H4IIE BIOASSAY

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The H4IIE rat hepatoma cell line bioassay (H4IIE bioassay) is an *in vitro* test used to detect and semiquantify specific contaminants and classes of contaminants chemically extracted from environmental matrices such as sediment, water, and organisms (whole or specific tissues). The H4IIE bioassay measures the catalytic activity of cytochrome P4501A (CYP1A), a mixed-function oxidase (MFO) enzyme, as 7-ethoxyresorufin-*O*-deethylase (EROD) activity in cultured rat liver cells exposed to environmental extracts. EROD is induced by, and the H4IIE bioassay is consequently useful for characterizing, the presence of certain polycyclic aromatic hydrocarbons (PAH) and related compounds (e.g., nitrogen heterocyclics and sulfur-, oxygen-, nitro-, amino,- and alkyl-substituted PAH) and polyhalogenated hydrocarbons (PHH) in environmental samples. The PHHs include the highly toxic and persistent polychlorinated dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and naphthalenes, as well as the brominated analogs of these compounds. These classes of compounds induce CYP1A and hence EROD activity in cells by binding to the cytosolic aryl hydrocarbon receptor (AhR). This AhR-mediated mechanism of EROD induction is believed to be involved in many of the toxic effects associated with PHHs and PAHs (Poland and Knutson 1982) (Fig. 3). An overview of EROD induction is presented in "EROD Activity."

The H4IIE bioassay has advantages over traditional analytical chemistry techniques in that it reveals the cumulative biological activity of numerous structurally similar contaminants, each with differing potencies. This assay can also reveal the potential interactions that can occur between contaminants when they are present in environmental samples as complex mixtures. The H4IIE bioassay is valuable for environmental monitoring purposes because it enables the assessment and ranking of the potential toxicity of samples based on their ability to induce EROD as a surrogate for analytical determination of specific compounds. When based on tissue samples, cumulative H4IIE-derived potency estimates can be used to assess the risk to the organism(s) from which the extract was obtained. Such estimates can also be used to estimate the contaminant burden or dose that the organism could contribute either to higher trophic levels (via the food chain) or to its progeny (via maternal transfer). The H4IIE bioassay has a high degree of sensitivity (detection limit < 10 femtomoles 2,3,7,8- tetrachlorodibenzo-*p*-dioxin [TCDD]) and can be rapidly performed. These characteristics make the H4IIE bioassay an ideal tool for evaluating the toxic potential of the samples collected for monitoring programs.

Background

The continuous cell line, H4IIE, was derived from the Reuber Hepatoma H-35 (Reuber 1961) by Pitot and coworkers (Pitot et al. 1964). A decade later, induction of CYP1A catalytic activity in the cell line was demonstrated (Benedict et al. 1973; Niwa et al. 1975). The H4IIE cells are well-suited for the examination of EROD induction due to their excellent growth characteristics and the presence of low basal, but highly inducible, CYP1A activity. The H4IIE cell line is exquisitely responsive to 2,3,7,8-TCDD-based CYP1A induction. These characteristics prompted researchers from the U.S. Food and Drug Administration to develop and characterize a contaminant detection bioassay based on the H4IIE cell line (Bradlaw and Casterline 1979). This original assay was used to screen for the presence of CYP1A-inducing chemicals in foodstuffs as indicated by aryl hydrocarbon hydroxylase (AHH) activity, a catalytic measure of CYP1A (Trotter et al. 1982; Casterline et al. 1983). The assay was subsequently modified to examine EROD activity rather than AHH activity (Sawyer and Safe 1982) because the EROD assay employs a non-toxic substrate.

