September 22, 2003
Lecture 7: Neuroendocrine Toxicity (Endocrine System Effects—Part I)

I. How Do We Know About Hazards Caused by Environmental Contaminants or Naturally Occurring Chemicals?

A. Prior to beginning a discussion of hazards to the endocrine system (or neuroendocrine system), let's review the types of studies used to determine what the potential physiological (or other level of organization) effects are as well as the hazards on a population level.
   1. Three types of studies (or experiments) are used to assess hazard or potential biological effects:
      a. In-vitro
      b. In-vivo
      c. Epidemiological

B. <u>In-vitro Experiments</u>
   1. These experiments involve use of biological material (cell cultures, tissue cultures, enzymes, receptors, etc) isolated from the whole organism, but essentially kept functional
   2. A specific response of the biological material is measured relative to some increasing dosing regime
   3. An LC50 type parameter can be calculated depending on the endpoint
      a. If the endpoint is cell death, for ex, an LC50 would be appropriate, or if the exact dose (i.e., mass per unit of biological material) were known, an LD50 could be calculated
      b. Many times a simple change in response or activity of an enzyme or receptor is noted
         1. In these cases, use of an ED50 (the effective dose causing a 50% change in response) is calculated
         2. With enzymes and receptors, where activity might be inhibited or alternative stimulated, a K_M (Michaelis-Menton constant for affinity with substrate) or K_d (equilibrium constant for the binding reaction) can be calculated; alternatively, an I_{50} (concentration inhibiting 50% of the activity) may be determined.
            a. For example, OP insecticides; specific inhibitors of cholinesterase can be characterized by their I_{50} for inhibition of the enzyme in *in vitro* studies.
            b. Note in the table below, that I50 can vary among an analogous series of enzyme-inhibiting compounds; such variations give clues to the mechanism of toxicity.

Table 1. Cholinesterase (ChE) Inhibition and Toxicity of OP Insecticide and Metabolites to house flies (*Musca domestica*) (ChE is an enzyme in the central nervous system and at neuromuscular junctions that hydrolyzes

acetylcholine, a chemical neurotransmitter, thereby modulating nerve signals).

Compound	House Fly Head ChE Inhibition, I_{50} (moles x 10^6)	LD_{50} (μg/fly)
Malathion	20	Not Determined
Malathion oxon	0.0046	Not Determined
Demeton, thiono	220	Not Determined
Demeton, sulfoxide	3.60	2.0
Demeton, sulfone	0.83	1.2
Demeton oxon	0.024	0.7
Demeton oxon sulfoxide	1.10	8.7
Demeton oxon sulfone	0.12	3.7

C. <u>In-vivo Experiments</u>

1. These experiments use whole animals; determinations of the NOEL would use this type of experiment (although a NOEL can also be determined from in vitro expts.)
   a. In these experiments a typical response might be death, but could also include change in enzyme activity (for ex., blood could be withdrawn and various enzymes analyzed) or behavior or nerve functioning, etc.
      1. Responses other than death are classified as sub-lethal
      2. Ideally, these experiments would use a range of doses (which would be required if a NOEL was sought), but often an investigator will just use a control and one or two doses, usually very high relative to real exposure.
         a. A rationale for using high doses would be to derive all possible adverse reactions. In other words, a high dose study to find possible effects can be considered hazard identification. In hazard identification, ideally one would look for the most sensitive adverse effect (i.e., one produced by the lowest dose tested).
         b. Another rationale would be to explore the mechanism of toxicity
      3. Experiments employing high doses are useful for determining possible effects from occupational (worker) exposures
2. For examples of in vivo assessments of contaminants used in risk assessment, see the EPA web site for OP insecticides (http://www.epa.gov/oppsrrd1/op/)
   a. As one example of the kind of data collected, a subchronic oral exposure study was conducted with rats to determine potential for neurotoxicity of an OP insecticide (azinphos-methyl). Here is how the released document summarized the study.
   b. "…groups of 18 male and 18 female rats were administered the technical grade of azinphos-methyl in the diet for 13 weeks at nominal doses of 0, 15, 45, or 120 ppm for males (0, 0.91, 2.81, and 7.87 mg/kg/day mean intake) and 0, 15, 45, or 90 ppm for females (0, 1.05, 3.23, and 6.99 mg/kg/day mean intake). Twelve rats per sex per dose were used for

neurobehavioral evaluation, with half used for neuropathology. The remaining six per sex per dose were used for cholinesterase determination. A statistically significant (>20%) inhibition of red cell cholinesterase was observed at all dose levels tested in this study, as was a statistically significant inhibition (>20%) of plasma and brain cholinesterase at the mid and high dose. Decreased forelimb grip strength, motor activity, and locomotor activity were observed in both sexes at the high dose, but did not correlate definitively with any pathology of the nervous system. Based on the data in this study, the systemic LOEL = 15 ppm (~ 1.0 mg/kg/day) for male and female rats, based on a statistically significant (>20%) inhibition of red cell cholinesterase. The systemic NOEL was < 15 ppm and estimated to be 5 ppm (0.3 mg/kg/day) for male and female rats, based on extrapolation of cholinesterase inhibition data. Although significant signs of cholinergic toxicity were observed in this study, there was no definitive evidence of a neurotoxic effect for azinphos-methyl in this study."

D. <u>Epidemiology</u>

1. The objective of these studies is to relate the incidence of a disease or condition with exposure to some agent (microbiological, chemical) or activity (lifestyle, behavior, product use, place of residence, etc.);
2. The objective of these studies is to relate the incidence of a disease or condition with exposure to some agent (microbiological, chemical) or activity (lifestyle, behavior, product use, place of residence, etc.);
    a. Epidemiological studies of chemical effects are normally applied to chronic exposures and maladies best classified as chronic toxicity as opposed to acute toxicity (see endpoints below)
3. These studies are the most controversial because it is very difficult to control confounding factors (i.e., variables that may affect or influence the outcome of the results);
    a. Furthermore, epidemiological studies at best can make associations between exposure to an agent and an outcome, but this is quite distinct from concluding an agent caused an outcome
        1. Epidemiological studies rely heavily on regression analysis
4. Epidemiology grew out of the need to control infectious diseases, which have definable causes; furthermore, infectious diseases, which are essentially microbiological problems, can be directly tested using Koch's postulates, stated as follows:
    a. The infectious agent (microbe) must be present in every case of a disease;
    b. The microbe must be isolated from the disease and grown in pure culture;
    c. The specific disease must be reproduced when a pure culture is inoculated into a healthy susceptible host;
    d. The microbe must be recoverable again from the newly infected host.
5. Unfortunately, for most chemical exposures, unless they are acute (as opposed to chronic), Koch's postulates are not applicable.

6. Furthermore, in many chemical epidemiological studies that do not involve workers at a specific industry (or manufacturing site), exposure records are poor to nonexistent.
    a. Often exposure is deduced from interviews of "what was used" or "next-of-kin" interviews.

II. Introduction: State of the Problem Regarding Neuroendocrine Toxicity (or Adverse Effects on the Endocrine System)

A. Over the last 10 years, a hypothesis has come into the forefront of toxicological knowledge: numerous chemicals, both synthetic and naturally occurring can affect the normal functioning of the endocrine system.
    1. Usually, the effects of various chemicals on the endocrine system have been studied from the perspective of adverse effects. Thus, the chemicals in question have been called endocrine disrupters (sometimes endocrine disrupting chemicals or EDCs)
        a. In other words, in an experiment in which dependent variables (for example, circulating hormone levels in the plasma) are compared between animals either treated or untreated with a contaminant, any statistically significant differences tend to be interpreted as an adverse effect caused by the contaminant.
            1. In fact, the observed response may be negative, neutral, or in some cases beneficial to the organism.

B. Substances affecting the endocrine system usually cause physiological, developmental, morphological, or behavioral changes at doses that are not lethal or associated with cellular toxicity.

C. Numerous studies indicate that a number of contaminants can adversely affect individual aquatic and terrestrial species with regard to reproduction and development.
    1. While wildlife (including terrestrial and aquatic organisms) population declines have been noted, it is yet unclear whether contaminants that interact with the endocrine system are directly causal in the declines.
        a. One confounding factor is the tremendous changes and loss of habitat that alone can drastically affect population abundance of many wildlife species.
            1. Thus, we are back to the dilemma of affects on individuals that can be shown in laboratory settings, but we still have an uncertainty of what is happening at the population level, yet alone any ecological scale effects.

