NATIONAL WATER RESEARCH INSTITUTE

INSTITUT NATIONAL de RECHERCHE sur les EAUX

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J. Sherry

NWRI Contribution No. 93-83

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J. Sherry

Research and Applications Branch
National Water Research Institute
Burlington, Ontario L7R 4A6

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MANAGEMENT PERSPECTIVE

Screening tests for the detection of dioxins and dioxin like molecules such as furans, and polychlorinated biphenyls (PCBs) are reviewed. The tests are divided into two categories: ligand binding assays and bioassays. The ligand binding assays include immunoassays and receptor binding assays. The bioassays include in-vitro and in-situ MFO induction assays (mixed function oxidase) and bioassays that are based on toxic responses. Key methods in each group are described and illustrated. Examples of their application in topical environmental studies are provided. The screening tests can be used to lower overall analytical costs and can also provide useful information about the toxic potency and potential biological impact of the planar halogenated hydrocarbons. Shortcomings in the specificities of many of the bioassays is compensated by their ability to respond to a sample in proportion to its biological potency. The screening tests can be used to increase our knowledge of the distribution and importance of dioxin like environmental contaminants. The described methods can assist the Department in meeting its objectives under the Green Plan.

SOMMAIRE À L'INTENTION DE LA DIRECTION

On passe en revue les essais servant au dépistage des dioxines et des substances apparentées telles que les furanes et les biphényles polychlorés (BPC). Les essais sont divisés en deux catégories : les essais de fixation de ligands et les essais biologiques. Les essais de fixation de ligands comprennent les immunoessais et les essais de fixation à des récepteurs. Les essais biologiques comprennent les essais d'induction de l'OFM (oxydase à fonction mixte) *in vitro* et *in situ* et les essais biologiques basés sur une réponse toxique. On décrit et illustre les principales méthodes dans chacun de ces groupes, et on présente des exemples de leur application dans des études environnementales bien précises. Les essais de dépistage peuvent servir à réduire l'ensemble des coûts des analyses et peuvent également nous fournir des données utiles sur la toxicité et les effets biologiques potentiels des hydrocarbures halogénés planaires. Les problèmes de spécificité de nombreux essais biologiques sont compensés par leur capacité de réagir à un échantillon en proportion de son activité biologique. Les essais de dépistage peuvent nous aider à augmenter nos connaissances sur la distribution et l'importance des contaminants apparentés aux dioxines dans le milieu. Les méthodes décrites peuvent aider le Ministère à atteindre ses objectifs en vertu du plan vert.

ABSTRACT

An array of screening tests have been developed for the detection of dioxins and dioxin like molecules such as furans and polychlorinated biphenyls (PCBs). The tests may be divided into two categories: ligand binding assays and bioassays. The ligand binding assays include immunoassays and receptor binding assays. The bioassays include in-vitro and in-situ MFO induction assays (mixed function oxidase) and bioassays that are based on toxic responses. Key methods in each group are described. The screening tests can be used to lower overall analytical costs and can also provide useful information about the toxic potency and potential impact on the biota of the planar halogenated hydrocarbons. Many of the bioassays have broad specificity which makes them responsive to a variety of planar molecules that can mimic 2,3,7,8-T_4CDD. On the other hand the bioassays tend to respond to a sample in proportion to its biological potency. The screening tests can be used to increase our knowledge of the distribution and importance of dioxin-like molecules in the environment.

RÉSUMÉ

Toute une gamme d'essais de dépistage ont été mis au point pour la détection des dioxines et des substances apparentées telles que les furanes et les biphényles polychlorés (BPC). Les essais peuvent être divisés en deux catégories : les essais de fixation de ligands et les essais biologiques. Les essais de fixation de ligands comprennent les immunoessais et les essais de fixation à des récepteurs. Les essais biologiques comprennent les essais d'induction de l'OFM (oxydase à fonction mixte) *in vitro* et *in situ* et les essais biologiques basés sur une réponse toxique. On décrit les principales méthodes dans chaque groupe. Les essais de dépistage peuvent servir à réduire l'ensemble des coûts des analyses et peuvent également nous fournir des données utiles sur la toxicité et les effets potentiels des hydrocarbures halogénés planaires sur le biote. Nombre de ces essais biologiques sont peu spécifiques et réagissent à divers composés planaires qui peuvent simuler la 2,3,7,8-T_4CDD. Par ailleurs, les essais biologiques ont tendance à donner une réponse proportionnelle à l'effet biologique. Les essais de dépistage peuvent servir à augmenter nos connaissances de la distribution et de l'importance des substances apparentées aux dioxines dans le milieu.

I. INTRODUCTION

The quest to lower the cost of environmental analyses is driven by the unprecedented demands for data on a growing list of analytes, and by the realization that society has limited funds for such work. The encouraging emphasis on environmental science at all levels of education, continued concern about the adverse effects of pollutants on the biosphere, and the high profile activities of various "green" groups, should help to ensure that ecosystem health remains a top priority well into the next century. Consequently, there is little reason to anticipate a decline in the workload of environmental laboratories.

