LABORATORY MANUAL

BIOLOGY 317

ENDOCRINOLOGY

Fall 2017

Rooms: W-3-066 & W-3-068

Th 9:30-12:30

NAME: _____

Dr. Kenneth L. Campbell, Professor

(Office Hours W 11:00-1:00 or by appointment, 617-287-6676, ISC - 5720)

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Table of Contents

Aims and Objectives
Imperatives
Schedule
Grading
Data Analyses
General Methods of Approach to Unknowns
Hormone-Receptor Binding, Immunoassays, Hormone Dose-Response

Aims and Objectives

The laboratory is meant to reinforce and augment the materials presented in the lecture portion of this course. Hopefully it will illustrate a number of important concepts and experimental approaches in endocrinology, *e.g.*, chemical and biochemical characterization of regulatory molecules, dose-response, bioassay, immunoassay, mechanism of hormone action, chemical and physical interruption of feedback cycles, multiple levels of control exerted in the endocrine system, and exposure to modern proteomics. In a practical sense, this laboratory will give the participants experience in several chemical and biochemical methods, as well as opportunities for handling small laboratory animals, techniques useful in other physiology laboratories as well as in clinical or endocrine research laboratories.

This laboratory will involve solution of an unknown along with conduct of several experiments that are purposely not defined in detail – students will finalize experimental design. The unknown is meant as a multi-dimensional puzzle, perhaps more interesting than group exercises. Paperwork is minimal and emphasis in evaluations will be placed on experimental design, research in endocrine literature, and deductive **thinking**.

Many elements of the lecture and laboratory for this course are at http://kcampbell.bio.umb.edu so that students may access or refer to them whenever it is convenient. This site includes copies of syllabi, lecture notes and illustrations, locations of other web sites of potential interest, and an e-mail address for the course instructor. This lab is always an "experiment" in educational innovation. Hopefully the web site will prove useful to you and make your life a bit easier as you take this course. Let us know what you find useful, what works, what doesn't work, and what needs to be added; your help and cooperation are appreciated.

We use a variation on other laboratories you may have encountered that should be helpful to all of us. Specifically, the major laboratory report for the term, covering efforts linked to proteomic work will arise from a single study, will be subjected to "peer" review prior to being rewritten and submitted to the course instructors. Each student will write up the laboratory report, as they normally would for submission to the instructor, by the first deadline date in the syllabus. Three copies of these reports will be made and distributed to three other students in the lab. The choice of reviewers will be made by the instructor who will keep a list of reviewers. Each student will read and make anonymous editorial comments on three lab reports over the course of two weeks. These marked-up, edited, copies of the reports will be turned back to the instructor and then given back to the student who wrote the report.

That student will then rewrite the report before submission to the instructor at the time of the second deadline for that report. **The edited initial copies and the final draft will be turned in at the time of report submission. Grading will include an evaluation of both the report and the editorial work done by each student.** It is hoped this exercise will assist students in honing their written presentation skills along with providing a "real-world" experience in how reports and scientific writings are normally generated.

I will state at the outset that experiments can fail completely or in part. Such failure is something normally encountered in the course of research, particularly if the paradigm or hypothesis upon which a particular set of experiments is based has not been repeatedly challenged by experiment. The other main reason for failure is, however, more controllable. That is, the exercise of care in the conduct of the experiment, the collection of the data, and the evaluation of that data. I have too often observed investigators collecting notebook after notebook of data which proves to be useless because care was not exercised in its collection and organization: standards were not used to calibrate measurements; positive and negative control samples were not tested along with the test samples; instruments were used incorrectly; dates and/or concentrations were not included in solution labels; time-of-day was not noted when blood samples were taken; deviations from written protocols were used but not recorded. Such sins of omission make research more difficult and expensive than it needs to be. Please do not be guilty of such errors during this course.

In this course I also strive to expose you to the research process including independent thinking and evaluation of published material as well as written exposition of your own results. **You will be asked to build on information you should have encountered and acquired in past biology, chemistry, and mathematics courses. You will be asked to use, manipulate, and calculate with standard scientific units and notations during and outside the lab. This is material that should have been mastered by this time in your training. Do not assume everything will be provided as a simple recipe or set protocol; you will be asked to decide on your own, or with the help of your peers, what the order of some work should be, when things should happen, where to find information, how to make up solutions, what experimental parameters should be tested, and what experimental design should be used.** In addition to trying to get people to become more self-reliant and confident in their own knowledge, observational, and intellectual abilities, I hope to make people going through this class begin to ask questions about dogma presented in the literature as fact. And I hope to encourage people to suggest designs for the disproof of "accepted" hypotheses, *i.e.*, to go beyond questioning authority toward redirecting authority and uncovering "truth" (Karl Popper, *The Logic of Scientific Discovery,* 1934/trans1959/2002, Routledge: New York, NY, ISBN-10: 8130908115; ISBN-13: 978-0415278447; ASIN: 0415278449).

IMPERATIVES

All students must demonstrate responsibility for helping to keep the laboratory and ancillary rooms clean. The class is too large to function otherwise. This means policing these areas for debris, helping to keep equipment and animal caging clean, and keeping benches and floors wiped up. We do not have enough lab help to have all the housekeeping done by someone else. Moreover, we are sharing this lab with several other classes this term. The potential for biologically active chemicals like hormones or animal waste, dander, *etc.* to cause serious allergic problems, particularly in already sensitive individuals, is too great to ignore given the number of people who come into or near the lab during the

course of a term. Chemical solutions must be handled with respect to avoid endangering others; hormones are biologically active so small, unintentional doses can cause undesired effects. Please be as helpful and cooperative as you can. As part of grading is also based on participation, the instructor will note repeated noncompliance with these imperatives. I would hate to decrease someone's grade for acts of omission.

Cleanup in the lab will also be very important this term since you may each be doing something different on any given day. **Labels** on solutions and specific apparatus, *e.g.*, slide boxes, should include: ***contents, concentrations, date prepared, preparer's initials or name, and intended use*.** Be careful to store materials appropriately, as protein solutions or high dilutions of steroids, thyroid hormones, *etc*., are prone to ready degradation or denaturation; *be liberal in the use of refrigeration and careful in the use of freezers* (biological materials are better preserved in the cold but macromolecules are sheared during the freeze-thaw cycle when aqueous solutions are frozen and melted). Again, materials must be put away between uses and everything must be well labeled.

With preparation and cooperation I am certain this will be an enjoyable and instructive semester for all of us.

Best Wishes,

Kenneth L. Campbell

Note:

Both sections of Endocrinology 317 Lab meet at the same time. Both sections meet jointly in W-3-066 before each lab then occupy W-3-066 and W-3-068 during lab.

SCHEDULE

Laboratory Date	Report/Summary Due	Comments
09/07	*Roll calls, Introduction of the TAs, Location of the Lab Manual, Introduction:* Materials, Grading, Reports, Resources & Journals, Schedule, Expectations, Description & Presentation, Data, Units, Math; Lab Safety; Animal Handling Training (due 11/02)	**Do not skip this session if you want to keep a seat in the lab! Sections are full & the Add/Drop deadline is Tuesday, September 12 (before the next lab meeting).** *Introduction:* Distribution of Unknowns and course introduction/background review.
09/14	*Unknown Hormones I*: *Classifications, physical characterizations*	*Unknown Hormones Approach:* melting point, TLC (2% HOAc, 1:1 EtOAc:Hexane), and ultrafiltration (30k MW cutoff), simple qualitative end-point detection: UV absorbance or spectrophotometer.
09/21	*Unknown Hormones II*	*Unknown Hormones:* Characterization completed.