D. One recent concern has been the "estrogenic" effects of certain environmental contaminants, mainly including those that contain chlorine (but not limited to these); this effect is better described as an endocrine disrupting effect, and the chemicals involved (both natural and synthetic) are known as endocrine disrupters.
    1. One highly cited "authority" who strongly associates exposure to endocrine-disrupting chemicals with reproductive problems in wildlife and humans as well as breast cancer is Theo Colborn from the W. Alton Jones Foundation

and world Wildlife Fund in Washington, DC; in March of 1996, Colborn et al. released a book "*Our Stolen Future*," which has been hailed as the sequel to *Silent Spring*. This book is the popular version of what some scientists think is the most significant contemporary environmental and public health crisis;

a. Essentially the story says that synthetic chemicals released into the environment (and some natural ones too) mimic hormones of the endocrine system (mainly estrogen and testosterone), and because of their wide ranging effects, essentially threaten the human species (as well as everything else).

2. *Our Stolen Future* is a popularization of some of the hypotheses presented in a conference held in Racine, WI during 1992. This conference resulted in publication of the compendium entitled, "Chemically Induced Alterations in Sexual Development—The Human/Wildlife Connection," that coincidentally was also co-edited by Theo Colborn.
   a. The conference involved invited scientists, a number of whom had been working on the connection between the drug, DES (diethylstilbesterol), that was a therapeutic given to women in the late 1950's and throughout the 1960's as an anti-abortifacient to prevent miscarriages. The children of women taking this drug had a significantly higher rate of genital cancer and other reproductive system maladies. Several of the scientists were also wildlife specialists who presented data that they conclude gives evidence to similar maladies in wildlife, but they hypothesized the cause as exposure to persistent organochlorine pollutants (known as POPs), like DDT, PCBs, dioxin.
   b. A consensus statement was issued at the WI conference:
      1. "A large number of man-make chemicals that have been released into the environment, as well as a few natural ones, have the potential to disrupt the endocrine system of animals, including humans."
      2. While the evidence for endocrine disruption in humans was based almost solely on the problems of DES, and thus tenuous at best, the conference attendees believed that wildlife populations were already affected.

E. The National Academy of Sciences' National Research Council (NRC) issued a report addressing putative endocrine system disrupting chemicals. The NRC chose to "rename" endocrine disrupters to Hormonally Active Agents in the Environment (which is the title of the report and is published by National Academy Press, Wash., DC, 1999; can be read online at http://books.nap.edu/books/0309064198/html/R1.html)
   1. The NRC chose to use the term hormonally active agents (HAAs) because after studying the published literature, it became clear that some of the putative effects were not necessarily related directly to effects on the endocrine system. Furthermore, some of the effects should not be considered disruption per se.
   2. The NRC also concluded that the evidence for HAAs effect on humans from environmental exposures to contaminants was weak, at best; the one exception is the experiences with the drug, DES (diethylstilbesterol).

3. However, the possibility that wildlife populations in some instances and in some environments have shown effects from hormone mimics has more plausibility than the effects on humans.

F. Owing to the nature of the endocrine system, which is probably more appropriately described as the neuroendocrine system with respect to the putative wide ranging physiological processes regulated, endocrine disrupters can be viewed as a unifying force for literally all health effects. Some are skeptical, however, that this linkage for all health effects is inappropriate, as concluded by the NRC (mentioned above). Nevertheless, everything from adverse reproductive effects, to immunotoxicity, and even certain cancers have been associated with endocrine disrupters. Even behavior, especially behavior that is gender specific is putatively adversely affected.
   1. The reason for this association probably stems from the nature of the endocrine system itself.

III. Overview of Endocrine Physiology--Vertebrates

A. "The endocrine system is one of at least three important integrating and regulatory systems in humans and other animals. The other two are the nervous and immune systems" (U.S. EPA. 1997. Special report on environmental endocrine disruption: an effects assessment and analysis. EPLA/630/R-96/012, February 1997) Thus, the endocrine system is actually one physiological system that is linked to both the central nervous system and the immune system
   1. I like to metaphorically call the endocrine system one node on the body's internet.
   2. All cells communicate with one another through a system of feedback loops
   3. The internet controls all growth and physiological functions
   4. The mechanism by which the internet functions is through a system of chemical messengers that bind to receptors
      a. Endocrine system produces hormones
      b. Nervous system produces neurotransmitters
      c. Immune system produces cytokines

B. Hormones, the chemical messengers of the endocrine system are produced by specific glands (or tissues);
   1. Operational definition of hormones from the EPA
      a. "Hormones are natural, secretory products of endocrine glands (ductless glands that discharge directly into the bloodstream). Hormones travel in the blood in very small concentrations and bind to specific cell sites called receptors in distant target tissues and organs, where they exert their effects on development, growth, and reproduction in to other bodily functions" (EPA 1997).

C. There can be redundancy among glands in the production of certain hormones
   1. For example, estrogen (actually estradiol 17ß) is produced by the ovaries, but testosterone is oxidized to estrogen by a P-450 enzyme called aromatase which is present in the brain and the adrenal glands as well as the gonads. Thus males have estrogen and females have testosterone (and vice versa).

a. Another example: the adrenal cortex gland associated with the kidneys can synthesize androgens, the class of hormones to which testosterone belongs; and of course, testosterone is mainly synthesized in the testes.

D. "Hormones influence important regulatory, developmental, growth, and homeostatic mechanisms, such as reproductive structure and function; maintenance of normal levels of glucose and ions in blood; control of general body metabolism; blood pressure; and other glandular, muscle, and nervous system functions. Some of the major endocrine glands include the pituitary, thyroid, pancreas, adrenal, and the male and female gonads (testes and ovaries)" (EPA 1997).

1. The vertebrate glands primarily responsible for hormone synthesis and release and the functions of the hormone are shown in the following table.

Table 2. Vertebrate Endocrine Glands, Hormone, and Major Function (from Schmidt-Nielsen 1997. Chapter 12, Hormonal Control. Pp. 499-501 in *Animal Physiology. Adaptation and Environment*. Cambridge University Press)

Gland	**Hormone**	**Major Functions**
Adenohypophysis (pituitary) (anterior portion)	Adrenocorticotropic hormone (ACTH)	Stimulates adrenal cortex
	Thyrotorpic hormone (TSH)	Stimulate thyroid
	Follicle-stimulating hormone (FSH)	Stimulates ovarian follicle development; seminiferous tubuole development in testes
	Luteininzing hormone (LH)	Stimulates conversion of ovarian follicle to corpus luteum; stimulates progesterone and testosterone production
	Prolactin	Stiulates milk production; osmoregulation in fish
	Melanocyte-stimulating hormone (MSH)	Stimulates dispersion of melanin in amphibian skin pigment cells
	Growth-stimlating hormonee (GSH)	Stimlates growth (acts via liver)
Hypothalamus	Releasing and release-inhibting hormones acting on pituitary	Hormones delivered via portal circulation to pituitary
Hypothalamus (via neurohypophysis or posterior pituitary)	Antidiuretic hormone (ADH) (aka vasopressin)	Stimulates water reabsorption in kidney
	Oxytocin	Stimulates contraction of

		uterine muscle; releases milk
Liver	Somatomedin	Stimulates growth
Kidney	Renin	Increases blood pressure; stimulates secretion from adrenal cortex
	Dihydroxycholecalciferol	Affects calcium absorption and bone calcification
Heart	Atrial natriuretic factor (ANF)	Increases renal sodium excretion
Adrenal cortex	Glucocorticoids (corticosterone, cortisone, hydrocortisone, etc.)	Regulates carbohydrate metabolism
	Mineralocorticoids (aldosterone, deoxycorticosterone, etc.)	Regulate sodium metabolism and excretion
	Cortical androgens, progesterone	Simulate secondary sexual characteristics, predominantly male
Ovary	Estrogens (estradiol, etc.)	Initiate and maintain female secondary sexual characteristics; initiate periodic thickening of uterine mucosa; inhibit release of FSH
	Progesterone	Cooperates with estrogens in stimulating female sexual characteristics; supports and glandularizes uterine mucosa, inhibits release of LH and FSH
	Relaxin	Causes relaxation of pelvic ligaments before parturition
Testis	Testosterone	Initiates and maintains male secondary sexual characteristics
Thyroid	Thyroxine,m triiodothyronine	Stimlate oxidative metabolism; stimulate amphibian metamorphosis, inhibit release of TSH; role in brain development
	Calcitonin	Inhibits excessive rise in blood calcium