The halogenated aromatic hydrocarbons (HAHs), which are trace contaminants of many environmental matrices, are particularly difficult and expensive to determine. As hydrophobic molecules they tend to accumulate in oily matrices. Higher chlorinated congeners that contain chlorine in the 2,3,7,8 positions are resistant to biological oxidation and tend to bio-magnify as they pass up the food chain. Conventional methods for the determination of HAHs combine extraction of the analytes from the matrix with tedious multi-step procedures to separate the target compounds from interfering molecules. High resolution gas chromatographs and mass spectrometers (GC-MS) are then used to identify and quantify the target congeners: those instruments are expensive to purchase (0.75 - $1 million), maintain, and operate. As a result, the determination of polychlorinated dibenzo-p-dioxin/polychlorinated dibenzofuran (PCDD/PCDF) congeners costs from $1000 to $2000 a sample. In addition to their low sample throughput, high resolution GC-MS instruments are prone to "down" periods because of the need for continual tweaking and optimization. The GC-MS step is often the main cause of delays and back-ups in analytical laboratories. To make matters worse, sample sets often include many samples that are either dioxin free or contain negligible analyte levels. At present those negative samples must be passed through the entire analytical process.

The discovery of PCDDs/PCDFs in pulp and paper mill effluents in the mid to late '80s[1, 21] caused a sharp increase in the numbers of samples requiring analysis for dioxins and dibenzofurans. The aquatic ecosystems in the vicinity of the offending mills were added to the list of problem areas that had been identified in earlier surveillance studies; many of those sites must now be regularly monitored.

There are several ways to tackle the problem of sample overloads. Conventional solutions include the expansion of analytical facilities, and the miniaturization, simplification, and automation of the extraction and clean-up procedures. Alternatively, a screening strategy could be used before the critical GC-MS step to eliminate samples that contain less than a threshold concentration from the analytical chain. In most cases the various screening methods that have been developed for the detection of planar HAHs are proposed as complements to, not replacements for conventional analytical methods. All positive or dubious samples and a random selection of negative samples should be confirmed by GC-MS. Fig. 1 outlines how a screening test for an environmental analyte such as PCDDs could be incorporated into a routine analytical scheme.

The attraction of a screening strategy increases in proportion to the number of steps that can be eliminated from the clean-up process. Pre-screening tests, require no sample preparation, other than perhaps a simple extraction step, whereas screening tests often require some clean-up of the sample. For reasons that relate to the hydrophobic chemistry of the planar HAHs and their normally low levels in environmental matrices pre-screening tests have not been developed for the detection of HAHs in matrices other than in water. Even the undemanding epithelial cell bioassay, which shall be described later in the chapter, requires the separation of analyte and lipid molecules. That situation is unlikely to change, unless a highly selective extraction technique or a novel screening test is developed.

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[1]Review article

An effective screening technique for the detection of planar HAHs would have several advantages. The immediate benefit would be a reduction in analytical costs and an increase in sample throughput. That would facilitate the broad surveillance and monitoring studies that are needed to provide a more comprehensive picture of dioxin distribution patterns in the environment and in human populations. Screening tests for planar HAHs could also be used to systematically monitor known sources and sinks. The number of samples that must be analyzed for other environmentally important analytes has also risen to critical levels in recent years. Because the use of high resolution analytical instruments can enhance the determination of many of those analytes, a strategy that could lower the "dioxin" load would also help to improve an analytical laboratory's overall productivity.

The screening techniques for the detection of dioxins and related compounds can be divided into two main groups: ligand binding assays and bioassays. Ligand binding assays such as immunoassay (IA) and receptor binding assays have been successfully used in clinical laboratories to detect and monitor a range of diverse analytes in tissues and body fluids.[3-6] IA techniques are also used to screen food for the presence of mycotoxins and for the detection of steroid hormones and antibiotics in farm animals and meat products.[7-11] During the past decade a variety of IA based screening techniques have been developed for the detection of residues of agricultural chemicals and industrial contaminants in the environment,[12-14] and in agricultural produce.[15] Many of those assays are now distributed commercially. Bioassays are used to infer the presence of toxicants in industrial effluents or environmental matrices by the detection of an induced effect in a living system be it cell, tissue, or organism. Most environmental bioassays, such as the various fish, plant, daphnid, and microbial assays, are broad spectrum assays that respond to many toxicants or stressors. Bioassays have been developed that exploit induced responses in cells or organisms to infer the presence of dioxin like molecules.

The present chapter describes several of the screening tests for PCDDs and related compounds. The chapter also reviews the progress that has been made in applying

the methods to real world samples and considers some of the problems that remain to be overcome if those novel techniques are to become more widely used.

II. Ligand binding assays

A. LBAs background

Ligand binding assays (LBAs) exploit the ability of specialized biological molecules to selectively and reversibly bind organic molecules. The molecule that binds the analyte is the binder molecule; the target analyte and its derivatives are the ligands. The ligand molecules are usually labelled with a radioactive tracer, an enzyme marker, a fluorescent molecule, or some other marker so that the fraction of ligand molecules that have been bound can be estimated. In some IA formats the binder rather than the analyte is labelled. LBAs are widely used in clinical laboratories and have revolutionised routine clinical analyses during the three decades since their introduction. Without the LBAs many routine clinical analyses that are now taken for granted would not have been possible because of inadequate instrumentation, or, if possible, would have been exorbitantly expensive. LBAs owe their popularity to their low cost, ease of use, and rapidity. Those characteristics together with the ability to precisely detect small quantities of analyte prompted researchers at the National Institute of Environmental Health Sciences in the U.S.A.[16,17] to initiate the development of immunoassay techniques for the detection of PCDDs and related compounds in environmental and biota samples. At about the same time Poland et al.[18] at the University of Wisconsin developed a receptor binding assay (RBA) that can be used to assay receptor sites in tissue homogenates, and to measure the ability of various ligands to bind to those sites. The RBA can also be used to detect analyte molecules.