09/28	*Immunoassays I: Noncompetitive Assay* Discussion of Unknowns; Set up for Immunoassay labs; Introduction of Proteomics	*Immunoassays:* Noncompetitive hCG/LH enzyme-linked immunosorbent assay (ELISA) to be run on unknown synthetic urine samples using test strips. Standard curves and controls will be run.
10/05	**Lab Summary I: Solution of Unknowns Due.** *Immunoassays II: Competitive Assay*	*Immunoassays:* Solid-phase competitive estrone-glucuronide (EG) enzyme immunoassays (EIA) to be run on the panel of unknown urines (as above) plus standard curves to evaluate assays. This will use home ovulation kit materials. Results will be collated to the hCG/LH assays.
10/12	*Discussion of Immunoassays & Proteomics Lab (Major Report)*	*This is clarification for the second summary & necessary background for the Proteomics labs.*
10/19	*Proteomics of Hormone Fragments I* **This is the basis for the major lab report.**	*Proteomics:* Exploration using computer methods of the digestion of protein hormones by target cells, the generation of proteolytic peptide products, and the identification of known proteins that contain sequences that are identical or similar to such peptides. Students will explore an assigned protein hormone. The lab report on the proteomics labs will be peer reviewed and returned for rewriting based on peer comments.
10/26	**Solution of Unknown Urine Panel Due.** *Proteomics of Hormone Fragments II* **This is the basis for the major lab report.**	*Proteomics:* Continuation of the proteomics experiment, discussion of results and their presentation, plus introduction to the next 2 labs.
11/02	*Hormone Control Circuits I*	*Feedback Controls:* treatment of mice with methimazole, thyroid powder, or nothing followed by calorimetric evaluation of basal metabolism; baseline information should be collected at the beginning of thyroid treatment and after the course of treatment; comparisons of basal metabolic rate (O_2 consumed/g body weight/min) and/or thermal output (calories/g body weight/min) should be made between treatment groups and change over time of treatment (treatment response rate).
11/09	**Lab Report: Proteomics of Hormone Fragments. 3 Copies Due.**	*Mechanism of Hormone Action:* Oxytocin on mouse uterus contraction; add inhibitors, blockers, or 2nd message enhancers (*e.g.*, dbcAMP, IBMX, PMA, indomethacin, okadaic acid) in simple +/-/0 designs to

	Hormone Mechanism I	look at effects of modulating intracellular signaling. Choose the chemicals to be used, compute how to make up the solutions beginning with available stock materials, and make up chemical solutions.
11/16	**Anonymous Peer Reviewer Comments Due.** *Hormone Mechanism II*	*Mechanism of Hormone Action:* Oxytocin on mouse uterus contraction; add inhibitors, blockers, or 2nd message enhancers (*e.g.*, dbcAMP, IBMX, PMA, indomethacin, okadaic acid) in simple +/-/0 designs to look at effects of modulating intracellular signaling. Finish making up stock solutions, run the experiment, and collect the data.
11/23	**Thanksgiving Day**	**Holiday**
11/30	*Hormone Control Circuits II* **Mechanism of Action Summary Due.**	*Feedback Controls:* Continue work on feedback and discuss questions from other labs. Peer Reviewer comments will be redistributed to the authors of the reports.
12/07	**Final Lab Exam.** **Lab Report: Proteomics of Hormone Fragments. Final Copy and Peer Reviewed Drafts Due.**	---

Grading

Laboratory is worth 30% of your overall grade. Course work for lecture is worth 70%. (Keep in mind, however, that effort expended in lab and on reports is often the best way to learn facts and concepts covered in lecture; lab is synergistic with lecture. They reinforce one another.)

A total of 1000 points is awarded in lab:

Attendance & Participation	100 points
Summary I: Unknown Hormone (Decision Grid & Conclusions)	100 points
Solution of Unknown Urine Panel (Results & Conclusions)	100 points
Major Report	200 points
Summary II: Hormone Mechanism (Result Grid & Conclusion)	100 points
Peer Reviewer Comments	150 points
Lab exam	250 points

SOLUTION OF UNKNOWN URINE PANEL: Due early in the term, this puzzle solution is meant to force students to apply the basic rules and concepts described for immunoassays and assays generally and to stimulate students to apply those concepts to a typical forensic or clinical diagnostic task involving distinguishing males from females and females at different stages of their ovarian cycles using biological fluids and hormones as markers.

LAB NOTES: Part of the process of doing good lab work is keeping good notes. Good notes include clear definition of the purpose of each experiment or test you conduct, including: a definition of what you are trying to rule in or out as a possible unknown, for example; dates, initials, and page numbers on each page; and a brief summary in writing of how the test or experiment came out. In addition, the notebook should contain a list of the articles and materials you are reading as references and/or copies of those materials. All this will be useful and important when it comes to writing up your summaries and reports and the habit should come in useful later. Further considerations of what constitutes "Good Laboratory Practice," GLP, are covered at http://www.anachem.umu.se/cgi-bin/jumpstation.exe?GLP-GMP. Because of the importance of such considerations in industrial and biomedical arenas -- places where many students end up being employed -- students are encouraged to begin to familiarize themselves with this major quality assurance material.

LAB REPORT: 5% Format, 10% English, 10% Abstract, 10% Introduction, 10% Materials & Methods, 25% Results, 25% Discussion, 5% References

The format follows that in most scientific journals; they provide examples. The following outline is suggested and can be downloaded here. **All reports -- no exceptions -- must be typed or computer generated** as gaining computer literacy is a part of science education (and life) today.

TITLE

by

Name

Date (of submission, not drafting)

in partial fulfillment of

Endocrinology Laboratory, Biology 317

Department of Biology, University of Massachusetts at Boston, Boston, MA 02125

[No page number on cover page, all others at bottom center.]

Abstract

[The Abstract should be a single paragraph of no more than 200 words containing: a *statement of purpose or question addressed;* a *brief description of methods used*; presentation of the *seminal or central findings* obtained; and a *summary or conclusion based on the question posed and the results obtained*. It is usually written after the rest of the report.]

Introduction

[The Introduction should include a presentation of the *purpose of the experiments or question posed* as well as a *brief description of the background material providing the rationale for the experiments*. It should also include a *statement of anticipated results or alternative hypotheses being tested*.]

Materials and Methods

[Materials and Methods should *concentrate on deviations from the protocols given and definitions of the experimental groups handled or measured* by a given student.]

Results

[Results *presents the data collected*, including pooled data, *in a meaningful and coherent manner*. **Raw data is not presented without further analyses or comparisons.** This section *should include the tables, charts, graphs, figures and computations used to organize the data collected and to analyze it so that final conclusions could be reached*. *All* these elements *should be sufficiently detailed to tell some portion of the overall story when presented alone, in the absence of an accompanying text, as in a visual presentation. Tables, charts and graphs* should all *have titles*. *Tables and ch*arts should *contain explanatory footnotes defining the meaning of any numbers presented*. *Graphs* should *have explanatory legends and axes that are labeled and scaled in a logical manner*, don't start the y-axis at 1.37 simply because the first data point falls there -- start at 0 and *include units of measurement on the scale*. In the *textual portion of this section include highlights of what is to be found in the graphs, tables, etc.*, and try to avoid making judgements about the data in this section. **All graphical materials or figures must be introduced by a brief statement indicating why they are being presented and what the key observations are.**]

Discussion

[In the Discussion ***evaluate the results obtained*** *in light of those obtained elsewhere* but, more important, ***in light of the question(s) posed or the hypotheses being tested***. *Use your results to answer the question* then go on to address tangential issues and the needs for improvements in the protocol. (Reports containing discussions that draw no conclusions from data gathered, or that indicate an absence of any meaningful attempt to relate questions posed in the experiments to the results obtained, will be given as low a score as feasible. Effort counts.)]

References

[References should be *presented in a consistent format* and should be given *as they would be in an English paper*, *e.g.*, Campbell, K.L., 1985, Methods for Monitoring Ovarian Function and Predicting Ovulation: Summary of A Meeting, *Research Frontiers in Fertility Regulation 17*:1-15. In the text, *references should be cited by number or first author and year*. Avoid footnotes. **Reference lists limited to the course textbooks or the lab handout are not acceptable,** work in libraries is always valuable in science and use of primary journals and computerized databases, *e.g., PubMed* or *UNCOVER*, is to be

recommended. When citing unique Internet documents, indicate the full URL and any page numbers. Attempt to identify the author of the document and the location of generation; these may be on an associated Home Page or at the beginning or end of a document. **Published papers and abstracts located on the Web are not unique documents and must be cited as any other printed publication, preferably after being read in full in the original journal.** Free open stack access to the Tufts Medical Center (Sackler) library, the Boston University library, and the MIT library should augment stack access at the UMass (Healey) and Boston Public Libraries even if electronic access to full text versions of a journal article is not available via PubMed or other search engines.

SUMMARY: 50% Results, 50% Conclusions.

Summaries are shortened versions of the reports. They are meant to help organize the data for use in full reports and as a stimulus to analyze parts of what may be a much longer experiment. The Question or Purpose is meant to substitute for the Abstract and Introduction of the reports. It need not be detailed or go into depth as far as background is concerned. It may contain figures that help organize concepts or help pose the problem under investigation. The Results should include treated raw data, transformations of data such as rates or percent of control, graphs, tables, or charts that allow conclusions to be drawn, **along with appropriate explanatory text indicating what is contained in the graphics and why the graphics are presented**. The Summary or Conclusion is simply a statement of conclusions reached concerning the questions posed based on the results obtained. It does not need to include the sorts of comparisons with other data that are desirable in a longer report. References should also be included in the Summary.