Parathyroid	Parathormone	Increases blood calcium
Stomach	Gastrin	Stimulates secretion of gastric juice
Duodenum	Secretin	Stimulates secretion of pancreatic juice
	Cholecystokinin (=pancreozymin)	Stimulates release of bile by gallbladder; stimulates secretion of pancreatic enzymes
	Enterogastrone	Inhibits gastric secretion
Pancreas	Insulin	Reduces blood glucose; stimulates formation and storage of carbohydrates
	Glucagon	Increases blood glucose by mobilization of glycogen from liver
Adrenal medulla	Adrenaline, noradrenaline	Augment sympathetic function (vasodilation) in muscle, liver, lungs; vasoconstriction in many visceral organs); increase blood sugar
Pineal	Melatonin	Affects or controls daily rhythms

E. Summary of major functions under endocrine control and partial listing of the principal hormones that have a role (adapted from Schmidt-Nielsen 1997)

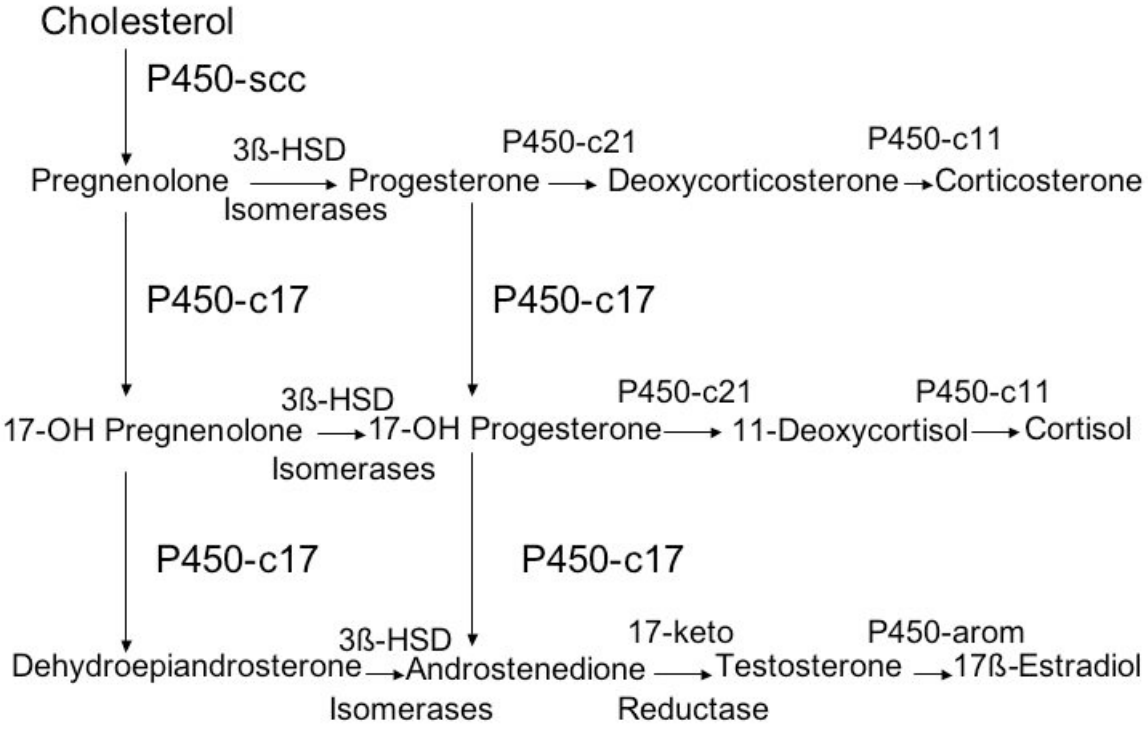
Table 3. Major functions of endocrine system and associated hormones

Digestion and Related Metabolic Functions	Secretin Gastrin Insulin Glucagon Noradrenaline Thyroxine Adrenal corticoids
Osmoregulation, excretion, water and salt metabolism	Vasopressin Prolactin Aldosterone
Calcium metabolism	Parathormone Calcitonin
Growth and Morphological Changes	Growth Hormone Adrenocortical androgens Thyroxine (amphibian metamorphosis)

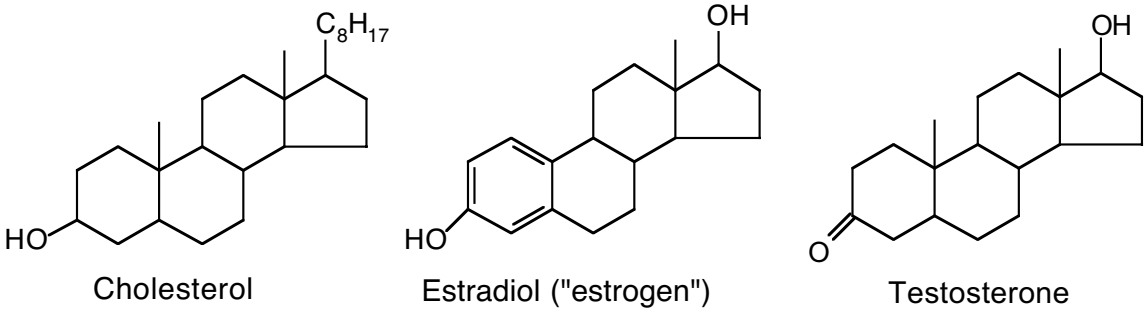
	Melanocyte-stimulating hormone (amphibian color change)
Reproductive Organs and Reproduction	Follicle –stimulating hormone Luteinizing hormone Estrogen Progesterone Prolactin Testerone
Brain Development *	Thyroid hormone

*Note: The idea that brain development is dependent on proper timing of release and titre of thyroid hormones is a fairly new concept in neuroendocrine physiology [Zoeller, R. T., A. L. S. Dowling, C. T. A. Herzig, E. A. Iannacone, K. J. Gauger, and R. Bansal. 2002. Thyroid Hormone, Brain Development, and the Environment. Environmental Health Perspectives Supplements Volume 110, Number 3, June 2002 110(Suppl. 3):355-361.]

F. Hormone Classification by Biochemical Type
   1. Steroid hormones
      a. Includes adrenocortical androgens, estrogens, progesterones, corticosteroids (cortisone and aldosterone)
      b. All derived from cholesterol in the following biosynthetic pathways (Figure 1):



c. Note that the P-450 enzyme isoforms are very important in synthesis of various forms from the precursor steroids
d. Slight changes in structure can result in big differences in physiological function. Note the similarity of estradiol and testosterone to cholesterol and the very close similarity of the former two hormone to each other (Figure 2).



2. Peptide and Protein Hormones
   a. Hormones released from the hypothalamus exert their action by causing release of other hormones from various endocrine glands. The hypothalamus hormones are peptides (3 – 14 amino acids)
      1. Examples: Thyrotropin releasing hormone (T-RH); Growth releasing hormone (GH-RH; Growth hormone release-inhibiting hormone (GH-RIH)
   b. Hormones released from the pituitary are proteins with several hundred amino acids
      1. Examples: Human growth hormone
   c. Some hormones are glycoproteins and contain a carbohydrate ("sugar") moiety or component in addition to the peptide chain
3. Tyrosine-derived hormones
   a. Catecholamines noradrenaline and adrenaline
      1. All synthesized from the amino acid tyrosine
   b. Thyroid hormones are synthesized from tyrosine (triiodothyronine; thyroxine)

G. "Certain aspects of endocrine systems are remarkably well conserved across phyla pointing, for example, to reproduction." "Based upon this observation, it was noted that endocrine-disrupting chemicals that act through specific receptors (affecting hormone synthesis, release and/or actions) could well be particularly amenable for extrapolation of prediction of relative risk across species. (from Ankley et al. 1997)
   1. In other words, although specific details may differ more or less among different classes of vertebrates, the functioning of the endocrine system and its integration with the central nervous system and immune system is very similar across all vertebrates. Thus, any chemical that interacts with the endocrine system in an animal from one phylum or class is likely to have a similar mode of action in a different phylum or class.