Use of the H4IIE assay to rank the toxic potency of

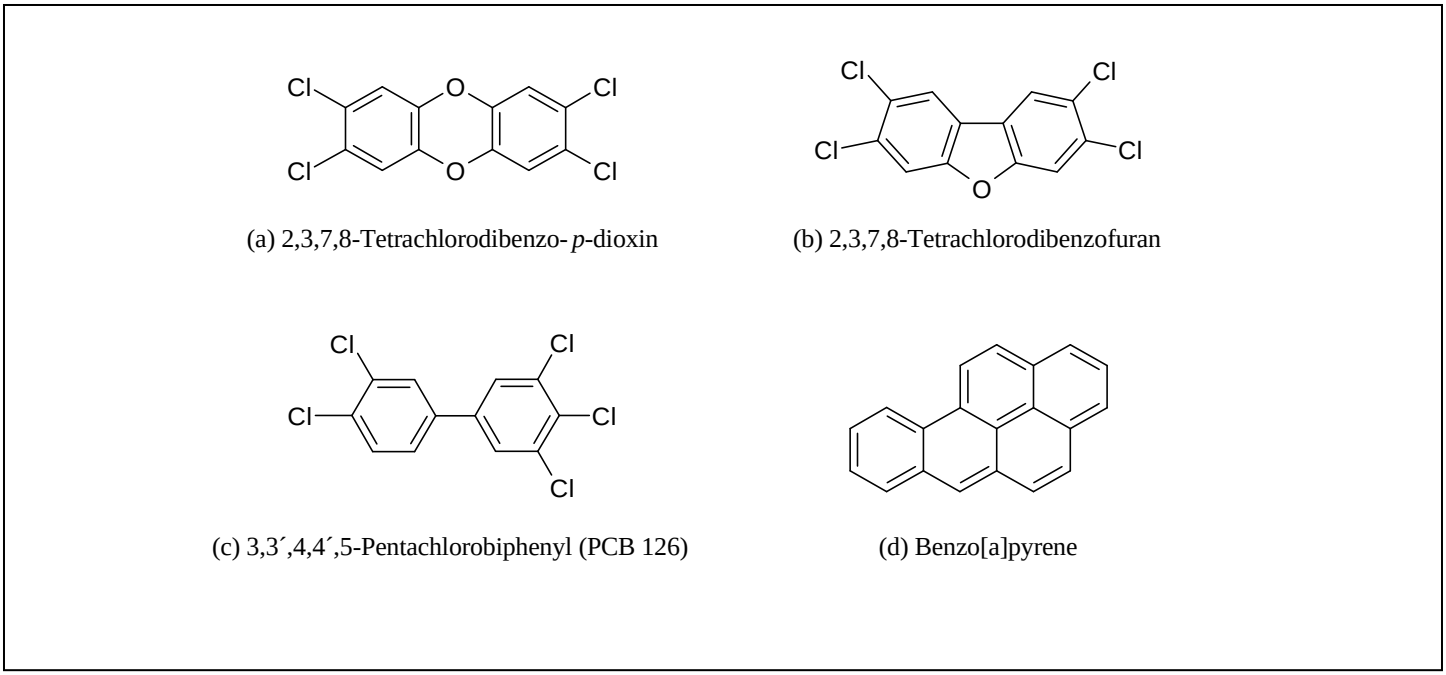

Figure 3. Representative AhR ligands. The molecules demonstrate the general structure of compounds in the following classes: (a) polychlorinated dibenzo-p-dioxins (PCDDs), (b) polychlorinated dibenzofurans (PCDFs), (c) polychlorinated biphenyls (PCBs) and (d) polycyclic aromatic hydrocarbons (PAHs).

individual chemicals based on the 2,3,7,8-TCDD equivalency (TEQ) concept was first proposed by Safe (1987). The TEQ concept was subsequently expanded and used to demonstrate the cumulative toxicity of mixtures of PAHs and PHHs in controlled laboratory studies and in extracts of environmental samples. TEQ values generated by the H4IIE assay provided a relative toxicity estimate for individual chemicals. The values can also be used together with analytical chemistry to evaluate the potential interactions of mixtures of CYP1A-inducing chemicals in biological systems. More recently, environmental assessments using the H4IIE bioassay have become more prevalent, mainly due to the systematic characterization of the assay by Tillitt et al. (1991). Additional modifications that have improved the bioassay (e.g., microtiter plates, use of live cells) have also been introduced over the past decades (Tysklind et al. 1994; Whyte et al. 1998; Bradlaw et al. 1982; Donato et al. 1992; Munkittrick et al. 1993).

