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# Hormone Analysis in Bats

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HORMONES ARE THE CHEMICAL MESSENGERS of the endocrine system that regulate and integrate numerous bodily functions. They play a role in maintaining homeostasis through their actions on osmoregulation, mineral balance, metabolism, growth, development, blood pressure, and reproduction. Many hormones also influence behavior. Hormones are classically known as those chemical messengers released from the ductless endocrine glands (e.g., pineal gland, pituitary gland, thyroid gland, parathyroid glands, adrenal glands, pancreas, ovaries, testes, and placenta); however, hormones also are synthesized and secreted from cells in the brain, gastrointestinal tract, thymus gland, heart, and adipose and other tissue. Hormones interact, in sometimes-complex ways, with other chemical messenger systems—the nervous system and the immune system. Although hormones may act locally, either on the cells that released them (autocrine action) or on neighboring cells (paracrine actions), all hormones circulate through the blood to interact with target cells at a distance.

To initiate a biological response, either inhibitory or excitatory, a hormone must bind to a receptor. Receptors are specific binding proteins either embedded in the cell membrane or located elsewhere in the cell that interact with a particular hormone. Receptors are characterized by their specificity and affinity for hormone binding. Because of the high affinity of receptors, many hormones are active at concentrations as low as 1 pmol, which makes their quantification difficult.

Depending on their structure, hormones have different chemical and biological properties. These different properties are important to understand, because they affect our ability to quantify hormone levels and activity. Steroid hormones are perhaps the best studied class of hormones in bats because they include the reproductively important gonadal steroids (testosterone, estrogen, and progesterone) and the metabolically important glucocorticoid hormones (cortisol and corticos-

terone). All steroid hormones are synthesized from cholesterol and are lipid-soluble, thus, they can diffuse through cell membranes and cross otherwise restrictive tissues, such as the blood-brain barrier and the placenta.

Biosynthesis of steroids begins within seconds of an appropriate stimulus, but increased blood levels of steroids are generally not detectable by current methods until 3 minutes or more after the onset of a stimulus. All steroid hormones are released as soon as they are produced because packaging lipophilic compounds in a membrane-bound secretory vesicle is not possible. They are highly stable molecules not subject to rapid degradation by heat, oxidation, or enzymes in blood. Many steroids are bound by a carrier protein in the blood, which increases their biological half-life, and which may present a complication in procedures for detecting hormones in the laboratory. The molecular structure of each steroid is conserved across taxa because all steroids are synthesized from cholesterol and follow the same biosynthetic pathways in all animals. Steroid hormones act by stimulating, or in some cases inhibiting, transcription of one or more genes in a target cell. The steroid hormone-receptor complex, therefore, falls in the category of a transcription factor. Because of the requirement for gene transcription, the actions of steroids are not usually manifest earlier than approximately 30 minutes, and often much longer following a stimulus. A notable exception is the recent discovery of a membrane-associated glucocorticoid receptor that acts via rapid G-protein activation in the rat hypothalamus (Tasker et al., 2005), which may explain some of the rapid central effects sometimes noted for glucocorticoid steroid hormones.

Despite the fact that steroid hormones (compared to other classes of hormones) have been relatively well studied in bats, the majority of vertebrate hormones are protein hormones (another term, "peptide hormones" is generally applied to those hormones of a few dozen or fewer amino acids in length, while monoamine hormones are derived from a single amino acid). They are water-soluble and thus do not easily diffuse through cell membranes, and can be stored in secretory vesicles for later release by exocytosis. Because they can be produced ahead of time, they can be released on demand and thus may appear in the blood with a shorter latency than steroids following a stimulus. As products of genes—produced by transcription and subsequent translation—protein hormones have been subjected to evolutionary changes, and therefore, the amino acid sequence of a given protein or peptide hormone may diverge considerably between species. This is a major complication in quantifying protein and peptide hormones in bats, especially species that are distantly related. The protein, peptide, and monoamine hormones (with the exception of thyroid hormone) all act on cell membrane receptors whose binding sites face the extracellular fluid. Their actions are mediated by one or more "second messengers" such as cAMP and occur more rapidly than those of steroid hormones.