H. After the hormones are released, they circulate to other tissues, adjacent or distant, and then associate with receptors either in the cell membrane or alternatively, they cross the cell membrane via a carrier protein or peptide and then are released within the cell where they diffuse to receptors in the nucleus.
   1. The hormones only associate with the appropriate receptors, thereby not stimulating cells (or tissues) lacking the receptors.

I. When the receptors are bound with the hormone, they turn on specific genes that make protein products necessary to cause a cell to grow or perform some physiological function
   1. Example: estrogen receptor, testosterone receptor, etc.
      a. The endocrine system is especially important during early fetal development
         1. Production of testosterone or lack thereof is key to masculinization and feminization of the fetus
         2. Early development can control future secondary sexual development (i.e., puberty)
      b. Receptors for estrogen also can be found in the brain, heart, and pituitary, indicating that estrogen is important for general physiological well being (both males and females produce estrogen; the difference between genders is related to the ratio of the two, with females having a higher estrogen to testosterone ratio than males)

J. Brain Control of Endocrine Function
   1. A constant interaction between the endocrine glands and the central nervous system
      a. Hypothalamus, a region of neurosecretory cells located at the base of the brain immediately above the pituitary gland, plays the dominant role in control of hormone release
         1. The hypothalamus controls the pituitary gland via neural connections to the neurohypophysis (the posterior lobe of the pituitary gland)
            a. The neurohypophysis releases:
               1. Vasopressin, which affects the reabsorption of water in the kidney so that a concentrated urine is formed (aka antidiuretic hormone)
               2. Oxytocin, which causes contraction of the smooth muscle of the uterus in the pregnant female at term.
         2. The hypothalamus controls the pituitary gland through blood vessels (known as the portal circulation) connected to the adenohypophysis (the anterior lobe of the pituitary gland)
            a. The hypothalamus secretes hormones through the portal system; the hormones are either releasing hormones (RH) or release-inhibiting hormones:
               1. Growth hormone RH, prolactin RH, melanocyte-stimulating hormone RH, corticotropin (ACTH) RH, thyrotropin (TSH) RH, luteinzing hormone (LH) RH, and follicle-stimulating (FSH) RH

2. For the growth hormone, prolactin, and melanocyte-stimulating hormone RHs there are release-inhibiting hormones (RIH); for example, prolactin RIH.

b. The hypothalamus is also under negative feedback control for release of the hormones, ACTH, TSH, LH, and FSH
   1. These hormones affect the adrenal cortex, the thyroid, and the gonads, respectively. Note that LH and FSH are gonadotropin hormones.
   2. When the above glands are stimulated, they release their hormones into the blood (respectively, corticosteroid hormones, thyroxine, sex steroid hormones [i.e., estrogen, testosterone]
   3. These hormones, when their blood concentration is high enough, in turn inhibit by negative feedback the secretion of the release hormones in the hypothalamus.

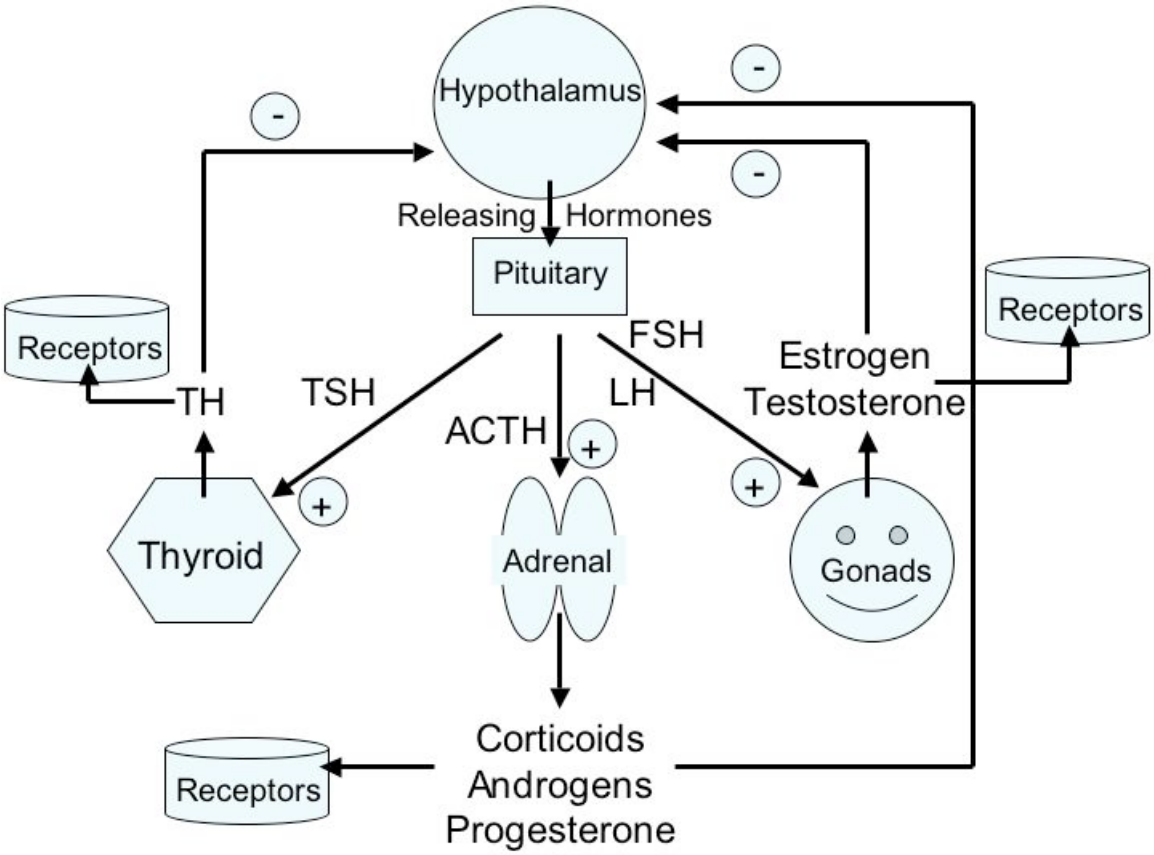


Figure 3. Schematic of positive and negative feedback control by the hypothalamus on the pituitary and by the distal endocrine glands on the hypothalamus

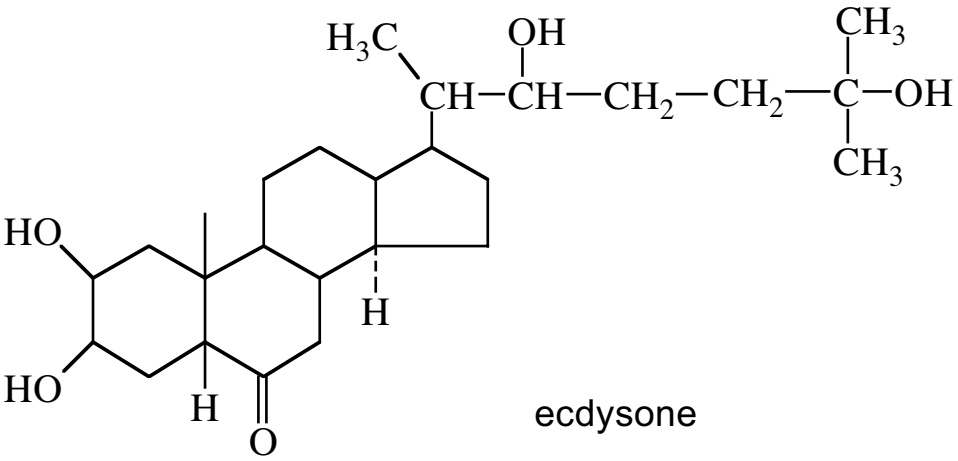
2. The hypothalamus has neural connections with different regions of the rest of the brain; thus, stimuli (both exogenous or environmental and endogenous or internal) affecting the nervous system has the potential to affect release of hormones from the hypothalamus. In short, the endocrine system is

essentially under control of the nervous system acting through the hypothalamus as a control center for all the other endocrine glands.