Performing the H4IIE Bioassay

Conduct of the H4IIE bioassay can be divided into three general stages (Fig. 5): 1) Extraction, cleanup, fractionation, and analytical characterization of contaminants in the tissue or other environmental matrix; 2) preparation of an extract dilution series with which the cells are dosed; and, 3) measurement of EROD activity and calculation of TEQs (expressed as pg 2,3,7,8-TCDD/g tissue). Extraction, cleanup, and fractionation methods depend on the contaminant classes of interest, but generally involve isolation of hydrophobic planar compounds such as PHHs (Feltz et al. 1995; Huestis et al. 1995). Whole extracts can be examined for general inducing potency. Alternatively, fractionated extracts can provide more detailed information on the contribution of different classes of contaminants (e.g., PCBs, PCDDs/Fs) (Gale et al. in press). Analytical detection techniques such as GC/MS or HPLC can identify specific chemicals in extracts potentially responsible for EROD induction in H4IIE cells, but the cost of this analysis is avoided if the bioassay is being used simply to indicate and quantify the cumulative presence of EROD-inducing compounds in extracts.

Prior to dosing the H4IIE cells, isolated contaminants are transferred into a solvent carrier suitable for delivery to the cultured cells (e.g., isooctane or DMSO) and a logarithmic dilution series is prepared (Tillitt et al. 1991; Smith et al. 1994). H4IIE cells, cultured under conditions described by Tillitt et al. (1991), are seeded in microtiter plates and grown for 24 h. Cells are dosed with extracts or a 2,3,7,8-TCDD standard, incubated (24 – 72 h) and assayed for EROD using a modified method of Pohl and Fouts (1980). The microtiter plate method allows for measurement of both EROD and protein in the same wells (Kennedy and Jones 1994). At the final stage of the assay, concentration-response curves are used to determine relative extract potencies (TEQs) by comparing EC50 or slope values of the extract to those of 2,3,7,8-TCDD (Mason et al. 1985; Tillitt et al. 1993).

Factors That Can Affect the H4IIE Bioassay

Because the H4IIE bioassay is a laboratory assay, many of the external modifying factors that can influence *in vivo* measurement of EROD in fish (e.g., sex, species, ambient temperature) do not influence EROD induction in H4IIE. Therefore, deviations from specified experimental conditions are the most likely source of variability in the H4IIE bioassay. Similar to the *in vivo* assay, variables such as reagent temperature and pH, and resorufin and ethoxyresorufin purity may influence EROD measurements. Other factors specific to cell culture (e.g., cell passage number, mycoplasma contamination) may also influence EROD measurements in H4IIE.

The assessment of environmental extracts with the H4IIE bioassay may also be affected by the presence of specific compounds in the mixture (e.g., certain PCBs). This is mainly caused by inhibition of the catalytic activity of CYP1A in H4IIE and can lead to erroneously reduced EROD measurements (Sawyer et al. 1984). An elegant solution to this problem was presented by Denison and coworkers (Garrison et al. 1996), who transfected a luciferase gene that is expressed upon AhR-complex binding to DNA in H4IIE cells. This results in induction of luciferase activity, which produces luminescence, upon exposure to CYP1A-inducing compounds. The luciferase activity is not influenced by substrate inhibition, resulting in enhanced sensitivity to AhR ligands and greater confidence in induction measurements. It is yet to be determined whether this modified technique will replace the traditional H4IIE bioassay.

Value and Utility of the H4IIE Bioassay in the BEST Program

The persistence and demonstrated toxic effects of PHHs are of great concern to the U.S. Fish and Wildlife Service and other natural resource management agencies (U.S. Fish and Wildlife Service 1993). The H4IIE bioassay is a rapid and highly sensitive test that can indicate the presence of PHHs and related compounds in environmental samples and in some situations can estimate the potential cumulative toxicity of these compounds to organisms. The bioassay can also provide information on the biological interactions of PHHs when used in concert with analytical chemistry methods. The H4IIE bioassay is also of value because of its ability to relate the EROD-inducing strength of PHHs to potential toxic impacts in whole organisms. Relationships between CYP1A enzymatic activities in H4IIE cells and deleterious effects in live rats have been demonstrated for individual PCDD (Bradlaw et al. 1980), PCDF (Mason et al. 1985; Bandiera et al. 1984), and PCB (Sawyer and Safe 1982; Leece et al. 1985) congeners. The strong correlation observed between AHH induction by these congeners in cultured cells and weight loss or thymic atrophy in whole organisms supports the use of the *in vitro* bioassay as a tool for predicting toxic potency (reviewed by Safe 1990).