Hormones have been studied in bats for many reasons, including the study of reproductive processes in a number of species (see many chapters in Crichton and Krutzsch, 2000, especially Martin and Bernard, 2000, and Kwiecinski and Damassa, 2000). Additionally, research has focused on the role of the endocrine system in meeting the special challenges of seasonally limited food resources and subsequent hibernation in temperate bats. This chapter will first address how to collect biological samples for hormone analysis and then turn to the larger topic of how to analyze the hormone samples.

## COLLECTING SAMPLES FOR HORMONE ANALYSIS

A number of different sources of biological material can be used to determine hormone concentrations. Historically, the majority of studies have used blood samples, measuring the concentrations of circulating hormones. More recently, a number of studies have validated the use of urine and feces for determining hormone activity in vertebrates, by measuring either the hormone of interest or its metabolites. Hormone levels also can be determined in saliva, although the concentrations of hormones are a fraction of those found in the blood. Lastly, hormone activity can be measured hair (not discussed further here, but see Koren et al., 2008), and *in vitro* in tissue samples or cultured cells. Each type of biological material has advantages and disadvantages for the measurement of hormone levels, as each sample type must be collected and often processed in different ways. Additionally, some sampling methods are more feasible for use in bats than others and this may vary, depending upon body size and whether captive (see Barnard, this volume) or free-ranging individuals are being studied (Reeder et al., 2004a).

Regardless of the type of sample collected, the timing of sample collection is critical. Many hormones are known to show significant circadian and seasonal rhythms, which can vary with the sex, age, reproductive condition, and the ecological and social environment of the animal (Wingfield et al., 1997; Martin and Bernard, 2000; Romero, 2002; Anestis and Bribiescas, 2004; Reeder and Kramer, 2005). Samples must be collected consistently at the same time of day (unless circadian variations are of interest), and seasonal, age (see Rossinni and Wilkinson, this volume), and reproductive effects (Racey, this volume) must be considered. On an acute temporal scale, several widely studied hormones, including the glucocorticoid hormones cortisol and corticosterone, as well as the protein hormone prolactin, are known to increase in response to a stressor, including capture and handling (Widmaier and Kunz, 1993; Widmaier et al., 1994; Wingfield and Romero, 2001; Reeder et al., 2004a, 2004b; Reeder and Kramer, 2005).

Elevated levels of these hormones can then affect other aspects of the endocrine system, resulting, for example, in the suppression of other hormones such as testosterone (Reeder et al., 2004b; Boonstra, 2005). In order to study basal levels of these hormones, blood samples must be collected within three minutes of capture in either captive or free-ranging conditions (Romero, 2002; Reeder and Kramer, 2005), although there may be a short window of time on either side of three minutes that differs between species, as has been shown in birds (Romero and Reed, 2005). Even when samples are collected within this three-minute window, it is still possible that the presence of researchers and/or capture protocols will influence bat physiology prior to capture. Once samples are collected, proper storage methods must be followed, which may vary by sample type and by the properties of the hormone(s) being investigated.

## Blood Samples

Blood samples can be collected in a variety of ways, depending upon the species of interest and the conditions of the study. Most hormones are measured in plasma; however, blood can be processed to produce either plasma or serum (plasma with clotting proteins removed). If animals are being terminally sampled for other purposes, the easiest way to collect a blood sample is to rapidly collect trunk blood following decapitation. It should be noted, however, that decapitation followed by collection of trunk blood may result in contamination of blood with cerebrospinal fluid, and possibly even with stomach acids. With this method, trunk blood can either be collected directly into a microcentrifuge tube or other blood collection tubes, or drawn into several capillary tubes containing an anticoagulant.

When collecting blood samples, each sample should be placed on ice or refrigerated until ready for centrifugation to obtain plasma. Cold temperature slows the rate of proteolysis and oxidation of proteins, both of which can result in artificially low hormone measurements. Whole blood should not be frozen, as this causes hemolysis, which is known to interfere with some assays. Following centrifugation to separate the plasma from the cells, plasma is removed with a pipette and frozen at −20°C or lower (generally, samples that will be stored for more than one month and that will be assayed for protein hormones are better stored at −70°C to ensure stability of the molecule).

