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This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement
This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (https://scholarworks.iu.edu/dspace/handle/2022/7911).

SHORT PROTOCOL FOR DIRECT RADIOIMMUNOASSAY OF CORTICOSTERONE FROM SMALL AMOUNTS OF PLASMA
(A.K.A. LITTLE B)

This protocol has been copied and modified from protocols provided by Dr. John Wingfield at the University of Washington. This protocol can be used to measure the concentration of corticosterone in small plasma samples. It has been written for use in the laboratory of Dr. Ellen Ketterson at Indiana University, and therefore the details may require modification if the procedure is performed elsewhere. The general procedure, which takes 4 days to complete, is described below. Variations and additional information are provided in the appendices. Prepared October 1999 by Joe Casto, modified October 2003 by Jodie Jawor.

PRIOR TO START OF ASSAY

1. Double-check of materials.

Before beginning an assay, make sure that all of the following materials are available for use.

Nitrogen Gas - AirGas, 317-881-1076, Cust. # TFI57, PO#
DiEthyl Ether - Chemistry Stockroom
Phosphate Buffer - See Appendix C
10x75 Test Tubes - Fisher Scientific
Charcoal/Dextran Suspension - See Appendix C
Standard, Labeled Steroid, and Antibody for the particular hormone(s) being assayed - See Appendices E,F, and G
Ultima-Gold Scintillation Fluid - Packard Instrument Company, 800 Research Parkway, Meridian, CT 06450. (800-323-1891). Item # = 6013329 ($225/2x5L Containers)

All other materials that are needed throughout the assay can be purchased at the biology stockroom. Make sure that you have everything you need if you are going to work over a weekend or holiday when the stockroom is closed.

DAY 1

Preparation of plasma samples.

Set up plasma sample in glass centrifuge tubes (pointed bottoms, 12 ml). The first and last tubes are always blanks and contain only distilled H20. The second and second to last tubes are always standards into which measured amounts of the steroids being assayed are placed. This measures the accuracy of the current assay as well as the interassay variation, when samples may be spread over more than one assay. When all plasma samples have been measured, a small amount of labelled steroid (20 µl of corticosterone = 2000 cpm) is added to all tubes except the blanks. Total cpm is measured at the end to determine what percentage of labelled steroid is recovered, and assuming the unknown steroid behaves the same, the final dose of the unknown steroid is adjusted according to the recovery value.

1. Setting up plasma samples.

Set up a data sheet for plasma volumes numbering samples as follows: 1. blank; 2. standard; 3. ... samples; second to last = standard; last = blank. Mix plasma on a whirl mixer if you have more than needed. Measure 5-25 µl plasma using a Hamilton syringe (or a P20 or P100 pipette) for each sample and place in centrifuge tube. Rinse syringe with distilled H20 and add to plasma. Bring all tubes to100 µl by rinsing the syringe. When all tubes are complete go back and bring volumes up to 200 µl using distilled water and an Ependorf Repeater.

2. Preparation of blank tubes.

Blank samples provide a negative control for background radiation in the assay. The first and last tubes in the assay should be blanks. Add 200 µl of distilled water to two glass centrifuge tubes (pointed bottoms, 12 ml). Do not add hot steroid to the blanks

3. Preparation of standard tubes.

Standard samples consist of a known amount of the steroid being assayed. They are used to measure the accuracy of the assay and to provide a measure of interassay variation. The second and second-to-last tubes in the assay should be standards. Additional standard samples may be placed throughout the assay if desired.

The standard tubes should be prepared in glass centrifuge tubes (pointed bottoms, 12 ml). Place 50 µl of cold (non-radioactive) corticosterone in each standard tube using a Hamilton Syringe (or P100 pipette). This will be 1000 pg of corticosterone (B). Bring the volume up to 200 µl with distilled water.

4. Setting up the recoveries.

A small amount of labeled steroid is now added to each sample and standard tube. Total cpm for each sample will be measured at the end of the assay to determine the percentage of labeled steroid that is recovered (i.e. not left behind during the extraction step), and the final concentration of steroid in each sample will be adjusted accordingly.

Add 20 µl (2000 cpm) of labeled corticosterone to all samples and standard tubes (but not to the blanks). An Eppendorf repeater pipette set on 2 with a 0.5 ml tip can be used. A yellow pipette tip (designed for the Eppendorf P200 Pipetman) can be fit over the end of the 0.5 ml tip to reduce drips. Also, add 20 µl of hot corticosterone to a scintillation vial. Add 2.5 ml Ultima-Gold scintillation fluid to this scintillation vial, vortex, cap, label, and store in a dark place. The total cpm of these vials will be measured to help determine the percentage recovered in each sample. Whirl mix all tubes and refrigerate overnight to allow the hot steroid to equilibrate with the plasma lipids and binding proteins.

DAY 2

1. Extraction of steroids from plasma samples.

- The diethyl ether must be freshly opened to avoid the formation of peroxides.

-Add 4 ml of diethyl ether to each sample. This can be done with an Eppendorf repeater pipette .

-Vortex each sample for approximately 10 seconds.

-Let the samples sit for 20 minutes to allow for complete phase separation. While waiting prepare a snap freezing bath of dry ice in methanol.

-Snap freeze each sample and pour off the unfrozen supernatant into a 13 x 100 test tube.

-Evaporate the supernatants under nitrogen gas in the 40ºC water bath.

- Add 550µl buffer (PBSG) to each tube. The buffer can be added using the Eppendorf repeater pipette. Whirl mix the racks of tubes, cover with parafilm, and refrigerate overnight. (To save a day, you may place the racks on the shaker for at least 45 minutes and proceed to set up the radioimmunoassay.)

