 197:675


Houslay MD, Gordon LM 1983 The activity of adenylate cyclase is regulated by the nature of its lipid environment. Curr Topics Memb Trans 18:179


Huhtaniemi I, Martikainen H, Tikkala L 1978 hCG-induced changes in the number of rat testis LH/CG receptors. Mol Cell Endocrinol 11:43


Kaltenbach CL, Graber JW, Niswender GD, Nalbandov AV 1968 Effect of hypophysectomy on formation and maintenance of the corpus luteum of the ewe. Endocrinology 82:753


Knobil E, 1973 On the regulation of the primate corpus luteum. Biol Reprod 8:246


Kimelberg HK 1975 Alterations in phospholipid-dependent (Na⁺+K⁺) ATPase activity due to lipid fluidity. Biochem Biophys Acta 413:143


Lee AG 1976 Interactions between anesthetics and lipid mixtures amines. Biochim Biophys Acta 448:34


Ling WY, Marsh JM 1977 Reevaluation of the role of cyclic adenosine 3',5'-monophosphate and protein kinase in the stimulation of steroidogenesis by luteinizing hormone in bovine corpus luteum slices. Endocrinology 100:1571


Marsh JM, Butcher RW, Savard K, Sutherland EW 1966 The stimulatory effect of luteinizing hormone on adenosine 3',5'-monophosphate accumulation in corpus luteum slices. J Biol Chem 241:5436


McCaleb ML, Donner DB 1981 Affinity of the heptic insulin receptor is influenced by membrane phospholipids. J Biol Chem 256(2):11051

McCrae J 1971 PGF2α and corpus luteum regression. Ann NY Acad Sci 180:456


Muller C, Shinitzky M 1979 Modulation of transferrin receptors in bone marrow cells by changes in lipid fluidity. Br J Haematol 42:355

Muller M, Ucer U, Engel W 1983 Effect of neuraminidase on luteinizing hormone/chorionic gonadotropin binding to the ovarian and testicular membranes from different mammalian species. Biochim Biophys Acta 762:135


Neufeld ND, Cobro L 1982 Increased fetal insulin receptors and changes in membrane fluidity and lipid composition. Am J Physiol 243:E246

Niswender GD, Sawyer HR, Chen TT, Endres DB 1980 Action of luteinizing hormone at the luteal cell level. Advan Sex Horm Res 153

Norman AW, Demel RA, DeKruyff B, Geurst Van Kessel WSM, Van Deenen LLM 1972 Studies on the biological properties of polyene antibiotics: Comparison of other polyenes with fillipin in their ability to interact specifically with sterol. Biochim Biophys Acta 290:1


Papahadjopoulos D 1970 Phospholipid model membranes. III Antagonistic effects of calcium and local anesthetics on the permeability of phosphatidylerine vesicles. Biochim Biophys Acta 211:467

Papainannou SA, Cospodorowicz D 1975 Comparison of the binding of human chorionic gonadotropin to isolated bovine luteal cells and bovine luteal plasma membranes. Endocrinology 97:114

Parola AH, Kaplan JH, Lockwood SH, Uzgiris EE 1981 Activation of human lymphocytes by concanavalin A or purified protein derivative results in no alteration of fluorescence polarization of lipid probes although the electrophoretic mobility of the cells is unchanged. Biochim Biophys Acta 649:616


Quinn PJ 1981 The fluidity of cell membranes and its regulation. Rec Prog Molec Biol 38:1

Rajaniemi HJ, Midgley AR, Duncan JA, Reichert LE 1977 Gonadotropin receptors in rat ovarian tissue III. Binding sites for luteinizing hormone and differentiation of granulosa cells to luteal cells. Endocrinology 101:898


Rao CV, Mitra S 1979 Gonadotropin and prostaglandin binding sites in nuclei of bovine corpora lutea. Biochim Biophys Acta 584:454


Rasmussen H 1970 Cell communication, calcium ion, cyclic adenosine monophosphate. Science 170:404


Rodbell M 1980 The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature 248:17


Rojas FJ, Asch RH 1984 Differential effects of ethanol upon stimulation of adenyl cyclase in human corpus luteum membranes. 7th Int Congress Endocrinol Abstr 2109

Roth J 1978 Receptors for peptide hormones. Endocrinology Vol 3: 2037


Sauerheber RD, Esgate JA, Kuhn CE 1982 Alcohols inhibit basal and insulin-stimulated glucose uptake and increase the membrane lipid fluidity. Biochim Biophys Acta 691:115


Scatchard G 1949 The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660

Schlessinger J 1980 The mechanism and role of hormone-induced clustering of membrane receptors. Tr Bioch Sci 210


Seeman P 1972 The membrane actions of anesthetics and tranquilizers. Pharmacol Rev 24:583


Shlatz L, Marinetti G 1972 Hormone-calcium interactions with the plasma membrane of rat liver cells. Science 176:175


Skou JC 1954 Local anesthetics. VI Relation between blocking potency and penetration of a monomolecular layer of lipids from nerves. Acta Pharmacol Toxicol 10:325

Smith ICP 1979 Organization and dynamics of membrane lipids as determined by magnetic resonance spectroscopy. Can J Biochem 57:1


Stadel JM, deLeon A, Lefkowitz RJ 1982 Molecular mechanisms of coupling in hormone receptor-adenylate cyclase systems. Adv Enz Rel Areas Mol Biol 53:1


Stouffer RL, Nixon WE, Hodgen GD 1979 Disparate effects of prostaglandins on basal and gonadotropin-stimulated progesterone production by luteal cells isolated from rhesus monkeys during the menstrual cycle and pregnancy. Biol Reprod 20:897
Stouffer RL, Tyrey L, Schomberg DW 1976 Changes in $^{125}$I-labelled hCG binding to porcine granulosa cells during follicular development and culture. Endocrinology 99:516


Suter DE, Fletcher PW, Sluss PM, Reichert LE, Niswender GD 1980 Alterations in the number of ovine luteal receptors for LH and progesterone secretion induced by homologous hormone. Biol Reprod 23:205


Ticku MK, Burch T 1980 Alterations in γ-aminobutyric acid receptor sensitivity following acute and chronic ethanol treatments. J Neurochem 34(2):417

Tolkovsky AM, Levitzki A 1978 Mode of coupling between the α-adrenergic receptor and adenylate cyclase in turkey erythrocytes. Biochemistry 17(18):3795


Wimalasena J, Dufau ML 1982 Water-soluble gonadotropin receptors of the rat ovary. Endocrinology 110(3):1004