If samples are collected and centrifuged in the field (Reeder et al., 2004a), the plasma can be stored on dry ice until transport to the laboratory. Processing blood as plasma, by definition, requires that the collection tube, the syringe, or the capillary tube used contain an anticoagulant. Heparin and ethylenediaminetetraacetic acid (EDTA) are the most frequently used anticoagulants, and which is preferable depends upon the hormone in question and the assay system being used. For example, heparin interferes with readings in some commercial RIAs for adrenocorticotropic hormone. For many hormone assays, however, the choice of anticoagulant does not matter, but samples within a given study must consistently use the same anticoagulant. Repeated freezing and thawing of samples should be avoided for protein and peptide hormones, but in our experience has no effect on quantification of glucocorticoids.

Clotting proteins can interfere with some assays, especially when assaying protein hormones, thus serum is sometimes preferable. When it is desirable to process blood as serum, blood samples must be collected without the use of an anticoagulant (e.g., heparin or EDTA), and must be allowed to clot at room temperature for 20 minutes or longer. Following clotting, blood is centrifuged to separate the serum from the clotted blood cells, serum is removed with a pipette and frozen as described above.

One problem with collecting serum is that samples must sit at room temperature (because cold slows clotting) for a prolonged period of time, which accelerates the rate of degradation of some protein and peptide hormones. From a sampling standpoint, processing blood as serum requires that blood be collected directly into a tube (as can occur with terminal sampling) or with a syringe, which can be difficult when nonterminal samples are desired from smaller bat species. For many hormones, whether plasma or serum is used does not matter, but one must be consistent because hormone levels can vary slightly between plasma and serum.

Many studies of smaller vertebrate species have collected blood samples using heparinized capillary tubes, which can be quickly sealed (at one end) with a clay or putty capillary tube sealant such as Critoseal until centrifugation (Fig. 26.1A). Following centrifugation, glass capillary tubes can be scored just above the cell/plasma interface with a file, then broken and quickly resealed to store the plasma within the capillary tube (see Fig. 26.1A). These portions of capillary tube can then be stored within cryovials and frozen for later hormone analysis. Capillary tubes containing EDTA do exist, but our experience is that they do not work well and are very expensive relative to heparinized capillary tubes. A small battery-operated field centrifuge can be used to process blood-filled capillary tubes in the field. Capillary tube samples can be collected from trunk blood, from the vein at the leading edge of the propatagium, or from the caudal vein in the uropatagium (see Fig. 26.1B). In smaller bat species (e.g., *Myotis lucifugus*), we have used a 26-gauge needle to puncture the caudal vein in the uropatagium and collected blood as it came out from both the ventral and dorsal surface of the wing (Reeder et al., 2004a). Serra-Cobo et al. (2002) describe a method for collecting blood from the region of the radius' proximal epiphysis. In general, plasma or serum recovery will be about 50–60% of the total volume of blood collected, and many hormones can be assayed in 5–20 μL of

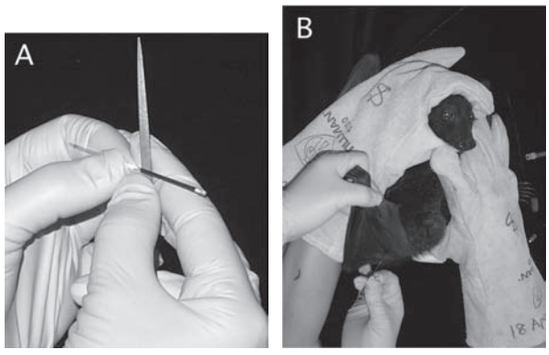


Figure 26.1. Using heparinized capillary tubes for blood samples. A, after centrifugation, the glass capillary tube is scored and broken just above the plasma/cell interface. The plasma portion of the tube is resealed with Critoseal and the sample is frozen. B, collection of a blood sample from *Pteropus vampyrus* is obtained by pricking the uropatagial vein with a needle and wicking up the blood from both the ventral and dorsal surface into a heparinized capillary tube.

plasma/serum (but this varies with the sensitivity of the assay and the concentrations of hormones in the blood). General veterinary guidelines for survival sampling recommend taking no more than 1% of an animal's body weight in blood in a given 2-week period, which translates to 10 µL/g (e.g., in an 8 g bat, no more than 80 µL of whole blood should be collected in any 2-week period). When animals are dehydrated (e.g., at emergence from the daytime roost), the hematocrit will be higher and less plasma/serum will be recovered from the sample. Additionally, in colder temperatures, obtaining a sufficient blood sample can be difficult due to vasoconstriction. This can sometimes be overcome by placing the bat on a warm surface (such as the small "hand warmers" found at camping supply stores). If anesthetics (which present a mortality risk and which themselves can alter physiology) are used, cardiac puncture may be a reliable sampling method.