IV. Endocrine System—Invertebrates

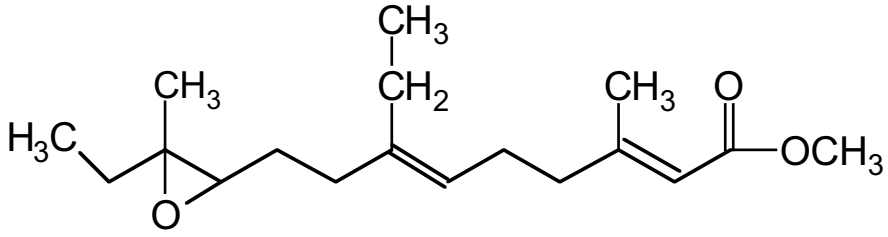
A. The more highly organized the organisms of an invertebrate Phylum, the more likely we are to find a "nervous system", neurosecretory cells (aka neurohemal organs, and endocrine glands as we do in vertebrates.
   1. Note that in higher invertebrates like insects, the central nervous system consists of the brain and a series of nerve cell clusters called ganglia that run to the posterior of the body.

B. Insects are probably the best studied group (and probably the most physiologically & morphologically organized) of the invertebrates.
   1. Two major clusters of glandular tissue:
      a. Prothoracic glands, which produce steroid hormones (Figure 4)



ecdysone

      b. Corpora allata, which produce terpenoid hormones (Figure 5)



juvenile hormone

   2. Because insects have a hardened exoskeleton, growth of tissues is limited; thus, insects moult or essentially shed the exoskeleton.
      a. Metamorphosis involves the changes from one stage (egg, larva, pupa, adult) to another; however, the larva will go through several stages or stadia (aka instars), with each getting bigger at each moult cycle.

1. Note that one large group of different insect Orders does not go through a pupal stage. These insects are hemimetabolous (for example, cockroaches, grasshoppers, bugs, lice).
    a. The larva is called a nymph and looks like a small adult, except it is wingless and not reproductively developed.
2. Other Orders of insects go through complete metamorphosis and are called holometabolous (i.e., well defined egg, larva, pupa, and adult stage; for example, flies, bees, wasps, beetle, moths, butterflies).
    a. The larval stage is usually "worm-like", wherein the legs are small and not developed as in the adult. The larvae completely lacks wings and its exoskeleton does not appear hardened, although it might be colorful with spiny hairs.

3. Moulting is initiated by secretion of ecdysone hormone from the prothoracic glands
    a. The prothoracic glands are under the control of a brain hormone called PTTH (prothoracicotropic hormone).
    b. Prior to moulting, the insect tissues in the larval stage have been growing bigger
    c. The target of ecdysone are the epidermal cells. When stimulated by ecdysone, the epidermis separates from the overlying cuticle.
    d. The change from the larval to the adult stage (which is reproductively competent and usually has wings) is under the control of juvenile hormone
4. Juvenile hormone controls development from one life stage to another;
    a. JH is secreted by the corpora allata;
    b. In the presence of high titers of JH, the insect retains its larval and nymphal characters from moult to moult.
    c. At the last larval or nymphal stage, the titer of JH drops and the tissues then start to develop adult characteristics prior to the last moult.
        1. For holometabolous insects, when the JH drops, the tissues of the larva degenerate and adult tissues being to develop. However, the pupal stage is an intermediate stage wherein the adult tissues are developing prior to the last moult. JH is completely absent in the pupa.
            a. Note that during the pupal to adult transformation, many pupa occur in hidden or protective environments, such as soil, within a cocoon, etc.

V. Indirect and Direct Mechanisms For Disrupting the Endocrine System

A. The concern about endocrine disrupters centers around the vulnerability of the fetus; some researchers point out peculiar effects in wildlife that forebodes possible effects in humans. Thus, the big concern are adverse effects on normal development, especially of the nervous system (brain) and the reproductive system.

B. Consensus indicates the following systems are most vulnerable to "attack" by HAAs (Ankley 1997):
    1. **Reproduction (highest priority)**
    2. **Growth/Development (highest priority)**

3. **Immunocompetence (lower priority)**

C. "**Two critical issues to address** when considering EDCs in the context of real-world scenarios where organisms are exposed to multiple chemical stressors during different life stages (Ankley 1997).
   1. "First, the organizational effect of a disruption during embryonic development might not be observed or expressed until much later in the animals life, perhaps not until activational hormone stimulus is received."
   2. "Secondly, unlike most mixtures (where additivity of toxic equivalence is generally considered to conservatively predict the total mixture toxicity), the potential for synergism may be high for endocrine disruption mechanisms."
      a. As we will see later, synergism among endocrine disrupting chemicals has not been proven with respect to interaction with receptors (especially the steroid receptor). Rather, additivity of effect has been most commonly observed.

D. The dynamics of hormone action can be summarized by the following graphic (adapted from Crain et al. 2000. Endocrine-disrupting contaminants and hormone dynamics: lessons from wildlife. Environmental Endocrine Disrupters. An Evolutionary Perspective. L. Guillette, Jr., and D. A. Crain, eds. Taylor & Francis, Inc., NY :pp 1-21.)

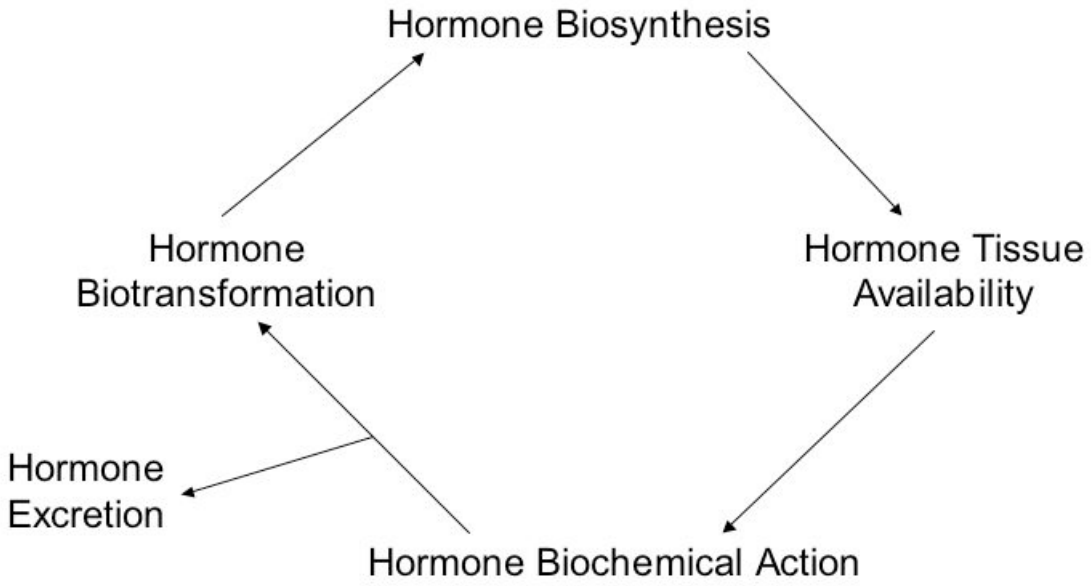


Figure 6. A model for the dynamics of hormone action

1. Each particular stage of action is vulnerable to disruption or interference by exogenous chemicals (natural or synthetic). Thus, chemicals can interfere

with the endocrine system at the level of (based on information in Crain et al. 2000):

