

**Appendix D - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL**

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PROJECT TITLE: Suppression of Pituitary-Adrenal Axis with
Dexamethasone: Comparison of IV versus IM

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Frank M. Andrews, DVM Faculty Mentor

Date: 30 April 98

Comments (Optional):

STRUCTURE, FUNCTION, AND USE OF CORTICOSTEROIDS

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University Honors Program

ABSTRACT

Dexamethasone, a powerful corticosteroid, is a frequently used drug in the realm of veterinary medicine. However, many times it is used without prior knowledge as to why or how it may produce the desired effect, or without consideration as to the best method of administration.

The purpose of this project is two-fold. First, it will consist of some basic research in order to clarify the mode of action of dexamethasone. In brief, corticosteroids are hormones produced by the adrenal gland, which is located adjacent to the kidney. The production of corticosteroids is subject to feedback regulation, where a build-up of corticosteroid will shut down the production of any further hormone.

The second phase of this project consists of a field study using six equines. Dexamethasone, a synthetic corticosteroid, was administered both IV (intravenously) and IM (intramuscularly) to determine the effects on regulation of the production of corticosteroids. The initial hypothesis was that blood cortisol levels would be equally suppressed in horses given dexamethasone IV as in those given dexamethasone IM. The final results of the study served to support this hypothesis.

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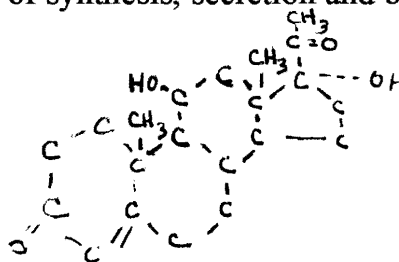
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CORTICOSTEROIDS: A LOOK AT BASIC STRUCTURE AND FUNCTION

INTRODUCTION

The human body is undeniably complicated. Yet in its complexity, it is incredibly efficient and accurate in its functional abilities. One example of this is its ability to synthesize and respond to a group of hormones known as glucocorticoids (or glucocorticosteroids), produced by the adrenal cortex. In this paper, the glucocorticoids will be introduced, covering areas of basic structure, pathway of synthesis, secretion and binding to receptors, and effects of the hormones in the body.

STRUCTURE



All of the steroid hormones (including glucocorticoids) have the same basic core structure, consisting of three six-carbon rings and one five-carbon ring. In order for them to have their anti-inflammatory and glucogenic ability, they must have a functional hydroxyl group (-OH) at carbon 11. Additionally, they must have a functional -OH group at carbons 21 and 17 in order to be maximally active. In this form, with an -OH at carbons 21 and 17, the steroid is said to be in its alcohol form. As such, it is classified as a lipid with the characteristic result that it is insoluble in water.

Because of its insolubility, a steroid will require a protein carrier in order to travel in an aqueous media (i.e. the plasma). The steroid will then be released into its target cells from the protein-hormone complex fairly slowly, in contrast to some synthetic steroids which are

water soluble, do not require a carrier, and are released quickly to their targets.

Additionally, there are different ways in which steroids can be chemically modified. Using hydrocortisone as an example, there is esterification at carbon 21. The addition of succinate or phosphate at this location will result in a compound that is truly water soluble. However, esterification at carbon 21 with butylacetate, acetate, acetonide, methyl acetate, dipropionate, or pivalate causes the formation of a water insoluble ester form of the lipid that traverses membranes much more slowly than the succinate and phosphate forms. However, since this causes the hydrocortisone to be absorbed more slowly, it also tends to increase the duration of its action.

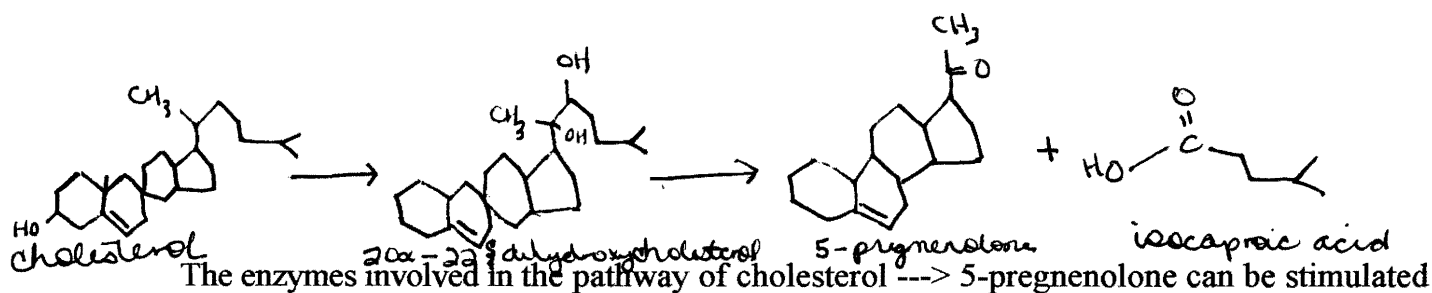
However, in order to be active, these esters must be hydrolyzed after crossing the membrane in order to convert the carbon at position 21 back to its active -OH form. This conversion, which takes place intracellularly, occurs most rapidly for phosphate and succinate esters, while other forms take longer.

There are also several alterations which can be made to the basic structure of hydrocortisone that produce changes in its activity. Forming a double bond between carbons 1 and 2, adding fluorine, adding a methyl group, and adding an -OH group all can lead to increased anti-inflammatory potency and increased gluconeogenic potency.

BIOSYNTHESIS

The biosynthesis of glucocorticoids begins with the breakdown of cholesterol. The pathway for the formation of cortisol, the most common glucocorticoid in humans, is known

to be cholesterol ---> pregnenolone ---> progesterone ---> 17-hydroxyprogesterone ---> cortisol. The conversion of cholesterol to 5-pregnenolone is carried out by a series of enzymes found in the mitochondria of the adrenal cortex. The first reaction results in the formation of a 20 α -hydroxyl in the side chain of the cholesterol molecule by a 20 α -hydroxylase enzyme, followed by hydroxylation at the 22 position by 22-hydroxylase enzyme, yielding 20 α -22 β -dihydroxycholesterol. Both of the hydroxylase enzymes require NADPH for the introduction of molecular oxygen to form the hydroxyl group. Then, a cleavage occurs between the hydroxyl groups at the 20 and 22 positions by a desmolase enzyme. This yields two products, 5-pregnenolone and isocaproic aldehyde. An interesting fact which will bear more importance later on in this paper is that the reaction sequence of cholesterol ---> 5-pregnenolone in the mitochondria has been considered to be one of the principal sites at which ACTH is able to regulate steroidogenesis.