## Urine and Fecal Samples

Because urine and fecal samples can often be collected non-invasively, the determination of hormone concentrations in both urine and feces is becoming an increasingly valuable technique (Whitten et al., 1998), especially for the analysis of steroid hormone activity. Hormones can be excreted from the body by both the kidneys and the gastrointestinal tract. Steroid hormones are conjugated by the liver, resulting in greater solubility prior to kidney excretion. It is important to note that while hormone levels determined in plasma samples are acute or discrete measurements, hormone levels determined in urine or feces represent the previous hormonal history of blood for a variable period of time. Because excretion rates of hormones can be highly variable and because each species may metabolize hormones differently, preliminary studies must be undertaken for each species to validate the collection and assay procedures. Excretion rates are often determined by administering a radiolabeled version of the hormone of interest, followed by intensive urine or fecal sampling. The relative amounts of the known metabolites of the hormone of interest can then be measured in these samples to determine the primary metabolite(s) excreted. Excellent examples of this methodology are provided in Möhle et al., 2002, and Wasser et al., 2000. In practice, determining excretion rates by these methods in small bats is challenging, and excretion rates may be approximated by measuring digestive tract transit time following feeding. For little brown bats, defecation following feeding occurred within one to two hours and samples collected within 1.5 hours of capture reflected baseline glucocorticoid hormone levels (Richardson, in writing). In addition to knowing the excretion rates of hormones, one must also control for variables such as recent feeding or fasting, age, sex, reproductive condition, and circadian and seasonal rhythms in interpreting fecal and urinary hormone levels.

Like most biological samples, hormones in urine and feces are stable if frozen at sub-zero temperatures. Thus, fecal and urine samples taken either in the field or in a captive setting should be frozen as quickly as possible. As is the case with plasma samples, urine or fecal samples can be rapidly cooled in the field by placement on dry ice or in liquid nitrogen. The collection of urine samples (see also Bassett, this volume), which is realistically only feasible for captive bats, can be achieved by holding a urine cup or a funnel attached to a tube under a bat during urination. Theoretically, bats should be able to habituate to the collection of urine samples, and it may be possible to train bats to urinate upon presentation of the urine cup or funnel. Urine samples can also be collected from bats by removing a bat from the roost or cage prior to arousal in the evening and holding the bat upright over a vial or other collection device. However, repeatedly handling a bat in order to collect a urine sample negates the benefits of this typically noninvasive handling technique. Chua (2003) reported on the use of plastic sheets laid under roosting sites and in caves to collect both urine and fecal samples from *Pteropus* species, but the ability to identify which sample belonged to which bat (and therefore, which hormone levels belong to which individual) with this methodology is extremely limited.

Hormones in untreated urine samples are subject to decomposition by naturally occurring enzymes and bacteria within a matter of hours. A preservative, such as 1% sodium azide can be added in small amounts to urine samples (e.g., 100 µL to 3 mL of urine; Schradin et al., 2003), which should be vigorously shaken prior to cooling. Care should be taken when handling sodium azide, as it is an enzyme blocker and thus is highly toxic. When rapid cooling is not possible, hormones in feces can be stabilized by the addition of 95% ethanol; some researchers also add a small amount of 1–3% sodium azide (Khan et al., 2002).