The Summaries will be shortened to a maximum of two pages in 2017; for the hormone unknown, the urine panel unknowns, and the mechanism of hormone action the summary will consist of a 1-2 sentence statement of the purpose of the experiment, a decision tree, a computed results grid, a statement of conclusions (not summary) based on the computed data, and a list of references cited in the report (not just read, but cited). All written portions of the Summary should be typed or computer generated. **Do not attempt to staple raw data collections to a cover sheet and expect that to serve as a Summary; if you don't wish to spend time generating the Summary, I refuse to spend much time correcting it or trying to justify giving a grade for what is not present.**

Peer Reviewer Comments: These are to be anonymous comments regarding the adequacy of your fellow student's work. Similar comments will be made concerning your efforts. You are being placed in the shoes of an instructor, or any other investigator, that might be attempting to replicate the studies being reported. Reports must convey enough information to allow such replication and/or to convince the reader that the summaries and conclusions reached by the author are reasonable and logical provided the results generated under the conditions described and given the background under which the data are reported to have been collected and evaluated. The big questions you must ask yourself as a reviewer are: *1) Is there a reasonable hypothesis stated that is being tested? 2) Is the experiment, as described, reasonable for addressing that hypothesis and is sufficient information provided that would allow the experiments to be repeated? 3) Are the results presented in a clear and logical manner that allows me to understand what information was generated and how it might be interpreted? And, 4) do the results and discussion provided allow me to reach the same conclusions regarding the original hypothesis that the author reaches (this includes being able to find background material in the references provided)?*

Comments should address the written document, not the author who wrote them. They can, and should identify both the weak and the strong points of the presentations: *change that, but keep this*. You are not assigning any numerical values to comments. These will be critiques of written work, they are not personal criticisms of the author and should not be interpreted as such. No one's work is perfect. Along with any good approaches you found when doing critiques, use the written comments on your own report to improve the report for final submission; that is the principle document being graded. Grading of comments will focus on how these appropriately attempt to improve other's work and how carefully and adequately they identify both good and bad elements in that work.

LAB EXAM: 1/3 practicum, 2/3 concepts, essay and data evaluation.

Data Analyses

A common problem encountered by students in this laboratory involves the critical evaluation and interpretation of data, *e.g.*, are two test groups, or two individuals, identical in their responses or even meaningfully different? In biological systems such questions are confounded by the tendency of each individual specimen to respond slightly differently from every other specimen or even differently from itself from one time to the next. Such variation must be kept in mind and expected when you examine the data generated in these laboratories. Do not expect all ovariectomized mice treated with estradiol to have uteri that weigh exactly the same amount.

[Please note that some of the equations below do not retain format well in going to HTML, they are best obtained from a statistics text or other source book. KLC]

But are the two results seen really different? The best way to examine such differences is to test them by the use of elementary statistics. For a group of 4 similarly treated animals the arithmetical average of any observed variable is the mean for that variable, $m = x = [\sum_{i=1-4}(x_i)]/N$, where $\sum_{i=1-4}$ means sum of i = 1 to 4, N is the number of observations, here N = 4.

The mean is attended by a degree of uncertainty as to whether it is the "true" mean, μ, *i.e.*, the one that would be observed if every possible subject were treated the same way and then observed. If the processes governing the observed variable allow random variation in all directions, the variation is distributed "normally" and the uncertainty is quantitatively represented by a measure termed the standard deviation, s. This is calculated as the square root, √, of the average of the squares of the differences between the group mean and the observed values:

$s = \sqrt{\{[\sum_{i=1-4}(x_i - x)^2]/N\}}$.

If N is small (<20), N is replaced by N - 1 in the equation to give a result more reflective of the true dispersion (Actually the adjustment is required to correctly reflect the number of "degrees of freedom" involved in the equation when differences from a mean are involved.).

The standard deviation, *s*, actually measures the width at the inflection points of the standard, normal, bell-shaped, Gaussian, distribution theoretically associated with the observed data. As such, 66.7% of all possible values of the observed mean should fall between x - 1 *s* and x + 1 *s*, if the sample used to

calculate x is not systematically biased in some way. Moreover, 95^{+}% of the possible values of x should fall within x - 2 *s* and x + 2 *s*.

The utility of *s* becomes obvious if there are several observations or measurements within a set that seem "way off" for unexplained reasons. Such "outliers" can be eliminated from means or comparisons by first generating x and *s* for the other observations involved. If the outlier lies more than 2 *s* above or below x you have a statistically meaningful reason to eliminate that observation from further consideration in your dataset. (*Caution*: This procedure cannot be applied repeatedly to the same group of results without violating the statistical assumptions underlying the procedure.) Likewise, in statistically testing whether two means are different or not you are effectively generating an x and *s* for all the data lumped together and then asking if the group mean's, x with a caret hat, for the various subgroups differ from the overall mean or from each other by more than 2 *s* (or some other chosen statistical distance), where *s* here is defined by the overall dataset.

One of the easiest ways to visualize comparisons among means is to draw line or bar graphs that have the means indicated as points and the standard deviations for each point indicated as lines or bars above and below these points. If variations around the means are quite uniform in size, then, if the standard deviations for two means do not overlap, there is probably a statistical difference between the means. The larger the N's are for each mean the more likely this is to be true.

There is a mathematical test that can be used in many cases to give an actual confidence level to the prediction of whether two means are actually different (or similar). It is called Student's t-test and generates a value for the difference between two means that can be compared to a standard table, a t-table, computed for several levels of probability or confidence and for several pairs of N_i values. If the value calculated for t for the difference between the means of the two groups exceeds the t value at, say, 0.05 then these group means have a 95% probability of being statistically different from one another even if every possible individual in each group was observed or measured. Computationally:

$$t = (x_1 - x_2)/[\sqrt{(s_1^2/N_1 + s_2^2/N_2)}]$$

Note, however, that the t-test is strictly appropriate only for the comparison of two groups that are independent of one another. It should not be used to compare the same animals before and after treatment, nor should a single control group be tested by this method against more than one experimental group in a given experiment. These latter "time series" and "multigroup" comparisons are better handled by application of other statistical tools such as ANOVA, analysis of variance, or Dunnett's multirange t-test. If time allows during our experiment discussions later in the term we may be able to describe some of these tests. They will not be fully expanded here; refer to a statistics text for more details, *e.g.*, http://www.richland.cc.il.us/james/lecture/m170/ or http://davidmlane.com/hyperstat/index.html.

If we have conducted an experiment and suspect that two test groups differ, we want to discern whether the apparent differences between the groups is a real phenomenon or simply due to random variations in, for example, subject selection, measurements, or experimental conditions. The approach to statistically testing for a difference is based in logical argument. Because it is easier to disprove a statement by finding a single exception to the statement or to show that contributing factors may give rise to apparent differences, the usual starting, or null, hypothesis, H_0, put forward when group differences are suspected is that the groups in question do not differ. That is, that the only contributors

to any apparent difference are from random, extraneous factors, rather than that factor being evaluated. The alternative hypothesis, H_1, in this case, is then that they do differ because of the factor being evaluated. If, using a statistical test, the groups are found to differ, the experimental data allow rejection of the H_0 hypothesis and acceptance of the H_1 alternative. Conversely, if the groups do not differ, H_0 cannot be rejected and the alternative H_1 is not supported. Note that, because the probability of failing to reject H_0 when it is false is often larger than the probability of rejecting H_0 when it is true, we can normally be more certain that H_1 is true if H_0 is not than we can that H_1 is not true if H_0 appears to be true. The inference structure is asymmetric, so conclusions are more definitive when H_0 is rejected than when it is accepted. This means more experimental progress is made when null hypotheses are constructed so that tests results allow their clear rejection. If a starting hypothesis is rejected, we can design a new experiment to test a new hypothesis. If the starting hypothesis is not rejected, we are forced to replicate or expand the old test or move in another experimental direction. Note that in biological systems we often have a mechanistic picture or model that allows the construction of the hypothesis, H_0. If we fail to reject H_0 we know that, at least under the conditions studied, the model appears to work. This is not terribly informative in that it may simply mean we chose the wrong conditions to show where the model's faults lie. If we reject H_0, we know, however, that something in the model is wrong, though not necessarily what. The idea of a good experiment is thus to allow the most specific test of a model possible to allow individual elements of the model to be tested and eliminated or supported.