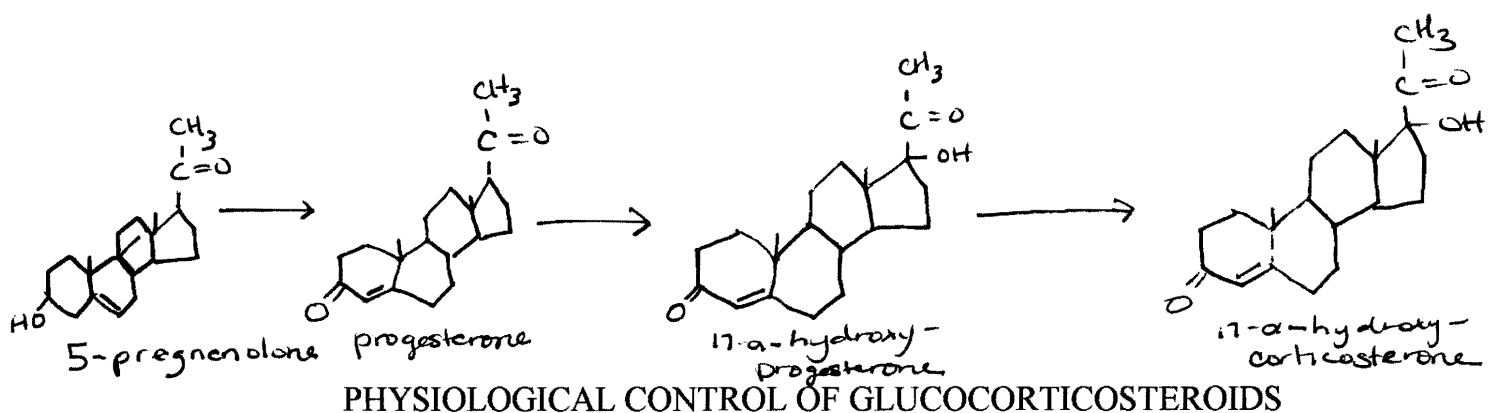


by the metabolism of citric acid cycle substrates such as isocitrate, malate, and succinate, which generate reducing equivalents for the respiratory chain leading to steroid hydroxylation. They can also be stimulated by an increased rate of reduction of NADP⁺ generated from the glucose-6-phosphate dehydrogenase reaction found in the pentose phosphate pathway. Additionally, high levels of ACTH in the blood can also stimulate the

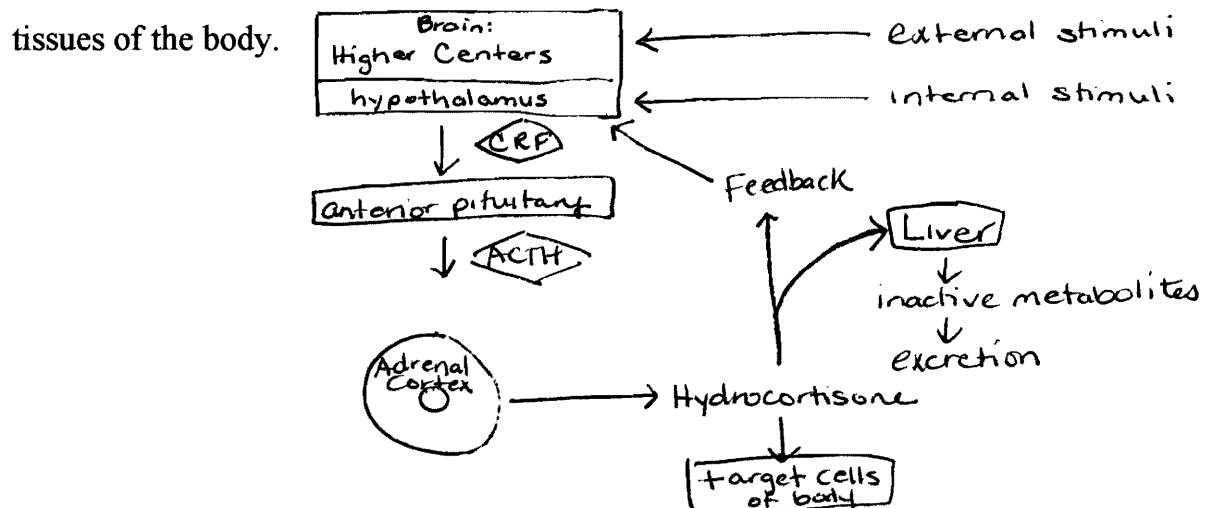
conversion of cholesterol to pregnenolone.

The next step in the sequence of steroidogenesis sees the chemical conversion of pregnenolone into progesterone, a series of reactions which also occur in the mitochondria of the adrenal cortex. The first reaction is the removal of two hydrogens from the -CHOH group at position 3 by a 3-hydroxysteroid dehydrogenase specific for pregnenolone, which uses NAD⁺ as an electron acceptor. Thus, the enzyme converts the 3-hydroxyl group to a 3-ketone. The NADH formed may be reoxidized in the mitochondrial electron-transport sequence. The second enzyme in this sequence is called Δ^5 -3-ketosteroid isomerase and results in the shift of the double bond from the 5 to the 4 position, yielding the product progesterone.

The final two steps in the formation of cortisol are less complex. Progesterone is converted to 17 α -Hydroxyprogesterone by the enzyme 17 α -Hydroxylase, which utilizes NADPH as an electron donor. The enzymes 21 and 11 hydroxylases then convert 17 α -hydroxyprogesterone into 17 α -hydroxycorticosterone (hydrocortisone). This completes the pathway of the conversion from cholesterol ---> hydrocortisone.



As the synthesis of hydrocortisone occurs in the mitochondria of the adrenal cortex, it is useful to examine the system by which this synthesis is controlled. The hypothalamus, the anterior portion of the pituitary gland, and higher centers in the brain all work together to regulate the activity of the adrenal cortex and thus the levels of glucocorticosteroids in the blood. In this physiological pathway, the higher centers of the brain may send nervous signals to the hypothalamus. The hypothalamus responds by releasing Corticotropin Releasing Factor (CRF) into the blood supply, allowing it to be carried to the anterior pituitary. The CRF then stimulates the anterior pituitary to release Adrenocorticotrophic Hormone (ACTH). ACTH travels in the blood stream to the adrenal cortex where it causes the release of hydrocortisone into the animals blood stream, thus being distributed to all



There are two primary control mechanisms whereby this system is regulated. The first one involves steroid feedback, where the level of hydrocortisone in the plasma regulates further hydrocortisone release from the adrenal cortex by a feedback mechanism. This feedback mechanism occurs at the level of the hypothalamus. Additionally, it is interesting to note that the hypothalamus cannot tell the difference between hydrocortisone and some of the

chemically synthesized analogues such as Prednisolone, Dexamethasone, or Flumethasone.