As the key reagent in any LBA, the binder molecule's properties tend to determine the overall characteristics of the final assay. The main steps in a typical LBA are outlined in Figure (2). In this competitive binding assay the binder molecule and an excess amount of labelled analyte are pre-incubated in a buffer solution. The target analyte is then added to the assay tube, either as a calibration standard, an unknown

sample, or a positive control. The analyte and binder molecules combine in a reversible reaction that obeys the law of mass action:

Binder + Analyte ⇌ Binder-Analyte.

At equilibrium K = [Binder-Analyte]/[Binder][Analyte].

Binder molecules of high affinity tend to push the equilibrium to the RHS and shorten the incubation time. Such binder molecules usually yield precise and responsive assays that have steep dose response curves. Some recent work suggests that the foregoing mechanism may over simplify the reaction between the dioxin receptor molecule (Ah) and its ligand.[19] That receptor-ligand complex apparently disassociates in a biphasic manner that has both fast and slow components with the former being dominant.

Once a state of equilibrium, or near equilibrium, has been reached the amount of labelled analyte that has been bound is quantified. Firstly, however, the free analyte must be separated from the bound fraction. That separation step is crucial to the success of most LBAs. A sloppily executed separation will result in imprecise and noisy data and an associated loss of sensitivity. An efficient and carefully executed separation usually yields precise data (mid-range CV of < 10 %) with a low detection limit - providing that the binder molecule has adequate affinity for the analyte. It is preferable to measure the amount of label in the bound fraction so as to optimize the detection of small quantities of analyte. If the free fraction is measured, the assay's response to small quantities of analyte can be masked by the larger absolute variations that occur in the response signal. The response data from the calibration standards are normalized with respect to an analyte free control (B_0) and are then plotted against analyte concentration. The amount of analyte in the unknown samples is interpolated from the calibration curve. Two types of LBAs have been developed for the planar HAH molecules: the immunoassays (IAs) and the receptor binding assays (RBAs). IAs use antibodies (ABs) to bind analyte molecules whereas the Ah receptor is used in the RBAs.

1. Advantages and disadvantages of competitive binding assays

IAs and RBAs have their respective strengths and weaknesses. Sensitivity and low detection limits are among the advantages that are common to both techniques, particularly when the techniques are used to detect hydrophillic analytes. Often only minimal clean-up of the sample is required - although that advantage usually only applies to polar analytes. Sample preparation costs become more important when LBAs are used to detect hydrophobic compounds, such as the HAHs, in oily matrices. Because the binder-ligand reaction occurs in the liquid phase, the analyte molecules must first be extracted from the matrix, separated from the lipid phase, and then solubilized in aqueous buffer. The extra steps are expensive, inconvenient, and can diminish the practical advantages of a screening test.

In general the LBAs tend to be highly selective. The RBAs detect only those molecules that can bind to the Ah receptor, whereas IA specificity can be tailored to suit analytical requirements by attention to the design of the immunogen, by careful screening and purification of the ABs, and by careful design of the reagent antigen. Both assay types are noted for their ease of use: they require neither expensive equipment nor highly trained operators. Moreover, many of the popular LBAs lend themselves to miniaturization and automation.

On the other hand, the LBAs can be confused by molecules that cross react with the binder or that impede the formation of the ligand - binder complex. Both types of interference reduce the measured response which generates a false positive signal. A matrix blank can be used to correct the influence of low level matrix interferences. The data from LBAs are semi-quantitative and as a safeguard all positive samples should be confirmed by conventional means. LBAs complement rather than replace conventional methods. In many cases, the need to confirm all positive samples can be relaxed as experience is gained with samples from a particular matrix or location. Once the development costs have been covered, the assays are inexpensive: when large samples sets are analyzed the cost per sample can be as low as $5 - $20, not including the cost of

sample preparation. Most LBAs allow the simultaneous analysis of multiple samples and permit a high level of replication. That capability not only lowers costs but also helps to improve assay precision.

III. Immunoassays

IAs are a blend of techniques from the analytical and clinical laboratory[20-21] and can detect virtually any organic molecule that has a molecular weight of greater than about 150. IAs owe their versatility to the immune system's ability to produce ABs in response to foreign organic molecules. All IAs are based on the sensitive and selective AB-antigen (Ag) reaction. The first quantitative IA was developed in the fifties by Yalow and Berson for the measurement of small quantities of human insulin in body fluid.[22] Since that breakthrough IAs have been developed for many analytes including pharmaceuticals, microbes, and environmental contaminants.[23-27] The original IA technique has been refined and modified over the years, so the analyst now has a choice of many assay formats.