Do not then be surprised if a model propounded by the instructor is rejected by the data you generate. Your task is to interpret your data in a meaningful way in the context of the model put forward and then to suggest an alternative model and/or experiment to test that model.

Lab Safety Videos

Before starting any work with chemicals or biological materials in the lab please view one or more of the safety videos found at: https://cls.ucla.edu/resources/videos.

General Methods of Approach to Unknowns

The unknowns distributed to you in this laboratory will, in the main, be highly purified materials with discrete chemical, biochemical, immunological and biological properties. They will be provided as dry powders in small numbered vials. You should make every effort to keep the stock material dry and cool, preferably refrigerated. Use the material sparingly. It is expensive. Do not be so sparing as to be incapable of determining what you have, however! I will retain the coding key that links you and the identity of your unknown. (And, if necessary, I can provide more material if that is required to complete the laboratory.) Unknowns may be duplicated; more than one person may receive the same hormonal unknown. The hormones distributed are drawn from the following list:

Progesterone
Estradiol-17ß
Testosterone
Gibberellin
Cortisol (hydrocortisone)
Thyroxine

Insulin
Human chorionic gonadotropin
Epinephrine
Indoleacetic acid
Oxytocin

The solution of these unknowns will involve several basic biochemical tests. Characterization of the class of compound will involve migration on thin-layer chromatography, melting point determination, molecular sizing with ultrafiltration, and spectral evaluation using a UV-visible spectrophotometer. Further characterization could involve acrylamide electrophoresis, chromatography on other thin-layer systems, binding to serum binding proteins, displacement of radioactive ligands in binding systems, inhibition of enzymes or metabolism by specific cells or bacteria, *etc.* Immunological characterization usually implies generation of a labeled-ligand displacement curve in an RIA or ELISA/EIMA that is parallel to that obtained with a pure known compound. Alternatively, serum binding proteins or crude target-cell receptor proteins could be substituted for the specific antisera in a competitive binding assay. For proteins, binding of multiple epitope-specific antibodies is further proof of identity as is binding of a specific band on a Western blot to an antiserum of known specificity. Further proofs of identity come from characterization of bioactivity in a living system. This may be done by using a known response in an intact system, *e.g.*, a specific growth response, or it may be accomplished by demonstrating the capacity to replace an ablated organ known to be the source of a given hormone. Ultimately, a quantitative version of this bioassay system may give parallel responses to a graded series of dilutions of the unknown and a supply of pure hormone. Lastly, if possible, some characterization of the mechanism of action stimulated by a given hormone would provide a definitive identification of the unknowns. This would involve demonstration that the hormone produced a predicted response in target cells either morphologically or biochemically. Often this is demonstrable using an immunoassay for the generation of a specific second-messenger, or the pharmacological blockade of a response by drugs with previously defined targets.

Your first summary will be a report on your solution of the identity of your unknown. It should briefly define your line of thought and intermediate hypotheses regarding the possible identity of the compound. I suspect the path will consist of a series of binary decisions based on negative inference (ruling out the possible compounds that do not fit the results you have gathered); this can be arranged in a table. The summary should be no longer than 2 pages, including the decision grid and cited references. I do suggest that one place to start finding pertinent information is in the library among the biochemistry books and journals or in similar online data sources (from reputable sites, not health food promoters or chatrooms). Learn to use the available *Science Search Databases* and various Web sources such as PubMed to help find out about specific subjects such as the properties of a given hormone or the assay systems that have been used to measure it. And keep good notes in the lab.

Rubric for Summary on Identification of an Unknown Hormone

Each experimental summary in Endocrinology will be no more than **2 pages long** and will be worth **100 points** in the lab.

It will follow the suggestions in a rubric issued for the benefit of the students and the TAs who will be grading these summaries.

All summaries will be typed and submitted as hard copies at the times indicated in the lab manual schedule. Late copies will be penalized 5 points per day, including weekends.

You must **list all the published and/or on-line references that you used in the summary. They need to be marked in the narrative and results sections as citations**; first reference is number one in the reference list at the end of the summary, second reference is number two, *etc.* **This counts for 15 points.**

All summaries will begin with a title and student name (we do not need, or want, your ID number). Unknown numbers should be indicated following your name. (In later labs you will also indicate which group you worked with and who the other members of that group were.)

Begin by stating your initial competing hypotheses. There may be many. These are worth a total of 10 points.

We do not need an introduction or a description of materials and methods.

You need to present your analyzed, organized, collected data and results; recognize TLC data needs to be presented as R_f values, not distances run on a TLC plate. It is suggested that you use some form of table to help you recognize which reference standard properties differ from those of your unknown. Something like the following might be used:

Property	Compound											
	T	E2	P4	Cort	IAA	Gibb.	Epi.	T4	Ins.	hCG	OT	Unk
Color												
Texture												
MP												
TLC 2% HOAc												
TLC 1:1 Hex:EtOAc												
Spec. pks												
MW?												
Other = ?												

You need to populate as much of this table as possible to allow you to rule out competing hypotheses. This section is worth 35 points.

Next you need to **state your rules for excluding possible competing hypotheses.** What are your assumptions and criteria for distinguishing one MP value from another one; same question for R_f values? How did you decide UV-visible spectra differed? **This section is worth 20 points.**

Next you need to **present your logic for eliminating competing hypotheses and drawing a final conclusion on the identity of your unknown.** Recognize the logic is much more important than whether you got the identity right or not. This is where that infamous "negative inference" plays a role. **This section is work 20 points.**

The summary should end with the numerical list of references that you cited earlier in the narrative (see the section on references above).

Briefly:

- Start with competing hypotheses.
- Collect and organize data to allow testing and eliminating competing hypotheses.
- Test the hypotheses against the data.
- If an hypothesis is not supported by the data, eliminate the hypothesis.
- If an hypothesis is supported by the data, it cannot be eliminated.
- Ultimately you should be left with only one viable hypothesis that is not eliminated by the data; that identifies the unknown (when you start with a limited universe of possibilities).

We look forward to seeing your summaries.

IMMUNOASSAYS

A key tool in the development and conduct of modern endocrinology is the immunoassay. It is now used to measure both small molecules such as steroids and large ones including proteins of many kinds including hormones and even nucleic acids. The competitive version of the assay involving one antibody and a radioactively labeled version of the analyte (radioimmunoassay, RIA) that was originally developed and described by Yallow and Bernstein for measurement of insulin has given way to a broad spectrum of assay variants. Small analytes/molecules most often are measured using assays very similar to the original RIA with the substitution of a colored, fluorescent, enzyme-labeled, or particle-labeled version of the analyte being used to compete with the analyte in the sample for binding to a limiting amount of high-affinity antibody either suspended in solution and subsequently precipitated by addition of a secondary anti-antibody preparation or bound to a surface such as a plastic bead or plate. While large molecules can be measured in competitive IAs, they are now most often measured in some variety of non-competitive immunoassay or immunometric assay (IMA) where one antibody directed against a part of the molecule surface is first immobilized in a non-limiting amount onto a surface so it cannot move. Analyte containing solutions, either standard controls or unknowns, are incubated with the immobilized antibody so the analyte can bind. A second antibody that is modified to make it visible or quantifiable, *e.g.*, radiolabeled, enzyme labeled, particle labeled, fluorescently tagged, *etc.*, is then added in non-limiting amount and allowed to bind to the analyte molecules present. If all the analyte molecules have been adsorbed to the first immobilized antibody, the second antibody-conjugate will form a "sandwich" in which the analyte is the limiting "cheese" in the sandwich. A wash of the plate to remove non-bound second-antibody conjugate then allows the detection and quantitation of the amount of immobilized conjugate topping the "sandwich." By inference, that only occurs when analyte is present so that measure is an index of the amount of analyte present in the sample. By running samples that contain known masses of analyte in these assays in wells that are separate from, but otherwise identical to, those used for unknown samples the analyst can generate a standard curve for that analyte in that assay. Since the results for the standards describe the universe of possible results for the same analyte run under the same conditions in the same assay, the response for any unknown containing that same analyte should fall on the same curve. So long as we know what the volume and dilution of the original sample was placed into the assay, we can ascertain what mass is found in unknown well and what mass and concentration exists in the original unknown sample. This

information can then be compared to tables of published values to ascertain if the sample came from a subject with normal or abnormal health, from a male or a female, from a young or old subject, from a pregnant or non-pregnant subject, *etc.* Note that many of these same questions arise in human medicine, veterinary medicine, environmental research, forensics, basic biological research, pharmaceutical development, and regulatory biology.