Thus, injection with any of these drugs will lead the hypothalamus to conclude that levels of hydrocortisone are high, thus decreasing production of CRF. An interesting study published by Altemus et al. in 1997 examined the reduced sensitivity to glucocorticoid feedback and reduced glucocorticoid receptor mRNA expression in the luteal phase of the menstrual cycle. The results indicated that reduced glucocorticoid feedback regulation of the hypothalamus-pituitary-adrenal axis may play a role in the generation of the common premenstrual symptoms of irritability and dysphoria.

The second control mechanism used involves the nervous system. As the nervous system is sensitive to changes in environment, it may respond by sending stimuli to the higher centers of the brain, beginning the pathway that leads to the production of hydrocortisone. By this means, animals are able to adapt to stress. Harmful stress stimuli such as fear, noise, allergic reactions, and trauma turn this system on and increase the release of hydrocortisone.

GLYCOLYSIS AND GLUCONEOGENESIS

One of the important functions of glucocorticosteroids in the body is their role in the production of glucose from short-chain precursors by gluconeogenesis. In this process, glucocorticosteroids act to inhibit protein synthesis in peripheral tissues, thus releasing free amino acids from muscle and other tissues to the liver. Once in the liver, these amino acids then undergo transamination and other reactions to form pyruvate or oxalacetate, and then eventually are converted into glucose by the gluconeogenic pathway. The nitrogen from the

amino acids is converted to urea by the liver. Furthermore, glucocorticosteroids also increase the breakdown of lipids in adipose tissues, thus releasing free fatty acids and glycerol. Glycerol is a precursor of glucose in the liver, whereas free fatty acids inhibit the utilization of glucose by the liver. Thus, the initial effects of glucocorticosteroids is to increase amino acid precursors for synthesis of glucose and, due to an increase in free fatty acid from adipose tissue, to decrease the activity of enzymes involved in the breakdown of glucose.

There are some additional early effects of glucocorticosteroids, although these seem to be secondary. Firstly, they induce the synthesis of transaminases which are involved in the formation of keto acid precursors from amino acids. Additionally, they seem to stimulate the synthesis of gluconeogenic enzymes concerned in the synthesis of glucose from the keto acids.

However, it should be noted that the action of the glucocorticosteroids can also have some disastrous effects if used in excess. A study performed in 1997 by McKay et al. demonstrated that some common side effects of corticosteroid therapy include muscle weakness and atrophy, which in part are mediated by the induction of glutamine synthetase. Additionally, reabsorption of bone matrix along with calcium, possible increased blood sugar, and increased glycogen deposition in the liver are also seen to occur. Furthermore, some of the glucose which is produced in excess of the body's needs may be converted into fat.

When the levels of sugar in the blood become low, two hormones are released. Glucagon,

the first hormone indirectly activates the enzyme phosphorylase α in the liver, leading to the breakdown of glycogen to form glucose. It also activates liver lipases for an increased breakdown of liver lipid with a release of free fatty acids and glycerol, the effects of which have already been examined. Epinephrine, the other hormone released in response to low blood sugar, leads to an increase in phosphorylase α . Like glucagon, it also stimulates lipase in the adipose tissues, thereby releasing free fatty acid and glycerol. But is also has an effect in stimulating the release of lactate from muscle, thus providing some precursors for gluconeogenesis. In addition, the most pertinent point is that epinephrine also is apparently a mediator in stimulating the release of ACTH from the anterior pituitary in response to a decrease in blood glucose. This increase in ACTH stimulates the adrenal cortex for an increased production of corticosteroids.

GLUCOCORTICOSTEROIDS AND THE IMMUNE RESPONSE

In addition to the effects of the steroid hormones on gluconeogenesis and glycolysis, the glucocorticosteroids are also important in their antiallergic and antiinflammatory actions. They are able to cause an initial lysis of the lymph nodes with increased release of antibodies. However, there is ultimately a decreased production of antibodies. The overall effect is the suppression of the function of the lymphoid tissues, the tissues responsible for the production of antibodies. Additionally, the glucocorticosteroids decrease the cellular response to infection by decreasing the phagocytic ability of neutrophils and lymphocytes, thereby reducing the inflammation without clearing up the infection. However, the actual

neutrophil count usually increases, although their activity is decreased. Additionally, glucocorticosteroids are able to decrease the formation of histamine and histamine-like substances in cells, thereby reducing the allergic response. Since they also cause a decrease in the eosinophil count, they can lead to a decrease in inflammation. Eosinophils are abundant at sites of immediate hypersensitivity reactions, and in this setting they contribute to tissue injury and inflammation. By lowering their count, glucocorticosteroids help decrease visible inflammation.

Furthermore, glucocorticosteroids have been shown to increase the stability of cell membranes and lysosomal membranes, thus decreasing the release of inflammation enhancing toxic products from injured cells and from lysosomes, as well as decreasing the cellular insult of toxins. By stabilizing these membranes, the activation of lipases and phospholipases is prevented, thus preventing the supply of free arachidonic acid that triggers the formation of eicosanoids. Finally, glucocorticosteroids inhibit prostaglandin production by macrophages. Prostaglandins, which are part of the 20 carbon containing atoms known as “eicosanoids”, are known to stimulate inflammation, and thus their inhibition would partially account for the decrease in inflammation observed upon injection of an individual with glucocorticosteroids.

Finally, it is also known that glucocorticoid hormones are able to inhibit T-cell growth and activation. A recent study performed by Migliorati et al. has suggested that the down-modulation of the T-cell receptor expression is due to the diminution of T-cell receptor gene products in cells treated with exogenous glucocorticoids.