Complex mixtures of PHHs have also been examined using the H4IIE bioassay and have been used to assess toxic impacts in wildlife. For example, hatching success of double-crested cormorants (*Phalacrocorax auritis*) in Great Lakes colonies was strongly correlated with TEQ results from the H4IIE bioassay (Fig. 6), whereas conventional contaminant analysis correlated poorly with hatching success of eggs from the same colony (Tillitt et al. 1992). Mammalian studies have also revealed the utility of the H4IIE bioassay to predict toxic effects. Mink (*Mustela vison*) fed a diet containing increasing percentages of fish from a contaminated bay in the Great Lakes

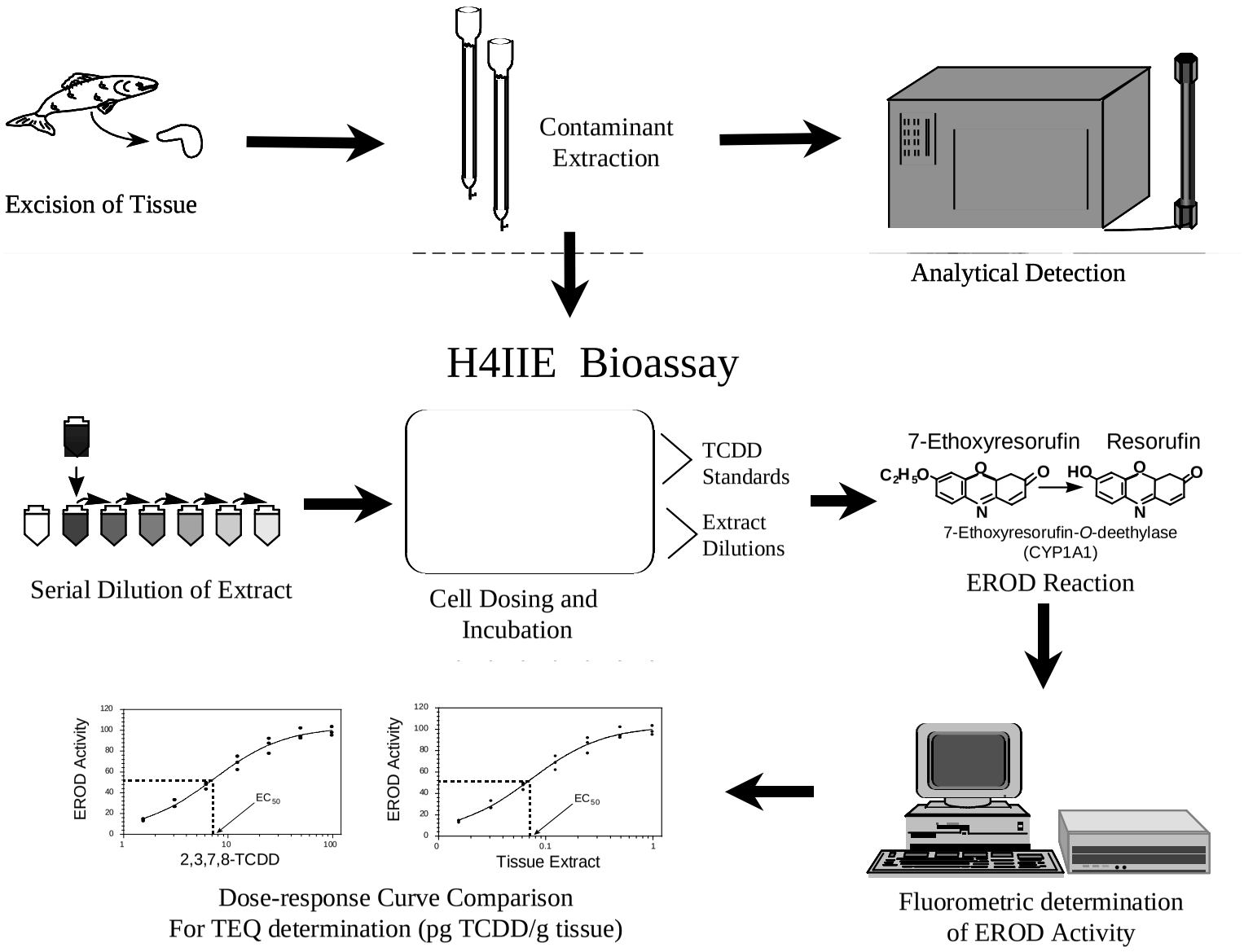

Figure 5. Application of the H4IIE cell line bioassay to assess the toxic potency of environmental extracts. Extract EROD-inducing potency in the cells is compared to that of 2,3,7,8-TCDD to generate a TCDD equivalent (TEQ) concentration (pg TCDD/g tissue).

showed reproductive effects that were related to results from the H4IIE bioassay (Tillitt et al. 1996). By taking into account the differing biological potencies of the chemical components in an extract, the H4IIE bioassay is a good predictor of the toxic outcome of exposure to the extract.

The H4IIE bioassay is among the Tier I or "workhorse methods" recommended for screening environmental samples for dioxin-like activity in virtually all habitats and guilds likely to be sampled by the BEST program (BEST 1996). The assay can detect extremely low concentrations of contaminants that are a high priority for the BEST program (PHHs and PAHs; U.S. Fish and Wildlife Service 1993). Used in conjunction with other methods (e.g., *in vivo* measurement of hepatic EROD and instrumental analysis of total PCB