a. Hormone production (biosynthesis; aka steroidogenesis when steroid hormones are the products)
    1. The first step is production of pregnenolone by cholesterol
    2. Note that P450 isozymes (or isoforms) are involved in many of the transformations
    3. Mechanisms of altering hormone production:
        a. Altered availability of cholesterol to begin steroidogenesis
        b. Altered steroidogenic enzyme activity
            1. Some chemicals induce different types of P450
                (a) One possible effect would be increased transformation of a hormone from one form to another; the example used is decreased levels of testosterone due to induction of the P450-arom enzyme (known as aromatase) that catalyzes the oxidation of testosterone to estradiol.
        c. Alterations in feedback loops
    4. If hormone biosynthesis is inhibited or reduced, there will be less hormone circulating in the blood, potentially reducing or slowing physiological functions stimulated by presence of the hormone.
        a. Alternatively, reduction in circulating hormones could inhibit the feedback mechanism. Thus, the hypothalamus might continue to produce releasing hormones (RH).
b. Hormone Bioavailability; controlled by several factors:
    1. Plasma or tissue concentration if the hormone
    2. Sequestration of the hormone by binding proteins
        a. Sex hormone-binding globulin (SHBG)
        b. Corticosteroid-binding globulin (CBG)
        c. The binding proteins serve to reduce the amount of hormone actually reaching the cells. These may help control the titre and serve as a fail safe mechanism if feedback loops are not working properly
            1. It has been shown that certain exogenous toxicants do not bind to these proteins very well.
    3. Clearance rate (how quickly is the hormone taken up into cells from the plasma; can it be altered?)
    4. Hepatic metabolism (see below, item [d]).
        a. Liver P450 enzymes that are induced by other chemicals can alter the hormones or transform them at faster rates than normal.
c. Hormone Biochemical Action
    1. Some synthetic chemicals and natural chemicals (mainly the phytoestrogens, also known as phytosterols) can interact with the cellular hormone receptors and mimic the natural hormones.
        a. The hormone mimicking action could result in stimulating (or turning on) the receptor causing an agonistic reaction (i.e.,

stimulating the normal response, but not necessarily at the right time in the developmental stage)

b. The hormone mimicking action can result in inhibition of the normal hormone action causing an antagonistic reaction.
   1. For example, some compounds have been shown to be competitive inhibitors for testosterone on the androgen receptors. These compounds (DDE, the oxidative metabolite of DDT, for example) sit on the receptor but do not turn it on. They just prevent testosterone from getting to the receptor.

2. The ultimate effect of hormone agonists and antagonists will depend on the developmental stage of the organism.

d. Hormone Biotransformation and Excretion
   1. Induction of enzymes could result in decreased titres of hormone or altered titres
      a. The herbicide atrazine putatively induces aromatase, which has been hypothesized as a mechanism for low testosterone levels in animals (specifically frogs exposed to atrazine) Hayes, T. B. et al. 2002. Hermaplhroditic, demasculinezed frogs after expsoure to the herbicide atrazine at low ecologically relevant doses. Proc. National Acad. Sci. 99(8):5476-5480.)

VI. Chemicals Most Frequently Studied and Shown to Have Effects on the Endocrine System

A. Pesticides
   1. DDT, DDE
      a. Anti-androgenic activity
   2. Vinclozolin
      a. Anti-androgenic activity
   3. Methoxychlor
      a. Estrogenic agonist
   4. Atrazine
      a. Induces aromatase

B. PCBs (polychlorinated biphenyls)
   1. Actually the PCBs must be hydroxylated before they exhibit estrogenic activity

C. Dioxins
   1. Anti-estrogenic activity

D. Bisphenol
   1. Estrogenic activity

E. Nonylphenol
   1. Estrogenic activity

F. Tributyl Tin

VII. Testing for Hormonally Active Agents (HAAs)

A. Biochemical Basis for In-Vitro Tests

1. Interactions of HAAs with the estrogen receptor is one of the several mechanisms believed responsible for causing endocrine system related effects (see Figure 6); note, however, that there are other possible mechanisms.
2. For example, the interaction could be with testosterone mimics, and therefore androgen receptors
3. The interaction could be with enzymes that metabolize endogenous hormones and therefore reduce or change the titer in the body

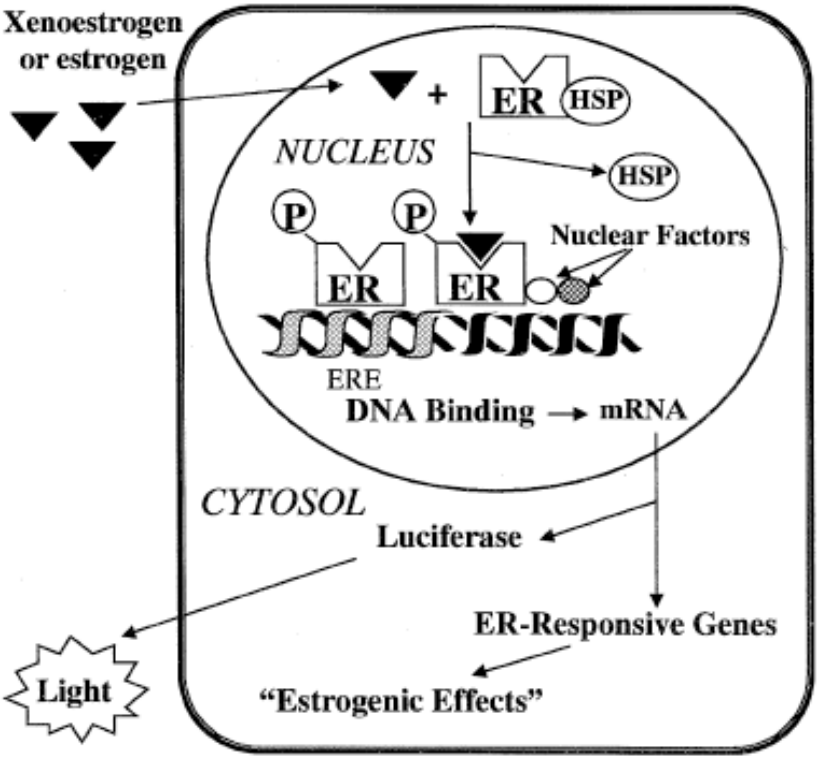


Figure 7. Model for interaction of estrogen or estrogen mimics (xenoestrogens) with cell and receptors and the development of an assay (from Giesy et al. 2002).

B. For those compounds that interact with the estrogen receptor (ER) the following discussion is from: Giesy, J. P., K. Hilscherova, P. D. Jones, K. Kannan, and M. Machala. 2002. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Marine Pollution Bulletin 45:3-16.
   1. ER is a nuclear receptor protein known as a transcription factor); it is associated with a protein called the Heat Shock Protein (HSP), and these two macromolecules cover up a DNA binding domain for estrogen . The binding domain is known as the ERE (estrogen responsive element), and it is located in the regulatory regions of estrogen-inducible genes.
   2. Estrogen (estradiol) and estrogen mimics (also called xenoestrogens) diffuse into the nucleus and bind to the ER. The HSP disassociates from the ER and the ER/estrogen or ER/mimic complex dimerizes (two units associate with one another and bind to the specific DNA coding region, or ERE).

3. ER complexes bound to an ERE recruit additional transcription factors, leading to increased gene transcription (i.e., synthesis of messenger RNA or mRNA, and thus synthesis of proteins required for expression of hormonal action.
4. Based on the biochemical principles discussed above, several ***in-vitro*** assays have been developed using recombinant DNA techniques that allow transfection of wild type cells (for example, a breast cancer cell line known as the MCF-7; yeast cells) with reporter genes that are under transcriptional control of the ERE.
    a. Reporter genes are genes that are turned on to produce a substrate that is easily measured, usually spectrophotometrically
        1. One example is the gene that codes for galactosidase, an enzyme whose activity is easily measured in the presence of the sugar (monosaccharide) substrate, galactose .
        2. One system involves the use of yeast cells and is called the YES system (Yeast Estrogen System).
            a. The human estrogen receptor has been cloned into yeast cells (a.k.a. the Yeast Estrogen System); the cloned gene also contains reporter genes; (Arnold, S. F. et al. 1996, A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens, Environ. Health Perspectives 104:544-548)
            b. When the human estrogen receptor is turned on, the reporter genes (which code for galactosidase enzyme, a.k.a. LAC Z gene) are also turned on
            c. By measuring the amount of galactosidase, one can estimate the degree of estrogenic activity a chemical might have
            d. All potential endocrine disrupting (i.e., estrogen mimic) chemicals are compared to estradiol