GLUCOCORTICOID RECEPTORS

At the cellular level, the many effects of glucocorticosteroids are mediated by the glucocorticoid receptor. These receptors appear to be located in the cytoplasmic compartment of the cell, and thus the first step in glucocorticoid action is for the hormone to gain entry into the cell. Generally, steroid hormone uptake is generally considered to occur by simple diffusion, but it is possible that specific carriers are involved in steroid hormone uptake. Following this entry into the cytoplasm, the hormone must bind to its appropriate receptor. The glucocorticoid receptor is a relatively nonabundant protein in cells, and is distinguishable by two basic characteristics. Firstly, it binds hormones with high affinity. This high affinity is necessary because of the low concentrations at which glucocorticoid hormones are generally present within the cell. Secondly, the glucocorticoid receptor binds hormones with a high degree of specificity, and thus has very low affinity for other classes of steroid hormones (i.e. androgens, estrogens). Additionally, the association between the glucocorticoid receptor and its ligand is noncovalent. An interesting fact is that several transition metals and their anions, including cadmium, arsenite, and selenite can inhibit glucocorticoid binding to receptors. A study published by Telford and Fraker in 1997 showed that zinc has an inhibitory effect as well. After this receptor/ligand binding takes place, the receptor undergoes activation to a DNA-binding form. In the absence of hormone, the receptor is unable to bind DNA, but the binding of the hormone converts it into its active form. Subsequently, the glucocorticoid receptor translocates into the nucleus of the cell. Because of its rather large size (approximately 97 kD), it is most likely that the receptor/ligand complex enters the nucleus via a facilitated transport mechanism rather than by simple diffusion. Finally, the glucocorticoid receptor will associate with specific sequences in the genome which are known as glucocorticoid response elements. These response elements regulate

the rate of transcription initiation from nearby promoters, causing an increase in the transcription of specific genes. For example, a study performed by Nuglozeh et al. In 1997 showed that glucocorticoids have transcriptional and posttranscriptional effects on rat mesenteric artery cells through glucocorticoid receptors. The glucocorticoid response element has a consensus sequence of TGTTCT, and has been detected as close as 39 base pair and as far as 2.6 kilobase pair upstream of the transcription start site, and between 100 base pair and 8 kilobase pair downstream from the initiation site. Additionally, there is also evidence that the glucocorticoid receptor is also able to act posttranscriptionally to influence the levels of mRNA present, either by altering RNA stability or by stimulating the transport of specific RNA species from the nucleus to the cytoplasm.

It is now known that the different regions of the glucocorticoid receptor have different functions, and thus are considered separate domains. These discoveries were made when the receptor was incubated with trypsin, resulting in three receptor fragments. The first two fragments arise from the carboxy terminal half of the receptor, where they bind DNA and hormone, respectively. The third fragment stems from the amino terminal of the receptor, and it reacts with most anti-receptor antibodies, which are often used in the purification of the protein. Because this third domain is able to bind to antibodies, it is thought that it constitutes the exposed surface when the receptor is in its native conformation.

As was already mentioned, it appears that the receptor is inactive and unable to bind DNA when in its unbound state. The process whereby hormone binds to its receptor and mediates a change to the DNA-binding state is known as “receptor activation”. Basically, there are two generally models of receptor activation. In the first, known as the oligomer dissociation model, the oligomeric structure renders the DNA-binding site inaccessible for interaction with DNA. On binding hormone,

the oligomer dissociates, and thus exposes the DNA-binding site on the receptor. The second model of activation is referred to as the conformational model. In this model, the function of the DNA binding region is repressed by the steroid-binding domain. When the hormone binds, the conformation of the receptor changes, rendering the DNA-binding site accessible to DNA. Generally speaking, these two models are extremely simplified, because there are additional factors involved in activation. One example has been termed “modulator”. Modulator is a phosphoglyceride that functions as an endogenous inhibitor of glucocorticoid receptor activation.

The intracellular concentration of glucocorticoid receptors is largely responsible for the magnitude of the cellular response to glucocorticoids. Studies have indicated that the intracellular concentration of glucocorticoid receptors is under autoregulatory control. Thus, many glucocorticoids are able to downregulate the level of their own receptor. This downregulation is relatively slow, taking at least 24 hours of steroid exposure to cause a notable decrease in receptor content. Furthermore, on removal of the agonist, the glucocorticoid receptor downregulation is reversed. During the period of receptor repletion, no altered forms of the glucocorticoid receptor have been identified. There are several possible mechanisms responsible for the autoregulation. In the first, the intracellular concentration of receptors may be determined by modulation of receptor half-life. Some studies have shown that the half-life of the glucocorticoid receptor appears to be shorter in the presence of hormone than in the absence of hormone. Secondly, the intracellular receptor concentration can be regulated at the transcriptional level. Thus, the level of receptor mRNA is decreased in response to glucocorticoids. It appears that the glucocorticoid receptor downregulation may be caused by an interaction of the receptor protein with its own gene. Thirdly, the receptor may be degraded by proteolysis, as there several calcium dependent enzymes that can

degrade receptors are known to exist at specific loci. Finally, it is possible that the receptor may exist in two forms, on an active hormone-binding state and one an inactive nonhormone-binding state. It is possible that the conversion to the steroid-binding form requires the hydrolysis of ATP. This was confirmed in an experiment which showed that the receptor binding capacity of the IM-9 line of human lymphoblasts was directly related to the ATP levels when cells were incubated under energy limiting conditions. This conversion from the nonhormone binding to the hormone binding form was both reversible and rapid. The exact function of the ATP is unknown. It is possible that the receptor may be phosphorylated by ATP in an enzyme dependent reaction. Recently, Miller et al. presented the hypothesis that the receptor is itself a kinase, and is capable of autophosphorylation in the presence of ATP. However, it remains uncertain whether phosphorylation actually controls binding capacity or whether the ability to bind steroids may depend more on the receptors ability to form a complex with ATP.

Additionally, a recent study published by Numi et al. examined the participation of tyrosine kinase in the regulation of the glucocorticoid receptor by using the tyrosine kinase inhibitor herbimycin A. Their findings showed that tyrosine kinase is necessary for maintaining the level of glucocorticoid receptor protein and its affinity of binding sites in the cytosolic fraction.

CONCLUSION

Consequently, it can be seen that glucocorticosteroids are able to play a large roll in the functioning of the human body, as well as in other animals. They are currently being used as therapy to treat such conditions as skin inflammations and hypoglycemia. As more becomes known about them and their beneficial and also possibly harmful effects, it

is likely that the medical and scientific worlds will receive great benefits. For example, work is currently being done to determine if prenatal stress might possibly impair the coping and regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Typical characteristics of prenatally stressed infants include attentional deficits, hyperanxiety, and disturbed social behavior. These symptoms are often associated with dysregulation of the HPA axis, characterized by decreased feedback inhibition of corticotropin-releasing hormone and prolonged elevation of plasma glucocorticoids in response to stress. It has been proposed that the impaired ability to deal with stressful situations by the prenatally stressed infants may be a result of the action of maternal hormones released during periods of stress. It is now believed that if enacted during a critical time in fetal development, gestational stress may increase the probability of developing this disorder. It is this type of research that will hopefully lead to a more useful knowledge of glucocorticoids in the near future.