residues in the carcasses of the fish), the H4IIE bioassay can provide information on the class or classes of contaminants in the samples inducing the MFO system without the expense of high-resolution chemical analyses. It should be noted that the *in vivo* and *in vitro* measures of EROD activity provide similar but distinctly different information. Hepatic EROD in fish indicates the presence of compounds that have already interacted with the AhR, whereas the H4IIE bioassay reveals contaminants accumulated in tissues that have the potential to bind to the AhR. Use of these two assays in concert and with specific fractionation schemes can yield critical information regarding the presence of CYP1A inducers that are easily metabolized or otherwise non-persistent. Perhaps most importantly, and in contrast to many biomarkers, TEQs in biological samples as estimated by the H4IIE bioassay indicate the presence of compounds that are known to exert toxic effects through a similar mode of action. Although questions have been raised about species-specific differences in response to PHH and PAH congeners (Clemons et al. 1997), extant information suggests that the mechanism of AhR-mediated CYP1A induction is similar among vertebrates (Stegeman and Hahn 1994), supporting the use of H4IIE in the assessment of toxic potency of contaminants accumulated in organisms such as fish. These attributes give the H4IIE bioassay predictive power in terms of risk to organisms.

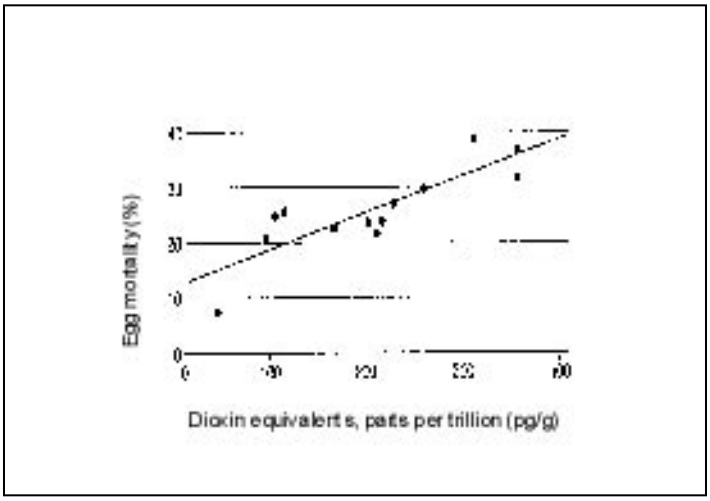

Figure 6. Hatching success in Great Lakes colonies of double-crested cormorants versus PCBs in eggs as dioxin-equivalent concentrations, 1987-1988 (modified and redrawn from Tillitt et al. 1992 by permission); each point represents the mean for a colony.