This laboratory will allow students to run both non-competitive and competitive immunoassays and to solve a biomedical/forensic puzzle while doing so. The class will work in groups. Each group will be issued a series of 8 tubes that have been synthesized by the instructor with one or more of the three possible analytes that will be measured. Each group will run two different assays; the second one will monitor two analytes with its results reflecting one or both of them. Each assay will involve measurement of a set of control standards of known concentration along with measurement of the analyte solution or several dilutions of that solution. The results for both the controls and the unknowns will be recorded for each assay. Then, using that information plus information students can find in the library or on the Internet, each sample will be labeled as to gender and possible physiological status (young, old; pregnant, non-pregnant; phase of menstrual cycle, etc.). The solution of the unknown grid constitutes the report for the exercise.

Sample & Standard Storage

Before starting the ovulation test assays label a 9x12 deep-well storage block with your group name. Place 1000 uL = 1.0 mL of each of the undiluted unknowns into one well of the first column of the block: so 1000 uL of unknown S goes into block well A1, 1000 uL of unknown T goes into block well B1, etc. Into the third column of your group's block put 1000 uL of each of the 5 pregnancy test standards (0, 3, 10, 30, 100 mIU hCG/mL), e.g., 1000 uL of 3 mIU/mL hCG goes into B3, 1000 uL of 30 mIU/mL hCG goes into D3, etc. Finally, into the fifth column of your group's block put 1000 uL of each of the 7 ovulation test standards: (10 ng EG & 10 mIU LH)/mL in A5; (50 ng EG & 10 mIU LH)/mL in B5; (50 ng EG & 50 mIU LH)/mL in C5; (50 ng EG & 150 mIU LH)/mL in D5; (150 ng EG & 10 mIU LH)/mL in E5; (150 ng EG & 50 mIU LH)/mL in F5; (150 ng EG & 150 mIU LH)/mL in G5. Place a plastic seal over this block and set it aside until testing the unknowns. Note these same blocks could be used to dilute the samples prior to testing if that were required.

TABLE I. Deep-well Plate Template for the Estrone Glucuronide/Ovulation Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	US		PS0		OS1							
B	UT		PS3		OS2							
C	UU		PS10		OS3							
D	UV		PS30		OS4							

E	**UW**		**PS100**		**OS5**							
F	**UX**				**OS6**							
G	**UY**				**OS7**							
H	**UZ**											

Notes:

PS# signifies the pregnancy or hCG control standard number used in each well; hCG concentrations are in mIU/mL. OS# signifies the ovulatory or estrone-glucuronide, EG, and LH standard used in each well: (10 ng EG & 10 mIU LH)/mL in A5; (50 ng EG & 10 mIU LH)/mL in B5; (50 ng EG & 50 mIU LH)/mL in C5; (50 ng EG & 150 mIU LH)/mL in D5; (150 ng EG & 10 mIU LH)/mL in E5; (150 ng EG & 50 mIU LH)/mL in F5; (150 ng EG & 150 mIU LH)/mL in G5.

The standards for the assays will be made up in advance and will be stored at the front desk. **Keep the order and orientation of the blocks as shown! If you do not you will not be able to match the results of the assay to the standard or sample that you used in each assay.** ☹

Noncompetitive hCG Assay

A non-competitive assay for hCG (and/or LH) will be run by using materials from home pregnancy test kits. For these the capture antibody is immobilized on one of two strips marked on a paper test pad glued to a plastic strip. The reporting, latex bead-conjugated, antibody is impregnated into a portion of the pad above the maximal fill position for the urine. Control standards are provided in a series of marked tubes. The protocol is as follows.

1) Use a marking pen to label each of 26 strips and test tubes with numbers from 1 to 26 plus the letter of your group (A,B,C,D,E,F, or G). Record the strip numbers in one column in your notes with room to also record an estimate of the results and to tape the finished strips onto the note page.
2) To each of the first 5 tubes add 0.5 mL of one of the control standards. Repeat this for tubes 6 to 10. You should have two tubes containing samples of each of the 5 control standards. To each of tubes 11 and 12 add 0.5 mL of unknown S. To each of tubes 13 and 14 add 0.5 mL of unknown T. Repeat for each unknown until you have liquid in all 26 tubes (1 & 6 = 0 mIU/mL hCG control, 2 & 7 = 3 mIU/mL hCG control, 3 & 8 = 10 mIU/mL hCG control, 4 & 9 = 30 mIU/mL hCG control, 5 & 10 = 100 mIU/mL hCG control; 11 & 12 = unknown S, 13 & 14 = unknown T, 15 & 16 = unknown U, 17 & 18 = unknown V, 19 & 20 = unknown W, 21 & 22 = unknown X, 23 & 24 = unknown Y, 25 & 26 = unknown Z.
3) Take the appropriately labeled test strips and place them into the concordant tubes. Keep your fingers off the test pads and make sure they are immersed in the liquid in the tubes to no higher than the indicated maximal fill line.

4) Incubate the strips in the tubes for 2-3 minutes. Be consistent for all the strips on the timing. Try to take them all off within a minute or two of each other at most.
5) Remove the strips and evaluate the test lines on the pads on each strip.
6) Place the control sample pairs next to one another (1 & 6, 2 & 7, etc.) and note the increase in color that should be apparent on the test line as the concentration of hCG increases (note that the antibodies used may also detect LH so you would get the same effect by using a series of LH controls). The 0 control is a blank and should not generate a colored test line.
7) Place the unknown sample pairs next to one another and note if each pair of strips generates a similar result. Then compare the unknown test lines with the controls and see which controls match or bracket the unknowns. Record your estimates of hCG (1 mIU hCG ~ 0.1ng hCG ~ 0.1 ng LH ~ 1 mIU LH) concentration in the unknowns.
8) Compare your estimates with the ranges previously reported for males and females, young vs old, etc. See if the data allows you to classify the unknowns.

Competitive Assays for Estrone Glucuronide and Non-competitive Assay for LH

Estrogen levels during the normal menstrual cycle are higher in females than the levels seen in males. During steroid metabolism and excretion many steroids are oxidized and/or conjugated to sugars or other compounds to make them more water soluble and more easily eliminated in urine. Estradiol-17β usually appears in urine as one of several conjugates, the most common of which is estrone-3-glucuronide. Estrogen levels in reproductive age women who are not using chemical contraceptives follow a pattern. They are low during active menstruation and the very early part of the follicular phase of the cycle, rise rapidly toward the mid-cycle and the time of ovulation, then fall back somewhat only to rise to a generally lower but still significant broad peak during the latter half, or luteal phase, of the cycle. If pregnancy occurs the levels rise throughout pregnancy to very high levels by the third trimester. For women taking combination steroid contraceptives the estrogen levels in blood and urine are quite measurable by many steroid assays. For those taking only progesterone mimics, estrogen levels are generally low as they will be in women who have not gone through puberty, have passed through menopause, are lactating, or have a cessation in cycles for unknown causes. The steroid patterns in males and females of most mammals exhibit variations that follow the general patterns outlined above.

Since steroids are small molecules two antibodies cannot bind simultaneously to individual steroid molecules. Their assays are normally done in a competitive format with a limiting amount of steroid specific antibody being allowed to bind to a mixture of the unlabeled analyte and a labeled or conjugated form of the analyte. This molecular version of musical chairs generates a signal from those conjugated analyte molecules that end up bound to the antibody after any unbound analyte or conjugate molecules have been washed away. Since binding to unbound steroid analyte would preclude binding of the conjugate, the signal strength is inversely proportional to the amount of unbound analyte present. Thus, in a competitive assay the assay signal strength declines with increasing unlabeled analyte concentration. It does not increase as in a non-competitive assay.