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1. Introduction

Corticosteroids are a naturally occurring hormone found in the equine body. They are released by the adrenal cortex following a multi-step pathway in which the adrenal cortex is stimulated to produce corticosteroids by the presence of Adrenocorticotrophic Hormone (ACTH) in the bloodstream. When levels of corticosteroids are elevated in the blood, endogenous production is decreased as a result of feedback inhibition which directly affects levels of ACTH in the blood.

Because of the complexities involved in the feedback mechanism, administration of exogenous ACTH or corticosteroids (i.e. dexamethasone) can upset the general balance. If corticosteroids are given over an extended period of time, production of ACTH by the pituitary will decrease, thus signaling the adrenal cortex to down-regulate its production of corticosteroids. This decrease in stimulation by ACTH will cause the adrenal cortex to atrophy, and corticosteroid production will either decrease or stop. If exogenous treatment is suddenly withheld, there is often a severe endocrine imbalance due to an inability to produce endogenous corticosteroids.

In this study, dexamethasone will be administered to horses in two ways, either IM or IV. One of the most prominently excreted forms of corticosteroids in the body is cortisol (hydrocortisone). However, the body cannot distinguish between cortisol and the different man-made analogues such as dexamethasone. Thus, dexamethasone will be recognized as a natural corticosteroid, leading to repression of the endogenous production of cortisol. By measuring the concentration of cortisol levels in the blood, the overall success of the corticosteroid suppression can be determined for the different routes of injection of dexamethasone. The initial hypothesis is that blood cortisol concentrations in horses will be equally suppressed in horses given dexamethasone intravenously as in horses given dexamethasone IM. Thus, either method of administration will lead to equal

suppression of the pituitary-adrenal axis in normal horses. As Pars Intermedia Pituitary adenoma with absent or mild clinical signs is currently diagnosed using a dexamethasone suppression test or a combined dexamethasone suppression/thyroid releasing hormone test, this study will attempt to prove that the two methods of injection are generally equivalent in their suppression of cortisol levels.

2. Materials and Methods

Six mixed breed horses between 4 and 17 years old and weighing between 398 and 629 kg (878 and 1,387 lb) were used. The horses were used in a random cross-over study where they were randomly assigned to treatment groups by drawing IV or IM from a hat. Blood samples will then be collected before dexamethasone injection and at three hour intervals following injection, concluding with a sample at $t=24$ hours.. A two week washout period will follow, after which the horses will be treated with the opposite route of dexamethasone administration, once again followed by collection of samples.

Jugular vein catheters were inserted at 8 AM, and the initial blood samples were drawn at 8:30 AM, whereby they were immediately placed into an evacuated purple top tube containing EDTA. Dexamethasone (40 micrograms/kg of body weight) was then administered. The remaining blood samples were drawn at the specified times, and also placed into a tube containing EDTA.

Following collection, the blood samples were centrifuged, and the plasma was collected and stored at -70°C until analyzed. Following thawing, cortisol concentration was then determined by use of the Count-A-Coat radio-immunoassay procedure produced by Diagnostic Products Corporation. In this procedure, I-125 labeled cortisol competes for a fixed time with cortisol in the equine plasma sample for antibody sites. Because the antibody is immobilized to the wall of a polypropylene tube, simply

decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled cortisol. Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the cortisol present in the patient sample.

Means and SEM for cortisol concentrations were estimated using proc MEANS in SAS. Analysis of variance was performed on data using the mixed procedure (PROC MIXED) in SAS to compare time and treatment interactions. Significant differences were determined a $P < 0.05$.

3. Results

The results showed that there was no difference in IM versus IV treatment ($P = .1878$). There was a significant decrease in cortisol over time ($P < 0.05$). However, there was no time and treatment interaction ($P = .9797$).

4. Discussion

As was expected, cortisol concentrations were seen to decrease over time (Figure 1). The results of this study showed that there was no significant difference in the overall suppression of cortisol when dexamethasone was injected IV and IM. Thus, both IV and IM injection of dexamethasone equally suppressed the pituitary-adrenal axis. In the future, this information will be useful to practitioners in determining which route of administration is the best for testing for Pars Intermedia Pituitary adenoma. Pituitary tumors are common in older horses and require blood testing for diagnosis. The current methods of diagnosing this disease require the administration of dexamethasone (either IM or IV) followed by IV administration of Thyroid Releasing Hormone. The

blood cortisol concentration is then measured at prescribed time intervals after dexamethasone injection. Previously, there was no information on whether the route of dexamethasone administration effected the interpretation of the test or whether there was a difference in cortisol blood concentrations depending on the route of dexamethasone administration. However, the results of this study have demonstrated that, in normal horses, the route of administration of dexamethasone does not affect the result of the test used to diagnose pituitary tumors.

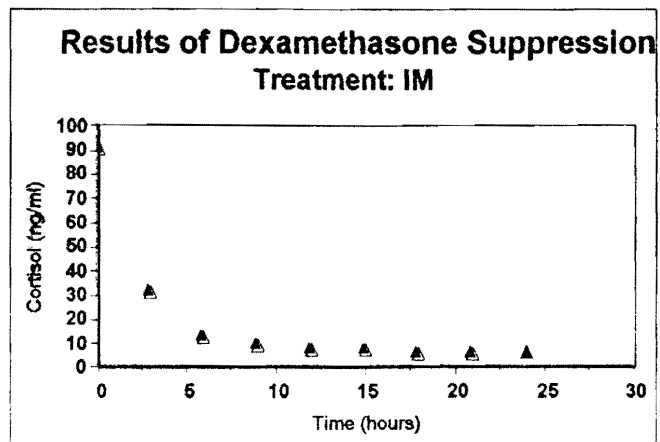


Figure 1: Treatment with dexamethasone IM.