Generally, fecal samples can be collected with a spatula or tweezers and placed directly into a plastic tube. Because many bats will defecate when handled, one can also directly collect fecal samples into a microcentrifuge tube while holding the bat in one hand. While not strictly noninvasive, if bats are being handled anyway for banding or measuring, collecting fecal samples at this time would be a convenient way in which to collect baseline fecal samples. If a bat does not defecate when handled, it could be placed into a clean holding bag or cage from which a sample could be later retrieved. Small boxes with Plexiglas tops and raised wire mesh bottoms (below which foil can be placed for sample collection) allow for observation and sample collection without disturbance. When collecting fecal samples, care should be taken to avoid urine or blood contamination. Samples can be collected in captivity directly from the floor of the enclosure, or in the field from a plastic sheet if the sample can be positively matched to a given subject. After the addition of a preservative, samples should be vigorously shaken or otherwise homogenized. Khan et al. (2002) found significant variations in steroid hormone levels in fecal samples preserved in 95% ethanol and stored at ambient temperature for prolonged periods. Because of this, they strongly recommend that samples already preserved in ethanol be moved from ambient temperatures to −20°C no more than a month (preferably less) following collection. Beehner and Whitten (2004) describe a technique for the field collection, processing, and analysis of fecal steroids that allows for the prolonged storage (up to 40 days) of samples at ambient temperature, which might be useful in assessment of hormone activity in free-ranging bats. Stability of fecal hormones stored over time should be independently determined for each species and storage technique. Prior to assay, fecal samples must be weighed so that the concentrations of hormones can be determined. Depending upon the protocol employed, this can be done at different times, for example, immediately after sample collection, after freezing (if no preservative was added), or after either pouring off or evaporation of the ethanol. It is also desirable to freeze dry fecal samples to control for differences in water content between fecal samples and to reduce variation in hormone measurements due to dietary fiber content (Wasser et al., 1993). For insectivorous bats, air dried fecal mass is very close to wet mass (Richardson, in writing), but water content may still be a factor in non-insectivorous bats.

### Saliva and Tissue Samples

Another less invasive sampling technique is the collection of saliva for hormone analysis. To the best of our knowledge, salivary hormones have not been measured in bats; however, bat saliva has been collected for other purposes from mouth swabs (see methods in Brookes et al., 2005). In order to collect saliva noninvasively, it might be possible to train captive bats to chew on sterile cotton swabs without being handled first, but care must be taken to avoid blood contamination in salivary samples (Kivlighan et al., 2004, 2005). Because hormone levels in saliva are generally only a small fraction of the levels found in plasma (Negrão et al., 2004; Randeva et al., 2003) and salivary samples from bats would be small, the utility of this technique in bats is questionable; thus the direct collection of plasma, urine, or fecal samples is preferable.

Hormone activity—including protein (and mRNA) levels and steroid hormone levels—is also sometimes assessed in tissues. Hormone levels can be determined in virtually any tissue in which the hormone is expressed and for which one has probes that will recognize the hormone. For example, Kronfeld-Schor et al. (2001) and Zhao et al. (2003) measured leptin secretion from adipose and placental tissue in *Myotis lucifugus* and *Eptesicus fuscus,* and Anthony et al. (1989) measured luteinizing hormone releasing hormone (LHRH) levels in the pituitary in *M. lucifugus*. Whether collecting tissue samples in the laboratory or in the field, tissues should be dissected immediately upon sacrifice. In the field, they must be placed in a container and immediately frozen in liquid nitrogen or on dry ice until transport to the laboratory; cycles of freezing/thawing should be avoided as many peptide and protein hormones are degraded under such conditions.

## METHODS OF HORMONE ANALYSIS

The vast majority of studies that have analyzed hormone levels employed an immunoassay of one type or another, and this review will focus on these methods. The development of the radioimmunoassay (RIA) and later of the enzyme immunoassay (EIA) made the study of actual hormone levels in a large number of samples feasible. Immunoassays are made possible by the fact that antibodies will competitively bind to antigens. For example, antibodies can be created that recognize the testosterone molecule (or any other hormone of interest) that will bind to testosterone with high affinity. Antigens (in this example, testosterone) can be labeled (radiolabeled or 'hot' in the case of the RIA and labeled with an enzyme in the case of the EIA) in such a way that the antibody used cannot discriminate between the labeled hormone and the native, non-labeled hormone.