Home fertility testing sometimes uses monitoring of estrogen metabolite levels as a means to track progression through the mid- to late part of the follicular phase of the ovarian cycle, the segment preceding the LH surge and ovulation near the middle of the normal menstrual cycle. The tests incorporate a limited amount of immobilized anti-estrogen and an enzyme or other labeled form of

estrogen-conjugate which competes with the estrogen-conjugate that is present in the biological sample being tested. As urine moves up the sampling stick it wets the deposit of labeled estrogen-conjugate and the substrate for the enzyme used, these mix with the sample and move up the strip to the zone where the anti-estrogen antibody is immobilized. Once the antibody binding sites are filled, any remaining labeled conjugate, unlabeled analyte, and enzyme substrate will move beyond the competition zone. This can be enhanced by putting a large excess of anti-estrogen antibody in a second zone further away from the dipping end of the sampling strip. The second zone draws the unbound labeled tracer away from the competition zone and allows verification that the enzyme in that conjugate is active, that the test is working. By reading the color intensity (or absorbance) of the competition zone using an LED lamp and a wavelength-tuned diode the result of the competitive immunoassay can be ascertained (we will be doing this visually without an electronic reader). Storing this information allows subsequent results to be compared with the initial test. This comparison allows upward or downward trends to be identified. In the test we will be using, a strong upward trend for estrogen triggers a decision by the test to stop monitoring estrogen and, instead, to follow LH which is usually being tracked using a non-competitive assay akin to the one used in the pregnancy tests we described above. In a normal mammalian cycle a rapid rise in estrogen above a minimal, species and individual specific, baseline stimulates the rapid and repeated release of LHRH followed quickly by the release of LH. When LH levels rise to near a peak they trigger physiological, biochemical, and morphological changes in the ovary and, soon thereafter (within 17 hours in the human) the release of the mature oocyte, which is now ready for fertilization. The test monitor expresses rapid rises in estrogen as flashing happy faces and near-peak and peak LH levels as stable smiles. These are semi-quantitative symbols of the underlying quantitative test results. (http://www.clearblueeasy.com/healthcare/clearblue-advanced-digital-ovulation-test.php)

Here each group will run an assay unit for estrone glucuronide and LH. The assays will be conducted per the manufacturer's instructions except that we will test a set of knows and unknowns as serial measurements with no more than 3-5 min per sample. The test strips will be dipped into one of the standards or unknown samples in the labeled deep-well blocks you set up earlier. **Do the strips one at a time as they must be read within a short period of time after sample exposure and each reading will take about 3 – 5 min.** The strips can be read by eye using similar strips that have been tested identically as comparison references. After reading each sample, record the result and prepare to read the next sample.

In each assay record in your notes the order and placement of your samples in the plate wells. Follow the grid in Table 1 (above) so that your readings can be compared to those of the standards.

The summary for this experiment will involve filling out the assay response grid we provide and then drawing a conclusion about the probable physiological states of the unknown sample donors. The summary should be no longer than 2 pages and should include references cited.

Rubric for Summary on Immunoassay Labs and Unknowns

Each experimental summary in Endocrinology will be no more than **2 pages long** and will be worth **100 points** in the lab.

It will follow the suggestions in a rubric issued for the benefit of the students and the TAs who will be grading these summaries.

All summaries will be typed and submitted as hard copies at the times indicated in the lab manual schedule. Late copies will be penalized 5 points per day, including weekends.

You must **list all the published and/or on-line references that you used in the summary. They need to be marked in the narrative and results sections as citations**; first reference is number one in the reference list, second reference is number two, *etc.* **This counts for 15 points.**

All summaries will begin with a title and student name (we do not need, or want, your ID number). Immunoassay Group Number and who the other members of that group were should be indicated following your name.

We do not need an introduction or a description of materials and methods.

Begin by stating your initial competing hypotheses. There may be many. These are worth a total of 10 points. You evaluated the responses of three assays, a noncompetitive immunometric assay for hCG in the pregnancy test, a noncompetitive immunometric assay for LH in the lower line of the ovulation test, and a competitive immunoassay for estrone glucuronide (E1G) in the upper line of the ovulation test. You used these to examine 5 concentrations of known, reference standards for each of hCG (0, 20, 60, 200, 600 mIU/mL), LH (0, 30, 100, 300, 1000 mIU/mL), and E1G (0, 9, 30, 90, 300 ng/mL) and 5 unknowns that mimic what might be found in one of 5 physiological states: 1) a child/male/or amenorrheic female; 2) a menopausal female; 3) a female at mid-pregnancy; 4) a non-pregnant female in the luteal phase (latter half) of her menstrual cycle; or, 5) a female in (very) early pregnancy. *Your main tasks were to determine if the hCG and LH assays were hormone specific and to identify which of the unknowns for your group matched each of the 5 possible physiologic states.*

Note, mIU/mL refers to the number of milli-International biological assay (bioassay) Units of a hormone (normally a protein) that is contained in each mL of solution. This unitage scale arose at a time when proteins were being extracted from biological preparations like serum, urine, or the soluble portions of homogenized tissues. Because hormones act in miniscule amounts, the hormonal activity in these preparations often represented only a small portion of the total protein present. You could detect it in a bioassay, but you could not assign it an exact weight of dehydrated protein. As purifications got better, the number of mIU/mg protein, or biopotency, slowly rose to a plateau that now comes close to the biopotency of pure protein produced by molecular cloning techniques, or even chemical synthesis. The unitage is a holdover because it has been commonly used in medicine and is preserved in textbooks.

You need to present your analyzed, organized, collected data and results; immunoassay data should be listed according to which known/reference concentrations an unknown sample falls between, e.g., if the unknown hCG value is darker than that for 60 mIU/ML but lighter than that for 200 mIU/mL, it should be listed as 60-200 mIU/mL. I suggest you use some form of table to help you recognize which reference standard properties differ from those of your unknowns. Something like the following might be used:

	Hormone															Unknown				
	hCG (mIU/mL)					LH(mIU/mL)					E1G (ng/mL)					U1	U2	U3	U4	U5
	0	20	60	200	600	0	30	100	300	1000	0	9	30	90	300					
Color intensity, pregnancy test																				
Color intensity, LH test																				
Color intensity, E1G test																				
~[hCG]																				
~[LH]																				
~[E1G]																				
Physiologic status?																				

You need to fully populate this table to allow you to rule out competing hypotheses. This section is worth <u>35</u> points.

Next you need to **state your rules and present your logic or arguments for eliminating competing hypotheses and drawing a final conclusion on the identity of your unknowns.** What are your assumptions and criteria for distinguishing one unknown physiological state from another? How did you decide the physiological statuses differed? Please recognize the logic is much more important than whether you got the identity right or not. This is where that infamous "negative inference" plays a role. **This section is worth <u>40</u> points.**

The summary should end with the numerical list of references that you cited earlier in the narrative (see the section on references above).

Briefly:

- Start with competing hypotheses.
- Collect and organize data to allow testing and eliminating competing hypotheses.
- Test the hypotheses against the data.
- If an hypothesis is not supported by the data, eliminate the hypothesis.
- If an hypothesis is supported by the data, it cannot be eliminated.
- Ultimately, you should be left with only one viable hypothesis for each of the unknowns; that identifies the unknown (when you start with a limited universe of possibilities).

We look forward to seeing your summaries.

PROTEOMICS OF HORMONE FRAGMENTS

This lab exercise arises from some recent work in my laboratory. While doing this study you will be contributing to the existing research data on this topic. Basically, the idea is that many proteins bind to their target cells, induce an initial signal inside the cells by currently known transduction processes, and then are cleared from the cell surface by the internalization and processing of the hormone-receptor complex. This processing often involves endocytosis of the complexes with gradual movement of the endocytotic vesicles toward the cell nucleus. The endocytotic vesicles may have multiple possible fates as they may be routed: back toward the cell surface where the receptor may be replaced into the cell membrane while the hormone or its degraded forms may be released from the cell; toward fusion with the Golgi Apparatus where the receptor and/or hormone may be post-translationally reprocessed or reactivated so that after exocytosis from the exocytotic granules generated by the Golgi the receptor may resume its actions and/or the hormone may resume its actions or its fragments may be released from the cell or be presented to adjacent cells as part of an immune antigen-like complex on the surface of the target cell; to fusion with primary lysosomes to form secondary lysosomes where the receptor and hormone are proteolytically degraded into fragments that may eventually be released to the exterior of the cell, may be stored as part of intracellular multi-vesicular bodies, or may be released to the cell cytoplasm via one of several mechanisms. Movement of the endocytic vesicles toward the cell nucleus is accompanied by declines the interior pH in these vesicles, starting from near 7.4 at or near the cell surface to as low as 4 in secondary lysosomes. The pH declines mean that the activities of any proteases within the endosomes or lysosomes varies so that proteases like cathepsin G are more active near the cell surface while others like cathepsin D are most active in the late stages of endosome/lysosome movement. If protein hormones are not broken down to very small peptides or single amino acids, the residual peptides may themselves possess hormonal or protein modulatory actions. Our studies are beginning to explore this possibility.