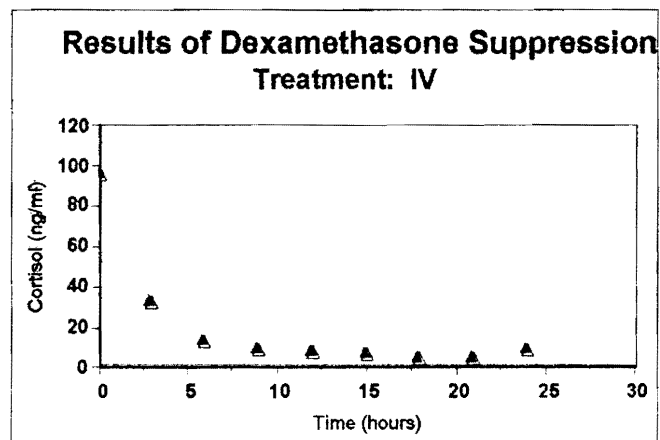


Figure 2. Treatment with dexamethasone IV

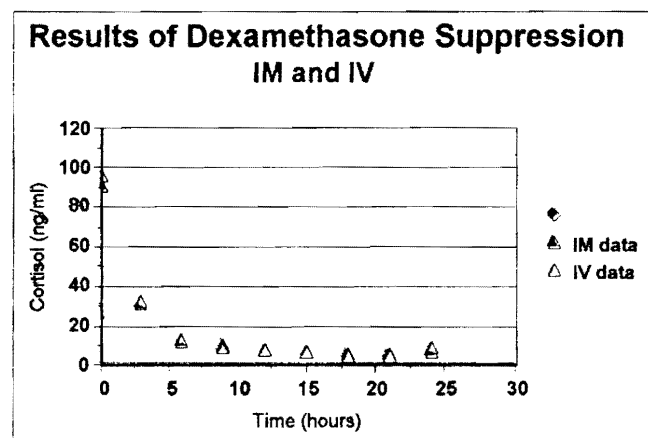


Figure 3. Combined results for dexamethasone IV and IM

PROTOCOL FOR USE OF LIVE VERTEBRATES THE UNIVERSITY OF TENNESSEE-KNOXVILLE

The cover page, part I, General Information and all applicable sections of part II, Detailed Information must be completed for all research and teaching activities.

Submit 3 typed copies to:

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HORSE ENDOCRINOLOGY

Title of Project/Course

Frank M. Andrews, DVM

Principal Investigator/Instructor

423-974-5704

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Department

(Our signature as P.I., Co-investigator, or Department Head on this application verifies that: (1) the information herein is true and correct and that you are familiar with and will comply with the legal standards of animal care and use established under federal and state laws and policies as well as university policies; (2) the proposal has received approval for scientific and/or educational merit by peer review; and (3) the activities do not unnecessarily duplicate previous experiments.

Signature of P.I./Instructor

Date

Signature of Co-Investigator

Date

Signature of Department Head

Date

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IACUC Number _____

Ethical Concern Category A B C D E

Assigned to: Full IACUC Review _____

Expedited _____

PART I.
GENERAL INFORMATION

To be completed for all Research and Teaching Activities.

A.

General Information

1. This is a ☒ New ☐ 3-year rewrite proposal for

☐ Research ☐ Teaching
2. Anticipated: start date March 9, 1998 end date May 1, 1998
3. ☐ Yes ☒ No This protocol includes the use of farm animals used or intended for use as food or fiber, or livestock or poultry used or for improving animal nutrition, breeding, management, or production efficiency, or for improving the quality of food or fiber.
4. Veterinarian responsible for clinical care
☐ OLAC Staff Veterinarians
☒ Dr. Dallas Goble
☐ LACS Ambulatory Service for the Department of Animal Science
☐ Other, list _____ If other, clinical veterinarian must follow the IACUC "Attending Veterinarian/Researcher Veterinarian" policy.
5. Funding Source: Large Animal Clinical Sciences

B.

Animal Housing Facilities

In order to provide assurance of humane care and use of laboratory animals, all animal housing facilities on the UTK campus will be operated according to federal laws, AAALAC and NIH Guidelines, and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching as appropriate. Housing of animals, including those actively on experiment, for periods longer than 12 hours, is restricted to facilities meeting such guidelines and laws.

Please check your preferred housing location.

- ☐ College of Arts and Sciences Facility in the Walters Life Sciences Building (WLS).
- ☐ College of Human Ecology Animal Facility in the Jessie Harris Building.
- ☐ College of Veterinary Medicine (CVM) Laboratory Animal Facility VTH/Cherokee
- ☒ CVM Large Animal Clinical Sciences in VTH/Cherokee Farm
- ☐ UT Medical Center Lab Animal Facility
- ☐ Brehm Animal Science Building Room _____
- ☐ IACUC Approved Satellite Facility List bldg/room # _____
- ☐ *Other List: _____

*Any newly established facility must be approved before housing animals.

In accordance with NIH Guidelines and Federal Law, the IACUC formally reviews all animal housing facilities and satellites semi-annually and files a report to the Office for Protection from Research Risks (OPRR) in Washington, DC and the United States Department of Agriculture (USDA).

Part I.
General Information
continued

Description of Animals

Common Name	Strain/Breed	Sex	Weight/Age	Source	#/project	Maximum per day
Equine		F	6-30 yrs	UTCVM	6	6

2. Animals will be removed from the animal housing facility:

☒ No

☐ Yes. Please answer the following:

1. Animals will be taken to (bldg/room number): _____
2. Animal manipulations that will be performed in the laboratory include: _____
3. Estimated total time period live animals will be kept in the laboratory: _____ hours
4. Animals will be returned to the facility YES NO

3. Disposal of animals after completion of activity:

☒ Return to production/breeding unit/facility inventory

☐ Slaughter, (must conform to the Humane Slaughter of Livestock, 9 CFR, part 313)

☐ Sold

☐ Transfer to another research project (if yes, project title and investigator)

☐ Euthanized

☐ Returned to owner

☐ Other (Please describe) _____

Non-Technical Summary

A complete non-technical summary is required for conformance with PHS Policy. Non-technical summary must be submitted as an attachment and should not exceed two (2) pages. Non-technical summary must be written to ensure comprehension by non-scientists on an 8th grade level.

It must contain the following:

- 1) objectives of the research or teaching activity;
- 2) species and number of animals;
- 3) methods used to reduce any experimental stress or discomfort
- 4) schedule of the course or the study procedures performed on animals during each phase
- 5) benefits, outcome and results expected in non-technical terms.

Pituitary tumors are common in older horses and require blood testing for diagnosis. Current methods of diagnosing this disease in horses requires the administration of dexamethasone either intravenously (in the vein (IV)) or intramuscular (in the muscle (IM)) and Thyroid Releasing Hormone IV and measurement of blood cortisol (steroid) concentration at prescribed time intervals after injection. Unfortunately, there is no information on whether the route of dexamethasone administration (IV vs. IM) effects the interpretation of the test or whether there is a difference in cortisol blood concentrations depending on the route of dexamethasone administration. The objective of this study is to determine if there is a difference in blood cortisol concentration in horses given dexamethasone IV versus dexamethasone IM. The data from this experiment will help determine if the route of administration affects the results of the test in normal horses so that this test can be used as a diagnostic test in horses suspected of having pituitary adenoma.

We propose to use 6 healthy 7-30 year old horses. This is the age range in which the disease occurs in horses.