The basic steps of an immunoassay are outlined in Figure 26.2. In the first step, antibodies to the hormone of interest are added to a reaction tube, which can be either a standard test tube or a well in a microplate. In practice, assay kits are available from a number of manufacturers; some kits include tubes or microplates that already contain antibodies bound ("coated") to the plastic, while others provide antibody solutions for the researcher to add. The second step involves the addition of the unlabeled

hormone (either a standard of known concentration or the sample that requires assay) to the reaction tube, which begins to bind to the antibody (step 3). Immediately following this, labeled hormone of a known concentration is added to the reaction tube (step 4). Subsequently, the unlabeled hormone and labeled hormone compete for antibody binding sites during an incubation period (step 5). The length of this incubation period is highly variable depending upon the assay protocol, as are the conditions under which the incubation is carried out (e.g., room temperature versus 4°C). In practice, the order of steps 2 and 4—whether the sample or the labeled hormone is added first—is variable, and theoretically, it does not matter because the competitive binding reaction that occurs during the incubation period reaches an equilibrium point. Following the incubation period, the unbound hormone (both labeled and unlabeled) is removed, either by simply decanting the tubes if the antibody is coated to the tubes or by a precipitation reaction in which the bound hormone is precipitated and the unbound portion is decanted. Lastly, the amount of labeled hormone is determined, either by counting radioactive particles (e.g., using a gamma counter to detect $^{125}$I, a common label in RIAs) or by adding a substrate that reacts with the enzyme label to produce a colorimetric reaction, which can then be detected with a microplate reader that reads the optical density of a particular wavelength (step 6). It is important to note that there is an inverse relationship between the amount of label measured and the concentration of the unlabelled hormone. To determine actual hormone concentrations, a standard curve must be constructed, from which concentrations in the samples can be calculated (step 7).

In practice, most assay protocols call for 5–8 standards that cover a wide range of possible hormone concentrations (e.g., in the cortisol RIA assay kit from MP Biomedicals, Irvine, CA, standards range between 10 ng/mL and 1000 ng/mL). A nonspecific binding (NSB) tube is also typically included, in which labeled hormone but no antibodies are added. Radioactive counts per minute (CPM, for RIA) or optical density (OD, for EIA) levels detected in the NSB tubes (which are typically very low) can then be subtracted from all other tube or well counts to control for nonspecific binding (binding to the tube or well rather than to antibodies). Standards and samples are nearly always run in duplicate within an assay, and sometimes even in triplicate for highly variable assays. Mean values for hormone concentration between the replicate tubes are calculated. Standard curves can be constructed by plotting the concentration of standards on the *x*-axis and the percent bound (e.g., CPM of standard or sample divided by CPM of zero standard 100) on the *y*-axis. The curve may be plotted on a linear, semi-log, or log-logit graph. A variety of curve fitting methods are used in different protocols, but in many, the standard curve can be determined by performing a linear regression through the points on a semi-log graph. The concentration of unknown samples can then be read directly from the curve.

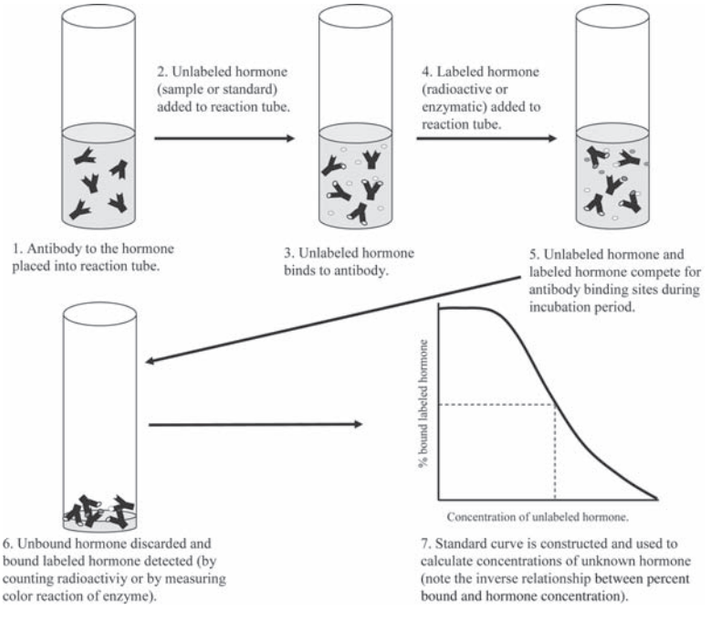


Figure 26.2. Schematic of standard immunoassay procedure (either radioimmunoassay or enzyme immunoassay).

The inclusion of control solutions in the assay is critical for determining variability both within and between assays. For a discussion of data reduction procedures and immunoassay quality control issues, see Robard, 1974; Robard et al., 1977; Chard, 1995; and Straume et al., 1998, and references cited therein.