DAY 3

This day is devoted to setting up the competitive-binding radioimmunoassay. Begin by placing the appropriate number of 10x75 test tubes in racks. First arrange 12 pairs for the standard curve, with a space between the 3rd and 4th pairs. Follow with a pair of tubes for each sample. (When you get to the charcoal step on Day 5, only 148 tubes may be centrifuged at one time. This translates into the standard curve plus 62 samples including blanks and standards. If you have more than 62 samples, a second spin in the centrifuge is required. This spin should have B2 and B3 tubes in it also; so between spin comparisons can be made)

1. Setting up the samples.

Vortex each tube before aliquoting. Using an Eppendorf P200, pipet 200 µl of each sample into duplicate assay tubes. With another Eppendorf P200, pipet 100 µl of the sample into a scintillation vial to count the recoveries. Add 2.5 mls Ultima-Gold scintillation fluid to these vials, cap, vortex, and count on the scintillation counter. (Remember to also count the 2000 cpm vial from Day 1.)

2. Setting up the standard curve.

The standard curve is used to determine the concentration of steroid in the unknown samples (See Appendix B for an explanation of the calculations.) The first three pairs of tubes in the standard curve are the B1-3. B1 measures the total cpm in 100 µl of the labeled steroid solution. B2 measures non-specific binding (background), and B3 measures the maximum binding of the labeled steroid with the antibody. The remaining 9 pairs of tubes (S1-9) generate the curve.

Add 200 µl buffer to the B1 and B2 tubes and 100 µl buffer to the B3 and S2-S9 tubes using the Eppendorf repeater pipette with a 5 ml tip and set on 1. (At this point do not add buffer to the S1 tubes.) Add 100 µl of cold steroid to each of the S1 and S2 tubes using a Hamilton Syringe (or P100). This will be 1000 pg for P, 2000 pg for B, and 500 pg for T, E2, and DHT. Vortex one of the S2 tubes, remove 100 µl, and add this to an S3 tube. By continuing this serial dilution the concentration will be halved each time. Discard the last 100 µl that is removed from the S9 tube. Repeat the serial dilution for the second half of the pairs.

Following the set-up of the standard curve add an additional 100µl of buffer to all B and S tubes to bring them to the same volume as the samples.

3. Setting up the radioimmunoassay.

Gently whirl mix (do not shake) the labeled steroid and antibody solutions prior to use. Using the Eppendorf repeater pipette, add 100 μl of labeled steroid to all of the tubes (standard curve plus samples). Add 100 ml antiserum to all tubes starting with the B3 tubes (do not add antiserum to the B1 or B2 tubes). Cover with parafilm, whirl mix by hand (do not vortex), and refrigerate overnight. Curves may be left in the refrigerator longer before the charcoal step is done, but overnight is the minimum time required.

DAY 4

1. Separation of bound and free counts.

Dextran-coated charcoal is added to all of the tubes except the B1's to adsorb any unbound steroid. The tubes are then centrifuged to force the charcoal-dextran to the bottom, and the supernatant, which contains all steroids (hot and cold) bound to antibody, is decanted. The total cpm's in the supernatant are counted. High radioactivity in the supernatant indicates a low concentration of endogenous steroid, while low counts of tritiated steroid indicate high levels of endogenous steroid. The exact concentrations of steroids in the unknown samples are calculated by comparing them to the standard curve (see Appendix B).

-Run the Beckman TJ-6 centrifuge through one complete cycle to cool it to 4ºC.

-Add 0.5 mls distilled water to the B1 tubes using an Eppendorf repeater pipette set on 2 with a 12.5 ml tip.

-Place some dextran-coated charcoal in a beaker, add a magnetic stir bar, and place on a stirring plate. The charcoal mix should be stirred continuously while adding 0.5 mls of it to all of the remaining tubes. This step should be done as quickly as possible to avoid heating of the samples and/or the charcoal mix and to ensure that all samples will be subjected to the charcoal mix for the approximately the same amount of time. It may be helpful to cool the beaker and the repeater pipette tips prior to use or to place the charcoal/dextran beaker in an ice bath while aliquoting.

-Whirl mix the racks of tubes and let stand for 12 minutes. Start the timer as soon as the charcoal mix is added to the last sample. This is a critical step, so set a timer and do not get distracted. Load the centrifuge while waiting, being sure to balance the tubes both within and between buckets.

-After the 12 minutes is up centrifuge at 2000 rpm for 10 minutes.

-Decant the supernatant into scintillation vials, add 4.5 mls Ultima-Gold scintillation fluid, cap, vortex and count on the scintillation counter. The centrifuge tubes with the charcoal pellets should be discarded into the radioactive waste.

-To calculate the steroid concentrations of the samples see Appendix B of full protocol.

AFTER COMPLETION OF ASSAY

All glassware that is used during the assay should be cleaned in a solution of Radiacwash detergent. Prepare a plastic tub with distilled water and the appropriate dilution of the detergent on Day 1. Glassware can be added to the tub as it is used after it is first rinsed with distilled water in the sink in the hood room. Be sure that all solid particles are removed prior to the soaking, and that there are no air bubbles in the submerged glassware. After all glassware has been soaked for at least a day, rinse three

times with the tap-distilled water, and then rinse once with double-distilled water. Place the glassware in the drying oven set at 40oC. Higher temperatures can cause adsorption sites to be formed on the glassware. If this happens the glassware should be rewashed.

Radioactive waste, including scintillation vials, should be disposed of properly. Ask either the radioactivity safety personnel or others in the lab to explain the correct procedures to you.