The horses will be placed in box stall during the 24 hour blood collection phase with feed and water to minimize stress. At approximately 8:00 am in the morning the horses will be placed in an equine stocks with a rope tied in front and behind. The horses will be lightly restrained with a lead shank and halter and area over the jugular vein clipped and prepared with provodone iodine sterile scrub. Lidocaine (approximately 3 cc) will be injected subcutaneously over the jugular vein to locally anesthetize the skin to the minimize pain of catheter placement. The intravenous catheter will be placed in the vein and sutured in place to prevent the horse from removal during the study period. The horse will then be place in the stall and blood sampling started at approximately 8:30 pm (T=0). After the first blood sample is drawn then the horse will be injected with a low dose of dexamethasone (40µg/kg, IV or IM). A blood samples will be taken 3 hour after injection, then TRH (1.0 mg, IV) will be administered. Blood samples will then be taken at 3.5, 6, 9, 12, 18, and 24 hours after administration of dexamethasone. Each horse will receive the dexamethasone IV followed by a two week rest, then receive the dexamethasone IM in the same manner. Some horses will receive the injections in reverse order to remove any bias due to effect of treatment. The blood samples will be analyzed later for cortisol concentrations.

We expect to determine if the route of dexamethasone administration effects the cortisol concentrations measured. This information will help practitioners in determining which route of administration is the best for using this diagnostic test to determine horses that have pituitary adenoma.

E. Flow Sheet or Table of Groupings

Pertinent information (i.e., numbers of animals, experimental manipulations) should be provided on a flow sheet. The Committee members should be able to follow every manipulation of the animal from experiment initiation to completion (e.g., day one, group one, treatment; day two. etc.). Please provide as a separate page.

See attached Page

Flow Sheet or Table of Groupings

Pertinent information (i.e., numbers of animals, experimental manipulations) should be provided on a flow sheet. The Committee members should be able to follow every manipulation of the animal from experiment initiation to completion (e.g., day one, group one, treatment; day two. etc.). Please provide as a separate page.

Flow Sheet

Day	Description of Treatment
1	8:00 am-horses placed in equine stocks and IV jugular catheter placed.
T=0	8:30 am-blood sample taken, Dexamethasone (40 µg/kg, IV) administered.
T=1	11:30 am-blood sample taken, TRH (1.0 mg, IV) administered.
T=2	12:00 pm-blood sample taken
T=3	2:30 pm-blood sample taken
T=4	5:30 pm-blood sample taken
T=5	8:30 pm-blood sample taken
T=6	11:30 am-blood sample taken
T=7 ²	2:30 am-blood sample taken
T=8	5:30 am-blood sample taken
T=9	8:30 am-blood sample taken
T=10	IV catheter removed and horse returned to stall or pasture at Cherokee Same protocol repeated 2 weeks later with IM injection of Dexamethasone.

F. Qualifications of Personnel

List all individuals who will be working with the animals on this project. Include all investigators, student employees, post-doctoral researchers, staff research associates and laboratory assistants who will actually work with the animals. If personnel do not have experience, state how they will be trained.

The **Occupational Health Program (OHP)** is available for all personnel who work with laboratory animals. Any personnel in a high risk category (e.g. handling primates, wild animals or any animals at risk of infection with rabies) must participate.

Name/Degree(s)	OHP*	Procedure(s)/Description of Relevant Experience
Dr. Frank M. Andrews		IV catheter placement, injection of pharmaceutical agents, collection of blood/Dr. Andrews has experience as veterinarian and previous experience with pharmaceutical agents used per UT-ACUC# 665.
Janell Kapp (Student)		Will assist Dr. Andrews in above procedures. She has been trained by Dr. Andrews in the above procedures.

*If an individual having animal contact is not currently enrolled in the occupational health program call the OLAC (974-5634) for information on enrolling.

G. Animal Welfare

1. This project involves pain or distress ☐ yes ☒ no
The USDA (Policy #12) requires that the principal Investigator must provide a written narrative of the sources consulted to determine whether alternatives or refinements exist to procedures which may reduce pain or distress.

If yes, the minimal written narrative should include:

- the databases searched or other sources consulted,
- the date of the search and the years covered by the search,
- key words and/or search strategy used,
- a narrative written in such a way that the IACUC can readily assess whether the search topics were appropriate and whether the search was sufficiently thorough

2. Does the proposed research duplicate any previous work? ☐ yes ☒ no If yes, explain why it is scientifically necessary to replicate the experiment.
3. Why have you selected the particular species proposed in this project?
To improve a diagnostic procedure for pituitary tumors in horses.
4. Provide an explanation of how the numbers of animals to be used were derived. If used in an experiment (test a hypothesis) numbers should be based on scientific and statistical requirements (power tests) to achieve objectives.
In previous experiments we have determined variability in plasma cortisol. Six horses are sufficient for this purpose.

PART II.
DETAILED INFORMATION

Check and complete those sections A - E that are relevant to your project.

REMOVE ALL SECTIONS NOT APPLICABLE TO YOUR PROPOSAL.

- A. Non-Surgical Procedures (Pertains to any experimental procedure - including non-surgical, pre-surgical and post-surgical procedures using animals)

☐ NO ☒ YES: COMPLETE SECTION A

- B. Surgical Procedures (Pertains to any surgical procedure, including non-survival surgery. If other procedures are done on animals prior to or after surgery, **complete section A** and other applicable sections.)

☒ NO ☐ YES: COMPLETE SECTION B

- C. Field Studies Involving Wild Animals (Request this section from the OLAC office)

☒ NO ☐ YES: COMPLETE SECTION C

- D. Hazardous Agents/Prescription Drugs/Controlled Substances

☒ NO ☐ YES: COMPLETE SECTION D

- E. Euthanasia

☒ NO ☐ YES: COMPLETE SECTION E

Part II
Section A

Non-Surgical Procedures

1. Sites of Study

- a. Site of housing prior to study: CVM Cherokee Farm
Bldg. Room/pen
- b. Site of experimental work: CVM Cherokee Farm
Bldg. Room/pen

2. Health Assessment:

How often and by whom will the clinical condition of animals be monitored?
Once before the experiment is conducted and daily during the investigation.

What are the anticipated health changes?
None

What methods will be used to monitor health changes?
Temperature, Pulse, and Respiratory Rate, general physical examinations and observation

What criteria will be used for removing animals from the study prematurely?
Any animal exhibiting any abnormal clinical sign, including increased heart rate, respiratory rate, or temperature.

3. Study Procedures : Please check the following items that apply and fill out appropriate parts of this section.

- ☐ Polyclonal Antibody production*
- ☒ Blood* or other body fluid withdrawal, tissue collection, injections
- ☐ Restraint with mechanical devices
- ☐ Projects involving nutritional deficiencies, tumor and disease models or toxicity testing
- ☐ Anesthesia or analgesia* (for non-surgical procedures)
- ☐ Behavioral Studies

* The IACUC has approved guidelines for these procedures. Deviations must be justified.