The ABs used in IAs belong to the immunoglobulin's gamma fraction (IgG) and are produced by mammalian lymphocyte B cells as part of the immune response to foreign molecules.[4,28] Differentiated B cell clones form in response to each of the immunogen's antigenic sites. Each clone produces ABs to a single site, with the result that induced sera contain a mixture of clonal ABs (PABs). The weak AB-Ag interaction involves non covalent bonds such as Van der Waals interactions and electrostatic bonds. IAs are exceptionally selective because of the "lock and key" nature of the interaction between the antigen and its binding site. That interaction is favoured by a close steric fit and a favourable alignment of oppositely charged ions. Because of their small size the planar HAHs must be linked to a carrier molecule, such as bovine serum albumin (BSA), if they are to be recognised by the immune system. Molecules that are rendered immunogenic in this manner are called haptens. Often a reactive group, such as $-NH_2$ or -COOH must first be introduced into the hapten. The design and preparation of the immunogen, in particular the location and type of linker arm that is used to separate the

hapten from the carrier protein, has a major influence on the assay's characteristics. The purified and characterised immunogen is used to induce AB formation in a suitable host animal - usually a rabbit. Sera are selected for use on the basis of their avidity and specificity for the target analyte.

The acceptance of environmental IAs by the analytical community will largely depend on the availability of a steady supply of well characterised and stable ABs. Those supplies can be enhanced by pooling acceptable sera from a large number of rabbits, or by the use of large host animals such as goats or donkeys. Alternatively, hybridoma techniques, can be used to segregate and perpetuate clones of lymphocyte B cells that each produce an indefinite supply of monoclonal ABs (MABs). Each MAB is usually specific for a single antigenic determinant.[4,29] MAB based assays are usually highly specific and have steep dose response curves. MABs, however, are more costly and time consuming to develop than PABs. For that reason many fine PAB based IAs continue to be reported for environmental contaminants.

In recent years enzyme activity (enzyme immunoassay (EIA)) has become more popular than radioactivity (radioimmunoassay (RIA)) as the most widely used IA response parameter; other tracers such as fluorescent molecules are potentially sensitive but are less common. For the more popular environmental IAs a decrease in the level of the measured signal relative to the analyte free reference tubes (B_0) is proportional to the analyte's concentration. RIAs are competitive binding IAs in which the reagents are usually adjusted so that 30 - 50% of the tracer is bound in the absence of analyte. The labelled hapten and analyte molecules compete for AB binding sites. Radio-ligands, especially ^{125}I can be rapidly, conveniently, and sensitively counted. Unlike some EIA formats, the tracer's activity is unaffected by non-specific interferences.[30,31] The RIAs that have been developed for planar HAHs rely on either a double antibody (DAB) technique or physical adsorption by dextran coated charcoal (DCC) to separate the bound and free phases. In the DAB technique, ABs raised against the primary host's IgG are used to

precipitate the AB-ligand complex. The DCC methods bind the free tracer and are rapid and inexpensive.

Concerns about the transportation, use, and disposal of radioisotopes led to efforts to develop non-isotopic immunoassays. Enzyme labels do not suffer from short half lives,[32,33] and are inherently safer than RIAs - furthermore EIAs can be used in the field. Most environmental EIAs use horse radish peroxidase or alkaline phosphatase as the enzyme label. So far, the EIAs that have been reported for the detection of planar HAHs are competitive heterogenous assays. Those assays are similar to conventional RIAs and are often called ELISAs (enzyme linked immunosorbent assay) because either the AB or Ag are immobilized on a solid phase. In direct competitive EIAs (Fig. 3) the ABs are adsorbed on the solid phase - which is usually a plastic tube or microtitre well. After the ABs , analyte, and labelled hapten have been incubated together, the unbound reagents are washed from the solid phase and the amount of enzyme activity that has been bound by the ABs is measured. A decrease in enzyme activity, compared to the control tubes, is proportional to the amount of analyte present. The co-incubation of the enzyme tracer and sample in the direct EIAs can expose the enzyme to harmful agents.[27] That possibility is eliminated in the indirect competitive EIAs (Fig. 4), in which the antigen is adsorbed on the solid phase. The primary AB and sample are added to the wells and then incubated. After a washing step an enzyme labelled DAB is used to measure the amount of AB that has bound to the immobilized Ag.

1. IAs for dioxins

Albro et al.'s[17] RIA for PCDDs was conceived and developed as part of an effort to reduce the number of samples that require analysis by mass spectrometry. The immunogen and a tracer for use in the RIA were prepared from a common building block: the 1-amino derivative of 3,7,8-T_3CDD. The adipamide derivative of the hapten (Fig. 5) was converted to a mixed anhydride using isobutyl chloroformate and then conjugated to carrier protein (thyroglobulin and BSA). The immunogen was extracted with chloroform so as to remove free conjugated hapten before it was used to immunize

a set of rabbits. This precaution is important because hapten molecules that dissociate from the carrier could be toxic to the host. The radioligand (^{125}I-valeramido-T_3CDD) was prepared by incubating the I-valeramido-T_3CDD (Fig. 5) with Na^{125}I for 60 h at 60 °C; the labelled hapten (specific activity of about 80 Ci/mmol) is then extracted from the reaction mixture and separated from the large amount of free ^{125}I by chromatography on silica gel. ABs to the immunogen's adipamide group were removed from the sera by affinity chromatography: the adipamide side chain was used as the immobilized ligand in the affinity column. The key to the solubilization of the hydrophobic ligand molecules is to render them accessible to the ABs without unduly interfering with the ligand-binder reaction. The non-ionic surfactants Cutscum and Triton X-305 (0.5%) were the most effective of 15 detergents that Albro's group evaluated for that purpose; the solubilization procedure is outlined in Fig. 6. Preparation of the detergent reagent in acetone prevents the detergent from creeping up the tube walls during the solvent removal step: a simple modification that removes some of the tedium from the solubilization procedure.[34] Assay reproducibility is helped if the assay tubes are ultrasonicated at a standard starting temperature. Dimethyl sulfoxide (DMSO) can also be used as a solubilization agent in the RIA for PCDDs.[35] DMSO simplifies the solubilization process; the incubation time for the DMSO based assay was reduced from 72 h to 24 h without any appreciable loss of performance. DMSO is, however, more prone to matrix overload effects than Triton.[36]