In practice, many hormone assays are more complicated than the one presented in Figure 26.2, and some employ more than one antibody in which the hormone is "sandwiched" between (that is, bound by) two different antibodies, which can improve the accuracy of an assay. In many cases, it is desirable to extract (i.e., purify) hormones prior to assay and there are a number of extraction procedures described in the literature. This is especially the case for urinary and fecal assays and a review of the extraction procedures is beyond the scope of this chapter. We strongly recommend that researchers review the literature for assays of their hormone of interest and request protocols from both manufacturers and other researchers. If assaying urine samples, the concentration of urine must be controlled for, which can be achieved by measuring creatinine levels (Taussky, 1954; Schradin et al., 2003). Likewise, if assaying fecal samples, the sample must be dried to control for variations in water content and weighed to provide a reference point for the hormone concentration of interest.

For nontraditional species such as bats, new assays or the use of established assays that have been validated for other species must be validated for the study species. As outlined below, there are a number of steps that can be taken to validate an assay for a particular species. In our experience, many assays can be proportionally scaled down to cope with the sometimes-small volumes available from bats. Proper procedures (see below) must be used to validate this modification and a discussion of increasing assay sensitivity can be found in Chard, 1995. Excellent examples of assay validation can be found in Kwiecinski et al., 1986; Billitti et al., 1998; Carlson et al., 2003; and Good et al., 2003; and readers are referred to Chard, 1995, and Wild, 2005, for general immunoassay issues.

## Parallelism

Parallelism is the linearity of diluted samples with the standard curve. To assess parallelism, 2–3 samples should be diluted (typically serially diluted, with dilutions varying depending upon expected values) and included within the assay. The corrected concentrations (e.g., the amount of hormone measured at 1:100 dilution multiplied by 100) are then plotted along with the standard curve. Curves for the diluted samples should roughly parallel the standard curve. If parallelism between diluted samples and the standard curve is found, one can assume that the antibodies used in the assay are binding to the hormones in the samples in a similar way as to the standards. The equality of their slopes can be determined with a t-test or by ANOVA; see Plikaytis et al., 1994, for an excellent discussion of parallelism in assay validation.

## Precision

Precision is the ability to obtain the same result on a repeated basis. It can be assessed by determining the coefficient of variation (*CV*), or the percent difference, between duplicates of the same sample, standard, or control. It can also be determined by calculating the correlation coefficient of a large number of replicates of the same sample within an assay. Measuring the same control solution twice within the same assay allows for the determination of intra-assay variability (see Robard, 1974 for calculations). When performing multiple assays within a single study, the precision of the assays compared to one another is also important. This can be monitored by measuring the hormone level of the same control solution (preferably an aliquot from a pooled sample of plasma, urine, saliva, etc. from one's study species) across multiple assays to determine the inter-assay variability.

## Accuracy

The accuracy of an assay can be determined by adding given amounts of known hormone to a sample of known concentration. If an assay is accurate, the total amount that would be expected given the known sample concentration and the concentration of added hormone will be detected. For example, if a sample was previously determined to have 63 ng/mL of cortisol, and aliquots of this sample are spiked with (1) 20 ng/mL, (2) 100 ng/mL, or (3) 200 ng/mL of cortisol, we would then expect to measure (1) 83 ng/mL, (2) 163 ng/mL, and (3) 263 ng/mL, respectively. For accurate measurements, it is critical to thoroughly vortex these spiked samples. Accuracy is calculated as the percentage of the observed final concentrations versus the expected final concentrations and levels should be within 10% of expected values. Poor accuracy suggests that other substances within the sample (beyond the hormone in question) are interfering with the ability of the antibody to bind to the hormone being assayed. Various extraction methods can be tried to purify the hormone and remove the extraneous interfering substances. Poor accuracy may also be due to the assay antibodies binding or cross-reacting to closely related hormones other than the hormone being assayed. Although manufactured kits will typically report the cross-reactivity of their antibody with other compounds, these kits are made for model systems (human, rhesus macaque, mouse, rat, dog, etc.) and it is possible that their antibodies will cross-react with other compounds in bat samples. Potential cross-reactivity can be determined by spiking a stripped sample from the study species with purified hormones that might cross-react with an antibody and subjecting these samples to the immunoassay procedure (e.g., bat plasma with all steroids extracted and purified cortisol added, subjected

to a corticosterone assay to determine the cross-reactivity of the corticosterone antibody to cortisol; Reeder et al., 2004a).