Polyclonal Antibody production

Describe procedure, antigen and adjuvants used, the ratio of antigen to adjuvant and routes and volumes of injection: (Vol. should be < 0.25 ml per injection site; subcutaneous route is recommended)

Blood or body fluid withdrawal/tissue collection/ injections

Describe in detail method(s), needle sizes, volume(s) collected or administered, and frequency of collection or injections:
Blood will be collected from the preplaced 16 ga 5 ¼ inch IV catheters using a syringe. 10ml samples will be collected at 0-time, 3, 3.5, 6, 9, 12, 15, 18, and 24 hours after injections of dexamethasone. Approximately, 190 ml of blood will be taken from each horse, which comprises less than 0.5% of the total blood volume of a 450 kg (1000 lb) horse. By preplacing the catheter we will avoid having to stick the horse with needles 9 times during the 24 hour period.

Part II
Section A - Non-Surgical Procedures
continued

Restraint with mechanical devices

Describe device, duration of restraint, frequency of observation, conditioning procedures and steps to assure comfort and well-being:

The horses will be lightly restrained in an equine stocks for intravenous catheter placement and housed in a box stall and restrained with a lead shank and halter for blood collections.

Behavioral Studies

Describe in detail types of behavioral manipulations, including placement in testing chambers or apparatus, use of aversive stimuli, duration of test periods, and frequency of test periods.

N/A

Nutritional deficiencies/Ascites tumor, monoclonal antibody production/Disease, neoplasia models/Toxicity testing

Describe methodology. State objective criteria used to assess physical condition and pain and distress during course of study. Include clinical signs or manifestations expected from the procedure. What criteria will be used to determine a humane endpoint before severe morbidity and death?

N/A

Anesthesia/Analgesia/Tranquilization (Other than Surgery Section B)

Adequate records describing anesthetic monitoring and recovery must be maintained and available to the attending veterinarian and animal care staff.

Drug	Dose	Route	Frequency	For which procedure

Name and qualifications of person administering drugs: _____

Describe: frequency and methods to be used to monitor level of anesthesia (e.g., blood pressure, heart rate, pedal reflex, jaw tone); the anticipated duration for recovery from anesthesia; how often will the animals be monitored and by whom.

RESULTS OF DEX SUPPRESSION TEST

SPORT	IM - 3/11/98	IV - 4/1/98
t=0	69.998	78.073
t=1a	21.2	26.75
t=1b	22.557	37.284
t=1 average	21.8785	32.017
t=2a	22.974	21.176
t=2b	18.48	29.888
t=2 average	20.727	25.532
t=3	12.531	13.261
t=4	8.5252	7.1824
t=5	7.3274	6.2296
t=6	6.0833	5.3764
t=7	5.5979	4.3809
t=8	4.1229	4.2942
t=9a	5.8712	3.7348
t=9b	4.2302	5.3456
t=9 average	5.0506	4.5402
t=10a	13.757	83.705
t=10b	12.785	20.421
t=10 average	13.271	52.063

BEE

IM - 3/13/98

IV - 4/1/98

t=0	46.154	75.915
t=1a	12.528	22.845
t=1b	12.653	22.325
t=1 average	12.5905	22.585
t=2a	11.114	19.112
t=2b	12.557	19.641
t=2 average	11.8355	19.3765
t=3	6.9263	11.001
t=4	7.3145	7.2408
t=5	7.3133	5.5151
t=6	6.8168	4.3959
t=7	4.5605	5.1187
t=8	8.4522	4.8861
t=9a	10.408	5.883
t=9b	7.0322	7.4805
t=9 average	8.7201	6.68175
t=10a	10.669	10.84
t=10b	9.557	12.442
t=10 average	10.113	11.641

97015

IV - 3/13/98

IM - 4/8/98

t=0	79.454	77.461
t=1a	29.553	53.144
t=1b	25.988	43.266
t=1 average	27.7705	48.205
t=2a	20.401	38.01
t=2b	17.288	40.286
t=2 average	18.8445	39.148
t=3	9.3124	13.228
t=4	8.8875	7.2369
t=5	8.9025	6.3026
t=6	14.227	7.765
t=7		5.7711
t=8	5.2055	4.5962
t=9a	10.825	5.7912
t=9b	10.901	5.0388
t=9 average	10.863	5.415
t=10a	11.765	12.312
t=10b	8.4247	12.858
t=10 average	10.09485	12.585

97008

IV - 3/13/98

IM - 4/8/98

t=0	114.26	127.02
t=1a	37.08	28.67
t=1b	34.077	26.536
t=1 average	35.5785	27.603
t=2a	32.16	21.636
t=2b	27.959	20.476
t=2 average	30.0595	21.056
t=3	16.377	11.424
t=4	10.152	8.7517
t=5	7.0485	9.3114
t=6	5.3818	7.319
t=7		9.3906
t=8	5.3385	6.0063
t=9a	7.0399	6.9891
t=9b	6.4836	5.1593
t=9 average	6.76175	6.0742
t=10a	10.746	7.9763
t=10b	8.4669	8.4617
t=10 average	9.60645	8.219

97009

IM - 3/13/98

IV - 4/15/98

t=0	129.24	64.426
t=1a	47.606	18.219
t=1b	38.678	23.173
t=1 average	43.142	20.696
t=2a	32.973	20.641
t=2b	29.192	17.293
t=2 average	31.0825	18.967
t=3	19.759	8.6819
t=4	16.39	10.369
t=5	9.973	12.011
t=6	7.4113	5.641
t=7		5.9264
t=8	8.5624	5.0389
t=9a	10.64	16.854
t=9b	7.9084	13.458
t=9 average	9.2742	15.156
t=10a	49.438	51.02
t=10b	52.093	55.188
t=10 average	50.7655	53.104

97018

IV - 3/13/98

IM - 4/15/98

t=0	163.19	95.919
t=1a	56.487	37.39
t=1b	51.607	34.373
t=1 average	54.047	35.8815
t=2a	45.155	28.714
t=2b	41.21	25.88
t=2 average	43.1825	27.297
t=3	20.556	12.586
t=4	9.5262	9.7436
t=5	10.155	6.3317
t=6	8.3578	8.3979
t=7		4.1931
t=8	55.39	5.2392
t=9a	54.402	4.3358
t=9b	41.587	3.7229
t=9 average	47.9945	4.02935
t=10a	99.443	27.97
t=10b	92.192	28.417
t=10 average	95.8175	28.1935