The RIA for PCDDs is a conventional competitive assay (Fig. 7). The ABs and analyte molecules are pre-incubated at 37°C to compensate for any tendency of the ABs to preferentially bind the tracer's linkage group. After the addition of the tracer the incubation temperature is changed to 4°C. Once so adjusted, the tubes should be kept at 4°C until the phase separation step is completed so as to prevent temperature induced alteration of the equilibrium. Albro's group showed that an assay based on Triton was more sensitive than a Cutscum version: probably because Cutscum inhibits AB binding (15 - 20%) more than Triton (1 - 2%).[37] The Cutscum based assay, however, had a wider working range (Table 1) and greater capacity. Typical calibration curves that were prepared using two selected sera are shown in Fig. 8: GC-5 and F-12 were raised against

the thyroglobulin and BSA immunogens respectively. Those antisera were selected from among others because they were selective, poorly recognised the immunogen's adipamide group, and had high affinity for the dioxin portion of the radioligand.[38] The RIA was highly specific for PCDDs and PCDFs: only the co-planar 3,4,3',4'-T_4CBP of several related compounds, cross reacted more than marginally in the assay (CR = 6%). The GC-5 serum had the broader specificity recognising several P_5 and H_6 congeners, which makes it suitable for screening applications. A DMSO based RIA which was evaluated with the GC-5 serum, had a working range of 20 pg to 2 ng of 2,3,7,8-T_4CDD (Table 1) and it had a steeper calibration curve when compared to Cutscum or Triton based versions of the RIA.[35]

The RIA has been validated using extracts of sandy soil (detection limit (DL) = 25 pg) and the lipid fraction from liver and adipose tissues.[17] There was generally good agreement between the RIA and GC-MS/GC-electron capture detector (EC) confirmation techniques. The authors suggested that the inclusion of extra cleanup steps in addition to acid treatment and chromatography on basic alumina would help improve the RIA's performance by reducing the influence of negative interferences.

A double blind study of the RIA's ability to detect 2,3,7,8-T_4CDD in minimally enriched extracts of human adipose tissue also indicated that more extensive cleanup of extracts is needed if the RIA is to be reliable near its DL of 25 pg.[39] The RIA had a reliable sensitivity of 100 pg ($P<0.05$) in a 60 mg sample which is equivalent to 1.7 ppb. The RIA was able to detect about 70 ppt of 2,3,7,8-TCDD in 300 mg equivalents of tissue when gel permeation chromatography (GPC), acid/base treatments, and alumina chromatography were used to prepare a Lake Trout extract.[36] Further improvements in the DL would probably require separation of the extract on carbon fibre. In theory, the carbon fibre clean-up step should allow a further increase in sample size with a consequent lowering of the practical DL. Water samples may not require such extensive cleanup, and heavily contaminated matrices may not demand low DLs, in which case the sample preparation could be reduced.

There are problems with the preparation of the ^{125}I-valeramido-T_3CDD tracer. Firstly the specific radioactivity of the purified tracer is low. Furthermore, the unlabelled hapten is unstable, tending to cyclize and loose iodine on storage which can lead to variable reaction yields. The labelling reaction must be repeated on a regular basis because of ^{125}I's short shelf life. Efforts to improve the RIA's sensitivity through the use of a high specific activity tracer became stalled by an inability to label a tyramine derivative of T_4CDD with ^{125}I - the problem may have been caused by the biphasic nature of the reaction mixture.[12] Tritiated 2,3,7,8-T_4CDD has been used as an alternative radioligand in the RIA for PCDDs primarily as a way of avoiding the tricky iodination reaction.[40] The [^{3}H]-2,3,7,8-T_4CDD based RIA had a working range of 20 pg - 2 ng of 2,3,7,8-T_4CDD (Fig. 9; Table 1). The assay used DMSO and DCC as the solubilization and phase separation reagents respectively. The [^{3}H] based assay was at least as precise as the [^{125}I] based assay's (CV of 15 % vs 20 %). The RIA was modified for improved performance at low analyte levels (Table 1) by radically reducing the amounts of tracer and ABs. A high activity tracer by permitting the reduction of both the tracer mass and AB levels without any loss of counting efficiency would probably further improve the RIA's performance at low analyte levels.