### Sensitivity

The sensitivity of an assay, also known as the least detectable dose (LDD) or the minimum detectable dose (MDD), is the lowest concentration that can be reliably detected by the assay. Two different methods for determining the sensitivity of an assay are typically found in the literature. In the first method, 10–20 replicates of the zero standard are assayed and the mean and standard deviation of the CPMs or ODs are calculated. One then subtracts two standard deviations from the mean of the zero tubes (remembering that the competitive binding of the immunoassay creates an inverse relationship between CPMs or ODs and the concentration of the hormone), and plots this number against the standard curve to determine the sensitivity. In the second method, the sensitivity is determined as the hormone concentration determined from the standard curve at 95% binding. In practice, the two procedures yield very similar results.

### Range

The range of an assay is defined as the difference between the least detectable dose and the highest detectable dose (typically the highest standard; the concentration found around 20% binding). This value is not a part of the validation of an assay *per se*, but establishing the range of an assay is important for determining the amount of sample needed for the assay and whether samples must be further diluted or concentrated prior to assay. Because immunoassays are most accurate when samples fall between 20 and 80 percent binding, knowing the range of the assay and the expected values of hormones in one's samples allows for the determination of optimum dilutions.

### Biological Validation

A number of researchers also include a biological validation of new assay procedures. For example, animals can be treated with a drug or administered a hormone that is known to cause a significant increase or decrease in the concentrations of the hormone being studied. If the expected response is detected in the new assay, it is likely that the assay will be able to reliably detect the hormone being studied. Biological validation is especially important when measuring hormone metabolites, such as those found in blood or urine.

## PROBLEMS IN ASSAYING PROTEIN HORMONES

As described above, genes for protein hormones are subject to selection and drift and can vary significantly in base-pair composition, leading to variations in amino acid sequence (and hence, even structure) between species. Thus, when one is using antibodies generated for another species (commonly mouse, rat, dog, rhesus macaque, or human), it is important to verify that the substance being detected in the assay is in fact the desired protein. Common methodologies for protein identification include gel chromatography and western blot (Carlson et al., 2003). In chromatography, authentic radiolabeled hormone (e.g., the label used in the assay system) and samples of the hormone to be assayed (e.g., bat plasma, tissue extract, etc., in approximately the same concentration as the radiolabeled hormone) are both loaded into columns (e.g., Sephadex) and eluted. Each elution fraction is captured and analyzed, and the elution profiles of the authentic hormone and the hormone detected in the sample should be very similar. In western blot, samples of both authentic hormone and those obtained from the animal being studied are separated by gel electrophoresis and labeled with antibody against the desired protein. The antibody used should detect protein bands corresponding to the known molecular weight of the hormone in both authentic hormone and sample hormone lanes. If possible, it is desirable to run western blots with the same antibody used in the assay system.

These tests may sometimes indicate that the readily available antibodies (usually heterologous antibodies that recognize hormones from traditional animal models) and standards do not work for bat samples, indicating that sequence divergences have occurred at the gene and protein level that affect the epitope in the protein. When this is the case, homologous antibodies can be generated against the protein in question in the study species. Briefly, this involves sequencing the cDNA for the protein of interest in the study species, and from this, the predicted peptide fragment can be created and antibodies can be commercially produced that are specific to the study species. If possible, it is ideal to sequence the cDNA for the coding region of the peptide fragment that varies between species.

## CONCLUSION

Given that approximately one out of five mammals is a bat and that hormones have only been studied in approximately 20 of the 1,116 currently recognized species of bats (Simmons, 2005), the study of hormones in bats is in its infancy. Moreover, given that bats exhibit a wide variety of life history strategies, behaviors, and reproductive tactics and that hormones influence most bodily functions and behaviors, the possibilities for examining endocrine function in bats are numerous. In this chapter, we hope to have laid the groundwork for future studies of hormones in bats, which we believe are excellent and valuable subjects for both field and captive studies of endocrine function.

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