The steps are initially quite simple and will be followed in this lab. 1) A protein hormone is chosen from a list of the several hundred possible. Each peptide chain is located in the existing national protein databases (e.g., NCBI, UniProt, PDB, or SWISSProt) and the full length amino acid sequence of the mature hormone is cut and pasted to a notebook. 2) This sequence is then explored for protease resistant peptides by testing it in one of several published proteolysis programs (often used to predict fragments when running mass spectral analyses of proteins) such as PROSPER established and run by a group at Monash University in Australia. We will show you how to run this program as well as to how to check the results by requesting results for only a single enzyme. Any peptides greater than 8 amino acids long that survive exposure to multiple proteases should be recorded, identified in the parent sequence, and used in the next step of the analysis. 3) The resistant peptides, if any, are used as the search sequences in classical protein BLAST searches of all existing proteins in the national protein databases. These are to be run using a local search paradigm along with the default parameters we will describe in the lab. Results for this step will produce a list of proteins that possess linear segments that are either identical to the peptides being queried or are similar in physicochemical properties as would occur by conservative amino acid substitutions during protein evolution. The longer the peptide being tested the more likely a meaningful match will be found among those proteins showing high identity/similarity values. The top 20 to 100 matches in these searches should be recorded (use cut and paste methods to capture results) and any groups of similar proteins noted (e.g., oxidases, transducers, chaperones, etc.). If matches to known peptide

hormones are found these should especially be flagged as they demonstrate the potential biological activity of the identified peptides. It will be most interesting if any patterns of peptides or protein matches arise among the group of protein hormones explored by this class.

Note that our research extends these results by looking not only for linear sequence matches but also structural sequence matches which involve three-dimensional modeling of the peptides involved and matching these to known x-ray crystallographic or similar structures for known proteins. If such matches (or even linear ones) exist for cytoplasmic or receptor proteins we would look for any protein partners that the identified proteins interact with or bind to. If the identified motifs match the sites of interactions for the identified protein and its complementary partner, it means the protein hormone proteolytic peptide may be involved in modulation of the action(s) of that pair of proteins, i.e., it may have a previously unknown biological action. Bench tests of those interactions can then be designed to test the strength or consequences of the presence of the protein hormone-derived peptides.

This exercise expands our database in this area and may lead to important new insights into how the endocrine system works!

ANIMAL EXPERIMENTS

Thursday, November 2, 2017 we will begin the first of 4 labs that involve handling animals or animal tissues. According to the rules now in place for the University as stipulated by the NIH, NSF, Department of Agriculture, NIOSH, and EPA the University must have an Institutional Animal Care and Use Committee, IACUC, which oversees and administers matters associated with animal care and use. There must be in place a set of protocols and procedures that all users of animals follow in research or training. Included in those procedures is the necessity that all individuals working with animals undertake appropriate training. This is normally satisfied by becoming certified by the CITI Program via a series of online training modules followed by minimal test questions to ascertain that the materials have been read and understood.

The attached .pdf files:
(New Users: https://www.umb.edu/editor_uploads/images/orsp/New_to_CITI_2_091616.pdf?cachebuster:72
Returning Users:
https://www.umb.edu/editor_uploads/images/orsp/Returning_User_to_CITI_2.pdf?cachebuster:83)

show you how to establish a certification account with CITI via the University of Massachusetts Boston organization, and then how to sign up to take the trainings for Responsible Conduct of Research, teaching activities that involve live animals or tissues, working with the IACUC, and working with mice (you do not need the rat module as we only work with mice in this class). If you have already completed these trainings via your associations with another class or research lab, please notify me of your account name, ID number, and the date of completion of trainings (or a copy of your training certificates). The modules are short and usually take less than 2 hours to complete in their entirety. Certifications are transferable, last for 3 years, and can be listed on your resume.

Note, these modules need to be completed before the November 2 lab. Completions will be transmitted by CITI to the IACUC office. I will need a copy to verify that you are eligible to work with the animals in the Endo lab.

HORMONE CONTROL CIRCUITS

This laboratory builds on a demonstration of the role of thyroid hormone in altering oxygen consumption and CO_2 production in mice. It will start 4 weeks prior to the final measurements of the experimental mice as this allows the time needed for the treatments to demonstrate their effects. Groups of mice will be treated with exogenous thyroxine or thyroid powder which should move them toward a hyperthyroid state, with methimazole, a goitrogen, which should move them toward hypothyroidism, or with nothing. The animals will be monitored for weight gain and basal metabolic rate using a computerized system that uses sensors to monitor both oxygen consumption (oxygen electrode) and CO_2 production (infrared sensor detects the IR signal of the C=O bond). Changes with respect to basal metabolic rate over time and type of treatment will be used to ascertain the degree of alteration of the feedback control circuits generated by the pharmaceutical agents. Details of the protocol and means of analyses will be arrived at during laboratory discussions. Students should come prepared to discuss what they have read concerning the control of the thyroid axis and its modification during periods of hyperthyroid and hypothyroid status. Links between thyroid status and general metabolic function should also be explored prior to the lab.

MECHANISM OF ACTION EXPERIMENTS

The protocol for these experiments will be discussed with the students in the lab. The actions of oxytocin on contraction of mouse myometrial muscle will be examined. Students are encouraged to search out information on this system and come prepared to share that information. The idea will be to attempt to elucidate the pathway(s) by which oxytocin is acting via pharmacological manipulations of intracellular signaling pathways. Drugs known to stimulate or inhibit specific pathways will be available. Decisions on designing the experiment will be made in classroom discussions.

Note: this lab requires students to make up solutions according to published bioactive concentrations. Be prepared to calculate and physically work with molar and/or weight/volume solutions. Be prepared to carry out dilutions from liquids already prepared as well as to prepare solutions from dry raw materials. Be ready to conduct serial dilutions. Think about how masses are measured and transferred, how volumes are measured and transferred, and how it is possible to produce very low concentrations of chemicals in solution without producing liter quantities of potentially bioactive stock solutions.

The protocol and design for this experiment are again fairly simple. The details and analysis required are more challenging.

Basically you will be trying to see if you can figure how oxytocin acts to stimulate smooth muscle contraction in short segments of mouse uterus by using the hormone alone or in combination with drugs that are known to stimulate or inhibit various steps in transduction cascades. To start, check your text for the current model of the mechanism of action for oxytocin:

OT → stimulation of a G-coupled receptor (Gαq) → stimulation of phospholipase – β → ↑ in IP3 & DAG → ↑ of intracellular Ca++ & activation of protein kinase C

Do note, however, that elevations of DAG are often accompanied by increased activity of phospholipase A2 which leads to release of arachidonic acid which is a substrate for cyclooxygenases 1 and 2 (Cox 1/Cox 2) which produce prostaglandins. Prostaglandins are known to stimulate muscle contraction and act by binding to G-coupled receptors of their own which can stimulate production of cAMP and activation of protein kinase A.

So what's most important here, activation of the pKC path by OT or the pKA path by prostaglandins?

You should use the drugs with and without added oxytocin to find out what steps are most crucial to contraction. We will make stock solutions of these drugs available for use in the lab (usually at micromolar or microgram/mL concentrations) along with media to allow you to dilute them to working concentrations. Before the lab you will need to look up what these drugs do and what concentration is needed for each of them to inhibit or stimulate the pathways involved. In using these compounds on tissues you are best advised to add the needed amount of drug in a total of 50 uL of media to the tissue already suspended in 5mL of unsupplemented media, *i.e.*, any drug solution you make from the stocks provided should be 100-fold more concentrated than the final, working concentration you are intending to expose the tissue to. If you decide to add a drug and oxytocin to the same mixture (*e.g.*, to see if the drug blocks the action of oxytocin), you should add them separately to the same dish containing basal medium and unexposed tissue. Do not attempt to reuse tissue fragments once they have been exposed to a drug and/or hormone. **Be very careful in working with the concentrated drugs and hormones. They are very potent! Think gloves and glasses minimally.**

You will again be working in small groups on a simple bioassay system. This time you will be looking at small segments of mouse uterus which contains myometrial smooth muscle that has been exposed to estradiol. The muscle is normally a target for oxytocin but becomes more sensitive to it under the influence of estrogens. The muscle responds to the presence of oxytocin by contracting. You will be able to observe a response within seconds of tissue exposure to the hormone and you can quantitate that response by taking before and after measurements of tissue length using a millimeter ruler or a caliper micrometer and computing the percentage of tissue shortening that occurred. Both measures are made more precise by doing the observation and measurement under a dissecting microscope.