Martin Vanderlaan's group at Lawrence Livermore National Laboratories raised MABs for use in an EIA for the detection of PCDDs. Their assay's success was attributed to the MAB's ability to react with both the detergent solubilized analyte and immobilized hapten (Fig. 11). Five MABs were generated that were each capable of binding free 2,3,7,8-T_4CDD.[41,42] Each of the five MABs favoured 2,3,7,8- substituted congeners with intermediate levels of chlorination.[43] Because the MABs recognize a chlorine atom in the -1 position as being similar to the immunogen's -NH group, the assay is more sensitive to 1,2,3,7,8-P_5CDD and 1,3,7,8-T_4CDD than to 2,3,7,8-T_4CDD. Apart from the co-planar 3,3',4,4'-T_4CBP, polychlorinated biphenyls (PCBs) were not recognised. The MABs are also highly sensitive for 2,3,7,8-T_4CDF and 2,3,4,7,8-P_5CDF[42] which is beneficial since those compounds are of toxicological concern. The EIA would probably be more suited to general screening applications if it were able to detect the

hexa - octa chlorinated PCDDs/PCDFs. The MABs were used in a convenient and rapid competitive indirect EIA which is outlined in Fig. 10 and whose performance characteristics are described in Table 1. In its present form the MAB based EIA for PCDDs seems to be less sensitive than the PAB based RIAs.

The EIA for PCDDs was validated for a set of badly contaminated industrial and soil samples. After extraction the samples were chromatographed on activated carbon and then passed through a combination column that contained acid silica and $AgNO_3$ treated silica.[34] The cleanup procedure which was designed to remove hydrophobic compounds that would tend to overwhelm the solubilization system, was adequate for the high (<1 ppb - 1750 ppb) analyte levels in the chemical and oil samples. The study showed that soil samples, however, need a more refined cleanup. With the exception of some of the soil samples, the samples gave additive responses in EIA, which demonstrates the assay's usefulness as a screening procedure for the more toxic PCDDs/PCDFs.

2. RIA for 2,3,7,8-T_4CDF

The NIEHS team used their previously proven approach to develop an RIA for 2,3,7,8-T_4CDF that closely resembled their earlier RIA for PCDDs (Fig. 7).[44] The assay had a working range of 20 pg - 4 ng of T_4CDF (Table 1), and being fairly specific for 2,3,7,8-T_4CDF is more suited to the detection of 2,3,7,8-T_4CDF than as a general screen for PCDFs. Unlike the anti-dioxin sera the ABs were influenced by the number of oxygen atoms in the analyte molecules: 2,3,7,8-T_4CDD cross reacted only 8.6 %.

3. IAs for PCBs

Most interest in the use of IAs for the detection of PCBs has focused on the various Aroclor preparations as target analytes. The Aroclors are complex mixtures of PCBs that vary in their chlorine content and in the average number of chlorine atoms per PCB molecule.[45,46] Antisera raised against haptens that contain few chlorine substituents should be more sensitive to Aroclors, that contain mainly lower chlorinated isomers. Luster et al.[37] developed antisera for use in an early and highly selective RIA. One of

the antisera was optimal for Aroclor 1254, however, the calibration curve was shallow, though linear: about 40 ng of analyte caused a 15% decrease in binding.

Newsome and Shields[47] followed up on Luster et al.'s observation that antibodies raised against amino derivatives of planar HAHs tend to also recognize a chlorine substituent in the same position. Those researchers raised an antiserum to 2-NH_2-2',4,4',5,5'-P_5CPB in anticipation that the serum would be sensitive to the higher chlorinated PCBs that predominate in Aroclor 1254 and 1260. The immunogen was prepared by coupling the succinamido derivative of the hapten to HSA via a carbodiimide reaction. A radioligand of high specific activity (2073 Ci/mmol) was prepared by the direct substitution of ^{125}I for the amino group in the hapten molecule. The proximity of the large iodine atom did not seem to unduly hinder the AB-ligand reaction. DMSO (25 %) proved superior to Cutscum as a solubilization agent. A classic RIA procedure was used with a main incubation period of 12 h at 4 °C. The inclusion of DMSO in the DCC phase separation agent improved the assay's sensitivity ten fold. Although the assay was most specific for the target congener it also responded well to Aroclor 1254 and 1260 (0.1 ng caused a 20 % response): in each case the calibration curves were sensitive and steeply sloped. Based on its performance at low analyte levels the assay warrants a full evaluation with environmental matrices. Solvent extracts of PCBs from blood and milk were prepared by chromatography on neutral alumina for analysis in the RIA. The RIA could detect as little as 20 ppb of Aroclor 1260 in milk and 2 ppb in blood. On average the RIA estimates were lower than those of a GLC confirmation method; the data from the two methods were well correlated, however.

Franek et al.[48] have devised an interesting micro-column RIA for the detection of PCBs in milk and animal fats (Table 1). After an overnight incubation the bound and free phases are separated by chromatography on home made mini-columns of Sephron gel. This innovative separation was used because calibration curves that were prepared using a DCC technique had poor reproducibility - the cause of that problem is unclear. An ultra-sonication step was included to minimize losses of the ^{125}I labelled tracer by

nonspecific binding to the walls of the glass assay tubes; DMSO was used as the solubilization agent. The assay's ability to measure Aroclor 1260 in milk samples (r=0.96) and fat samples that had been prepared by chromatography on florisil was successfully compared with that of a GC confirmation technique.

A rapid (<30 min) and practical EIA that is sensitive for Aroclor 1248 is now marketed as part of the EnviroGard line of IA test kits.[49] The assay uses a direct competitive format in which the ABs are coated onto the tube walls - a straightforward assay procedure that is suitable for field use. The ABs are specific for PCBs and can detect Aroclors other than 1248 such as 1254 and 1260. The kit developers recommend that the analyst use a calibration standard that matches the Aroclor whose presence is suspected in the samples. The kit is available in two versions, one with a nominal operating range of 5 - 50 ppm in soil and a DL of 3.3 ppm. A more sensitive version has a lower DL of 0.5 ppm and an operating range of 1 - 5 ppm. Millipore's IA development group have interfaced the assay with a solid phase extraction (SPE) procedure for the analysis of water and effluent samples (B. Harrison personal communication); that version of the assay has a working range of about 0.1 - 10 μg/L.