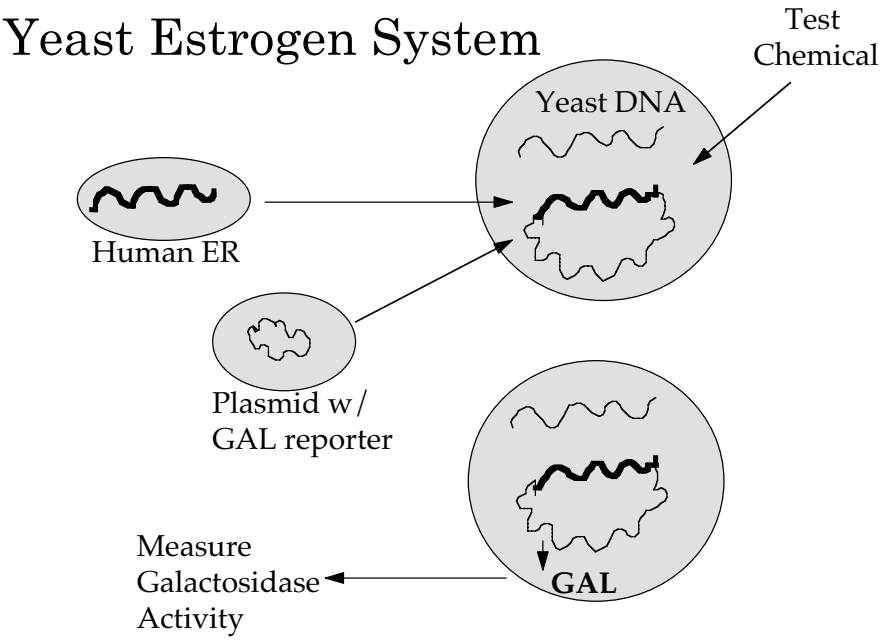


Figure 8. Yeast Estrogen Assay System for Estrogen Mimics

e. The YES system has been used by many research groups to reliably predict which chemicals can interact with the ERE via interaction with the ER. One study, however, came under controversy when it attempted to determine whether mixtures of weakly interacting chemicals can synergize an estrogenic response (Arnold et al., 1996, Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272:1489-1492)
   1. When endosulfan or dieldrin were tested in this system alone, their potency if virtually nil compared to estradiol; however, when added together in equal amounts, each of which has little activity alone, their potency increases by nearly 1000-fold; this response is synergistic
   2. However, note that the synergistic response was still ~ 1000 fold less potent than the natural estrogen; furthermore, the data shows evidence of a threshold (or NOEL)

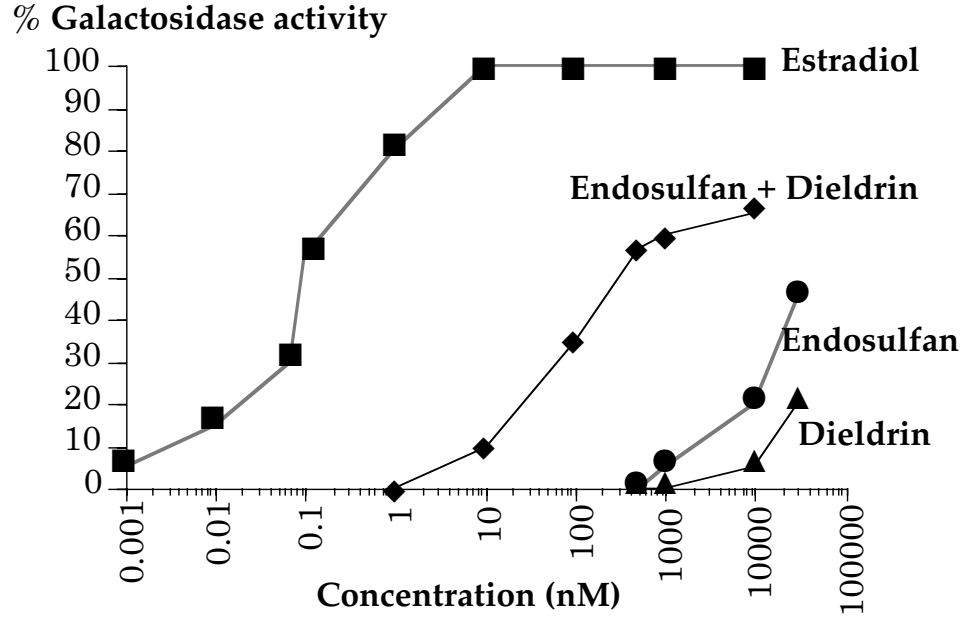


Figure 9. Results from Arnold et al. 1996, suggesting synergism between estrogen mimics

   3. The following summer (1997), in a letter to Science, McLachlan [research leader of the lab at Tulane Univ in which Arnold et al. were working] retracted their work showing synergistic interactions. They claimed they could not repeat the experiments. Others have confirmed that the interactions are additive rather than synergistic.

3. Another example of an in vitro system includes the gene for luciferase, which emits phosphorescence when turned on (i.e., synthesized in response to the binding of the ERE and estrogen or estrogen mimic)
   a. This assay was originally used for examining androgenic rather than estrogenic effects
      1. The human androgen receptor is cloned into a cell line and linked to a luciferase reporting gene;
      2. The receptor (i.e., the cells) is probed with the target chemicals, including testosterone as the control;
      3. When the receptor is activated by binding of the hormone (known as transcriptional activation), luciferase activity increases; luciferase catalyzes a chemical reaction that produces light, so light output can be measured by a spectrophotometer; light output is compared between treatments;

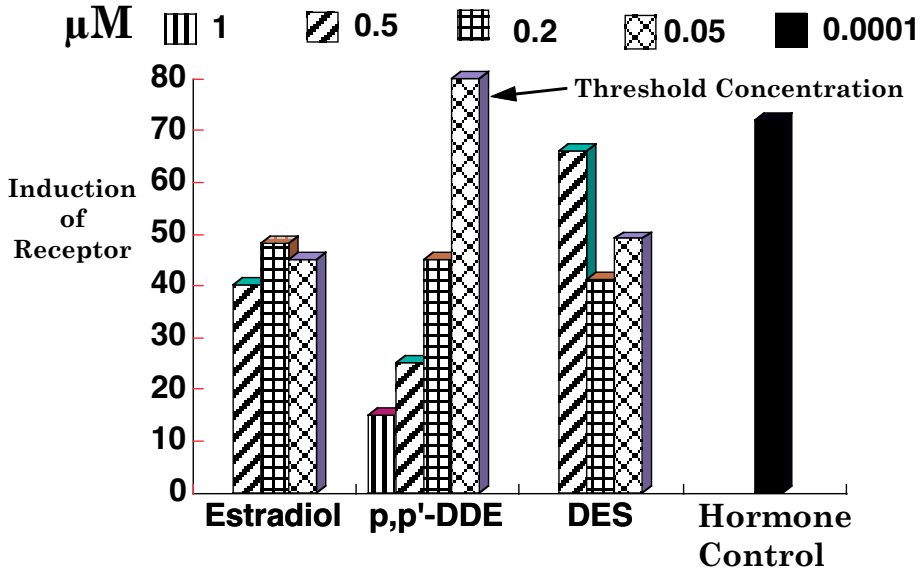


Figure 10. Results from the luciferase reporter assay testing different concentrations of estrogen mimics and androgen antagonists.