The tissues will be dissected on the morning of the lab from mice injected subcutaneously 3 days previously with 5 ug each of estradiol suspended in vegetable oil; several students may volunteer to help with dissections and/or injections. Dissections will be done in the surgical room in the animal care suite. The uterine segments will be placed into a simple culture medium such as Earle's or Hank's basal salt solutions. They will be delivered to the lab on ice and made available by the instructor as needed.

The approach is best designed as a simple treatment versus non-treatment grid with no more than 8 grid cells aimed at looking at one or two steps of the potential mechanistic pathway. If the class coordinates its efforts, you should be able, collectively, to say whether the pKA or pKC paths are more important in producing the contractions observed.

Sample treatment grid (one piece of uterine tissue per cell, test cells may be repeated if time and tissue allow it):

Uterine segment lengths in mm

	No drugs		+ inhibitor 1 [xx]		+ inhibitor 2 [yy]		+ stimulator 1 [zz]	
	before	after	before	after	before	after	before	after
Without oxytocin added								
With oxytocin added								

After length data are collected, compute any % changes in lengths, any averages and variances for replicated treatment cells, and try to make sense of how these results correlate with the suggested mechanisms of actions currently known for OT and prostaglandins. What drugs block or stimulate best? Try to answer the question about the relative importance of the pKA versus the pKC pathway.

The summary for this experiment will include: 1) the reasons for the drugs chosen; 2) the results grid reduced to % change in tissue length and a decision on the effect of each drug (e.g., stimulates alone, inhibits alone, no effect alone, synergizes with OT, blocks OT, no effect on OT); 3) a conclusion on the importance of the pKA versus the pKC pathway for OT action. The summary should be 2 pages long and include references cited.

Drug	**Known target & action**
Caffeine	Inhibits phosphodiesterase
Isobutylmethylxanthine (IBMX)	Inhibits phosphodiesterase
Ibuprofen	Inhibits Cox 1/2
Indomethacin	Inhibits Cox 1/2
Phorbol 12-myristate 13-acetate	Stimulates protein kinase C
Staurosporin	Inhibits protein kinases A, C, G
Phloretin	Inhibits protein kinase C
Genistein	Inhibits protein kinase C
Dibutyryl cAMP	Stimulates protein kinase A
Calcium ionophore plus EGTA in media	Decreases intracellular calcium
Prostaglandin E_1	Stimulates prostaglandin E_1 receptors
Polymyxin B sulfate	Inhibits protein kinase C

Drug	**Known target & action**	**EC 50**	**Working Stock [ ] = 100xEC50**	**Storage Stock [ ]**
Oxytocin	Smooth muscle contraction	50 ng/min human; 10 ng/L = 10 pg/mL	1 ug/L = 1 ng/mL	10-20 ug/mL
Caffeine	Inhibits phosphodiesterase	100 mg/L = 100 ug/mL	10 mg/mL	20 mg/mL
Isobutylmethylxanthine (IBMX)	Inhibits phosphodiesterase	5 mM = 5 umole/mL = 1111.2 ug/mL	50 mg/mL	50 mg/mL

Ibuprofen	Inhibits Cox 1/2	10 mg/L = 10 ug/mL	1 mg/mL	100 mg/mL
Indomethacin	Inhibits Cox 1/2	1 mg/kg = 1 mg/L = 1 ug/mL	100 ug/mL	10 mg/mL
Phorbol 12-myristate 13-acetate	Stimulates protein kinase C	1 nM = 1 pmole/mL = 616.8 pg/mL	61.68 ng/mL	10 ug/mL
Staurosporin	Inhibits protein kinases A, C, G	10 nM = 10 pmole/mL = 4.6653 ng/mL	1 nmole/mL	100 nmoles/mL
Phloretin	Inhibits protein kinase C	5 uM = 5 nmole/mL = 1371.35 ng/mL	137.135 ug/mL	10 mg/mL
Genistein	Inhibits protein kinase C	15 uM = 15 nmole/mL = 4053.6 ng/mL	250 nmoles/mL	250 nmoles/mL
Dibutyryl cAMP	Stimulates protein kinase A	1 mM/L = 1 uM/mL= 491.4 ug/mL	49.14 mg/mL	50 mg/mL
Calcium ionophore plus EGTA in media	Decreases intracellular calcium	1 uM & 5 mM = 1 nmole/mL & 5 umole/mL = 523.62 ng/mL & 1901.75 ug/mL	52.362 ug/mL & 250 mM	100 ug/mL & 250 mM
Prostaglandin E_1	Stimulates prostaglandin E_1 receptors	0.1 ng/mL	10 ng/mL	1 ug/mL
Polymyxin B sulfate	Inhibits protein kinase C	10 uM = 10 nmoles/mL = 13.8561 ug/mL	1.38561 mg/mL	100 mg/mL

Anticipated Results of Mechanism of Action Study

Drug	Known target & action	Expected Result	
Caffeine	Inhibits phosphodiesterase	**↑ cAMP & ↑ Contraction**	
Isobutylmethylxanthine (IBMX)	Inhibits phosphodiesterase	**↑ cAMP & ↑ Contraction**	
Ibuprofen	Inhibits Cox 1/2	**↓ Prostaglandin & ↓ Contraction**	
Indomethacin	Inhibits Cox 1/2	**↓ Prostaglandin & ↓ Contraction**	
Phorbol 12-myristate 13-acetate	Stimulates protein kinase C	**↑ Contraction**	
Staurosporin	Inhibits protein kinases A, C, G	**↓ Contraction**	
Phloretin	Inhibits protein kinase C	**↓ Contraction**	
Genistein	Inhibits protein kinase C	**↓ Contraction**	
Dibutyryl cAMP	Stimulates protein kinase A	**↑ Contraction**	
Calcium ionophore plus EGTA in media	Decreases intracellular calcium	**↓ Contraction**	

Prostaglandin E_1	Stimulates prostaglandin E_1 receptors	**↑ Contraction**	
Polymyxin B sulfate	Inhibits protein kinase C	**↓ Contraction**	

Rubric for Summary on Mechanism of Oxytocin Action

Each experimental summary in Endocrinology will be no more than **2 pages long** and will be worth **100 points** in the lab.

It will follow the suggestions in a rubric issued for the benefit of the students and the TAs who will be grading these summaries.

All summaries will be typed and submitted as hard copies at the times indicated in the lab manual schedule. Late copies will be penalized 5 points per day, including weekends.

You must **list all the published and/or on-line references that you used in the summary. They need to be marked in the narrative and results sections as citations**; first reference is number one in the reference list at the end of the summary, second reference is number two, *etc.* **This counts for <u>15</u> points.**

All summaries will begin with a title and student name (we do not need, or want, your ID number). Unknown numbers should be indicated following your name. (In later labs you will also indicate which group you worked with and who the other members of that group were.)

Begin by stating your initial competing hypotheses. There may be several. A figure may help here. These are worth a total of <u>10</u> points.

We do not need an introduction or a description of materials and methods except for which drugs you used and how you computed your % change in tissue length.

You need to present your analyzed, organized, collected data and results;

% Change in Tissue Length in 2 min Following Hormone and/or Drug					
Hormone?	***Compound***				
	Media	**Drug 1**	**Drug 2**	**Drug 3**	**Drug 4**
- Oxytocin					
+ Oxytocin					

You need to populate this table to allow you to rule out competing hypotheses. Raw data are not acceptable. This section is worth <u>25</u> points.

Next you need to **state your rules for excluding possible competing hypotheses.** What are your assumptions and criteria for distinguishing one measured change in tissue length from another one; a stimulation of the tissue by a compound alone, an inhibition of oxytocin action, an enhancement of oxytocin action? How did you decide a compound changed the action of oxytocin via the PKA pathway, the PKC pathway? **This section is worth <u>25</u> points.**

Next, **present your logic for eliminating competing hypotheses and drawing a final conclusion on whether the PKA or PKC pathways are more important for oxytocin action.** Recognize the logic is much more important than whether you get the identity of the path right or not. This is where that infamous "negative inference" plays a role. **This section is work 25 points.**

The summary should end with the numerical list of references that you cited earlier in the narrative (see the section on references above).

Briefly:

- Start with competing hypotheses.
- Collect and organize data to allow testing and eliminating competing hypotheses.
- Test the hypotheses against the data.
- If an hypothesis is not supported by the data, eliminate the hypothesis.
- If an hypothesis is supported by the data, it cannot be eliminated.
- Ultimately you should be left with only one viable hypothesis that is not eliminated by the data; that identifies the mechanism of action (when you start with a limited universe of possibilities).

We look forward to seeing your summaries.

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