EnSys, Inc. (Research Triangle Park, N.C.) have also developed a commercial EIA for PCBs. The assay has a DL of 5 ppm in soil.[50] The assay format resembles that of the tube based Envirogard assay with some useful modifications - one of which allows the kits to be stored at room temperature. The assay has been validated for soil samples. The IA yielded only 7% false positive and 2% false negative results out of 50 soil samples when a threshold concentration of 5 ppm was used in a field evaluation. The assay kit was designed so that the cut-off concentration of 5 ppm is located in the middle of the calibration curve; a design consideration that helps to minimize false negative results. Researchers at Ohmicron have added a sensitive EIA for PCBs (Aroclors 1254, 1260, 1248, 1262, 1242) to their range of RaPID IAs for environmental contaminants.[51] The assay is a direct EIA in which the ABs are covalently immobilized on magnetic particles. That format dramatically reduces inter well/tube variations in antibody levels,

prevents leaching of the ABs from the solid phase during the assay, and also permits close contact between the ligand and binder molecules. The result is a sensitive (DL 200 ppt in water without SPE) and precise (CV < 10%) assay that has performed well for the manufacturers when used to screen various surface waters. The water samples are diluted 1:1 with methanol to prevent adsorption of the analyte onto vessel walls - the assay can tolerate up to 55% MeOH in the sample without adverse affects. The Ohmicron assay appears to be the most sensitive of the IAs for PCBs (Table 1). Its performance characteristics and ease of use make it suitable for screening water samples that are contaminated with moderate levels of PCBs. Combined with SPE or super critical fluid extraction (SFE) the assay should permit the detection of trace levels of PCBs.

4. Some IA problems

Although the IAs for planar HAHs enjoy many of the inherent advantages of the LBAs, those advantages are counterbalanced by the difficulty and cost of developing a fully fledged and validated IA. For that reason it is important that the key reagents - haptens and sera - become more readily available, and at a reasonable cost, if the technique is to become more widely used. The development of those key reagents can be a stumbling block for the smaller laboratory. Because of their high development costs, IAs are most cost effective when used to screen large sample sets. The use of IA to detect small quantities of hydrophobic analytes such as the planar HAHs has presented special problems. In order to achieve a low detection limit for such analytes residual lipid materials must be removed from the extracted sample. That usually demands the use of a multi-step cleanup procedure which detracts from the IA's appeal. On the other hand, a reduction in the number of samples that require high resolution MS confirmation gains importance in proportion to the size of the sample set and the number of negative samples.

The detection limits (low fg) that can be achieved with state of the art GC-MS instruments are beyond what can be achieved by the current IAs for planar HAHs, even with extensive preparation of the sample extract. A possible solution would be to use a

threshold concentration below which samples could be considered negative. As of yet, there is little agreement as to what constitutes a safe level of PCDDs in environmental matrices.

5. IAs conclusions

The erroneous elimination of false negative samples from the sample chain of false negative samples is the most serious type of error that can occur in a screening assay. Such errors arise from either an inability of the ABs to bind the analyte molecules under the assay conditions, or poor assay sensitivity. Residual lipids can contribute to the former problem by solubilizing the analyte molecules in a manner that shields them from the ABs' binding sites. Shortcomings in either tracer activity, AB avidity, or phase separation systems can contribute to assay imprecision at low analyte levels with an associated loss of sensitivity.

The use of matrix blanks (+/- spikes), fortified samples, and the confirmation by GC-MS of a random selection of negative samples can help uncover any tendency of the IAs to generate false negative data. The suitability of the clean-up procedure can be tested by calibration of the IAs performance in the presence of the matrix. Any inadequacies should become apparent as a displacement of the assay curve or deterioration of the dose-response relationship.

False positive results are not as serious a problem. Most of the compounds that are known to cross react in the IAs are also of environmental or toxicological interest. In any case, positive samples should be confirmed by GC-MS; a precaution that should uncover any errors of that type.

Although many of the IAs for planar HAHs are currently able to detect moderate to high levels of their target analytes in environmental matrices, ultimately an IAs' performances at low analyte levels will determine whether it becomes widely used for routine screening applications. The sensitivities of the current assays need to be

improved if they are to detect their analytes at < 20 pg/assay in the presence of sample matrix. Albro et al.[17] have suggested that radioligands with high specific activity would help to improve the RIA's sensitivity. Bradfield et al.'s[19] successful use of a chloramine-T reaction to label a dioxin derivative with ^{125}I (Specific activity of product = 2176 Ci/mmol) suggests that the synthesis of a high activity ligand for use in the RIA for PCDDs is feasible. Such a tracer would allow a reduction in the mass of tracer that is added to each assay tube, which in turn, would reduce the effect of 'cold' displacements, which are not detected in the RIA, on the assay's sensitivity. Ohmicron's promising EIA design has the potential to detect HAH molecules at low ppt levels in water samples when interfaced with a simple solid phase extraction step.