4. Using this assay, Kelce et al. 1995 (Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist," Nature 375:581) reported that DDE, which was shown to have poor estrogenic activity, actually has moderately potent anti-androgenic activity;
5. In other words, DDE binds to the testosterone receptor blocking the binding of testosterone; however, unlike testosterone, the receptor is not activated;

6. Thus, in the graph, note that the induction of receptor as measured by light output is lowest for the highest dose of DDE; DDE would be considered a competitive inhibitor
7. Is there a threshold? Clear thresholds were found; i.e., no effect on transcription @ 0.05 μM (~16 ppb)

4. The MCF-7 Breast Cancer Cell Line is a reporter system that relies on cell proliferation as an endpoint. It has been used as a screen for estrogen mimics as well as a test to determine whether synergism is operational.
   a. The E-Screen--one of Theo Colborn's co-authors, Ana M. Soto (Tufts University) and her colleagues published a paper investigating the estrogenic effects of DDT, endosulfan, toxaphene, and dieldrin on human estrogen-sensitive cells using the E-Screen (1994, "The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells," Environ. Health Perspectives 102:380)
   b. They used cultured human breast cancer estrogen-sensitive MCF-7 cells; the biological response measured was cell proliferation in response to dosing
   c. Thus, when estradiol is put in the nutrient medium (which is actually filtered human blood serum), the cells divide; similarly, a response can be observed if a chemical acting like estrogen causes the cells to divide; in such a case the chemical would be considered a potential hormone mimic, xenoestrogen, or an endocrine disrupter (note that each of these words has been used in the literature and popular press)

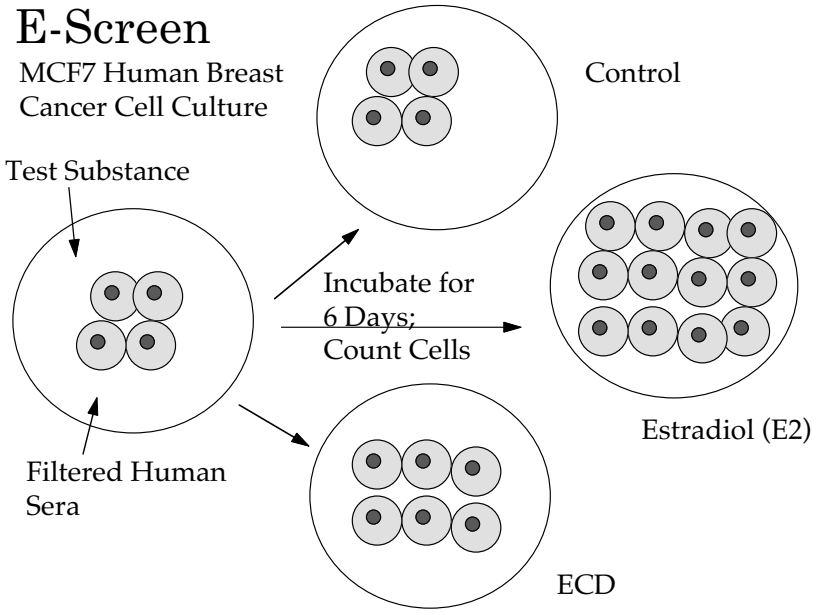


Figure 11. Schematic of the E-Screen assay

d. The data for cell numbers are plotted relative to dose of estradiol or the suspected EDC. The further toward the y-axis a curve is, then the relatively more potent it is; thus in the next graph, estradiol is about 1 million times more potent than the pesticides

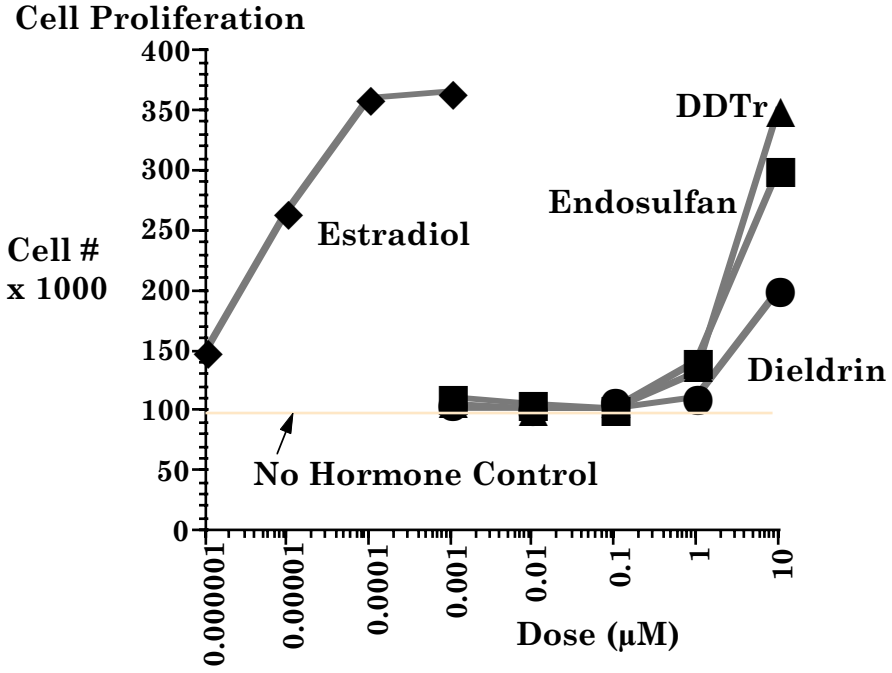


Figure 12. Dose-response curve for results from the E-Screen assay.

e. The results in the next table below show effective minimum doses to achieve a measurable effect and proliferative efficiency (ratio between highest cell number in the presence and absence of the "estrogen"):

Table 4. Effective minimum dose and proliferative efficiency of estrogen mimics in comparison to estradiol based on responsiveness in the E-Screen assay.

Chemical	Effective Minimum Dose	Proliferative Efficiency
estradiol	10 pM	3.68
o,p'-DDT	10 μM	3.17
endosulfan	10 μM	2.99
DDT	10 μM	2.93
dieldrin	10 μM	2.02
toxaphene	10 μM	1.91

f. Furthermore, a clear dose-response (threshold) was observed. For example, 10 nM of o,p'-DDT did not have a significantly different response than the hormoneless control

g. When a mixture of each of the pesticides at a concentration of 1 μM (for a total of 10 μM) was tested, the response was about 2/3 of the response of the estradiol treatment and about 2X the response of the hormoneless control; this result suggests additivity, perhaps by action at the same biochemical receptor.

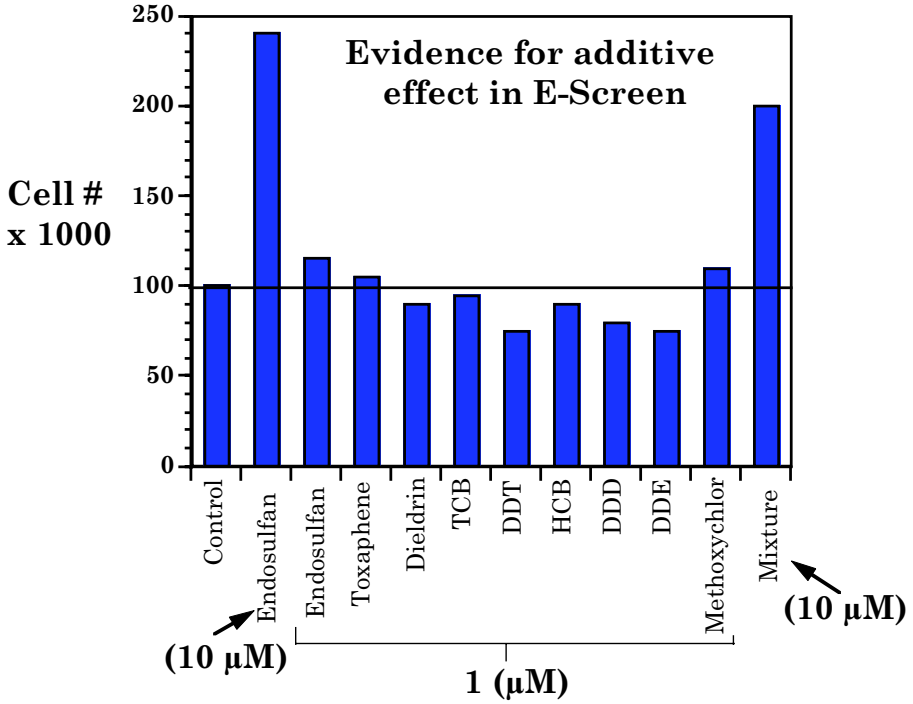


Figure 13. Additivity of estrogen mimics in the E-Screen assay.

C. Some examples of **in vivo systems** for HAA effects include:
1. Rodent uterine wet weight changes after feeding mice or rats with suspected endocrine active chemicals
2. Sperm quality and quantity assays
3. Reproductive toxicity studies
4. Feeding compound over two or more generations to rodents
5. Examine number of litters and assess effect on fertility
6. Behavioral assays that putatively measure gender specific behaviors
7. Morphological examination to check for skewed sex ratio