The extensive multi-step cleanup procedures that are needed to remove residual lipids and interferences have led some experts to suggest that the HAHs may be less than ideal targets for IAs. Many of the screening techniques cost and speed advantages are lost during the cleanup process.[52] However, Vanderlaan et al.'s work has shown that the amount of sample preparation can be reduced for samples that are highly contaminated or for analytes that need not be detected at ultra trace levels. Even when the analyte must be extensively enriched the screening technique can still reduce the number of samples that must be confirmed by high resolution GC-MS. The current IAs should be able to screen samples from badly contaminated sites or to screen serum samples from heavily exposed populations. The IAs for planar HAHs would benefit from the development of improved and efficient clean-up procedures, that would allow the amount of sample added to the assay tubes to be increased.

Validation of the ability of the IAs for planar HAHs to screen a variety of matrices such as serum, sediments, water, soil, and flyash extracts remains a challenge. The availability of commercial IAs, such as those for PCBs, should promote such studies.

C. Receptor binding assays

The RBAs for planar HAHs are based on the selective binding properties of the Ah receptor and are conceptually similar to the competitive receptor binding assays that are used to quantify steroid hormones in body fluids.[30] The Ah receptor, which is the soluble cytoplasmic protein that transports the bound HAH into the nucleus, is hypothesised to mediate the toxicity of planar HAHs at the molecular level.[53,54] It is now well established that the binding of planar HAH molecules to the Ah receptor is the key mechanistic step in their induction of detoxification enzymes such as aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin O-deethylase (EROD). The ability of planar HAHs to induce the formation of those enzymes parallels their ability to induce several toxic symptoms. The Ah receptor has been identified in many species,[53,55,56] including fish,[57] and tissues that are susceptible to the enzyme inducing and toxic effects of 2,3,7,8-T_4CDD.[56,58] Other planar HAHs, halogenated and non-halogenated polynuclear aromatic hydrocarbons (PAHs) such as benzo-a-pyrene (BaP) and 3-methyl cholanthrene (3-MC), and heterocyclic amines that meet the electronic and structural requirements of the binding site can also act as ligands for the Ah receptor.[18,59,54] Most compounds that bind to the Ah receptor can also induce CytP4501A1 activity.[18,60] So far, it is not known if there are endogenous ligands for the Ah receptor[61,62] or if the receptor evolved solely to deal with foreign compounds.[62-64]

The procedure outlined in Fig. 12 can be used to prepare batches of Ah receptor for use in the assay.[18] The various RBA assays closely resemble the conventional RIAs. The measurement of the amount of [^{3}H]T_4CDD that became bound to non-receptor components in the reaction mixture was a key step in the original RBA for 2,3,7,8-T_4CDD (Fig. 13)[18]. That estimate was made by blocking the Ah receptor's sites with a large (200 fold) excess of 2,3,7,8-T_4CDF and then separating the bound and free phases. The amount of non specifically bound (NSB) radioactivity is subtracted from the readings for each assay tube. Under standard assay conditions the NSB levels were reported to be 3 - 4 times less than the amount of specifically bound radioligand.

Several researchers, including Okey et al.[60] encountered reproducibility problems when using DCC as the phase separation agent in the Ah receptor assay. The problem was caused by the non-specific binding of the tracer to components of the cytosol mixture. The poor solubility of TCDD in buffer can also cause erratic test results under some assay conditions by causing the radio-ligand to crash out of solution -a phenomenon that also occurs in the RIA for PCDDs. Okey and co-workers[60] used a sucrose-gradient centrifugation step after the phase separation (DCC) to improve the reliability of the RBA assay. The additional step successfully separates the receptor bound from non-specifically bound tracer. The RBA has been adapted for the detection of 2,3,7,8-T_4CDD and related compounds in extracts of fly ash.[65] The cytosol preparations used in that study were stored for up to 2 months in liquid nitrogen without loss of activity. The assay had an EC_{50} of 3.21 ng and a detection limit of about 100 pg of 2,3,7,8-T_4CDD (Table 1).

In an effort to improve the sensitivity of the RBA, Bradfield et al.[19] prepared a high specific activity (2176 Ci/mmol) radioligand by labelling 2-amino-7,8,dibromo-p-dioxin with ^{125}I in a chloramine-T driven reaction. The amino group was removed after the completion of the labelling reaction. The high activity ligand was used in an exceptionally sensitive assay (Table 1).[66] The binder reagent were prepared by fractionation of the cytosol of C57BL/6J mice with ammonium sulphate (40 -55%). Isolated cytosol fractions were stable when stored at -80 °C; which is convenient. DMSO was used to assist the solubilization of the analyte. The assay was carefully optimised so as to maximize its performance at low analyte levels while maintaining the ability to solubilize the analyte and radioligand molecules. The standard assay mixture contained 8 pM of radioligand, 18-20 pM of Ah receptor, and 5 - 1000 pm of 2,3,7,8-T_4CDD. The reactants were incubated for 16 h at 4 °C after which gelatin coated charcoal was used to separate the bound and free phases. The assay had an excellent DL of 3.2 pg of 2,3,7,8-T_4CDD and a linear working range of about 10 pM - 200 pM with an EC_{50} of 41.3 pM. Reduction of the tracer and binder levels helped to improve the slope of the calibration curve and the assay's sensitivity. The assay DL was further improved to 0.8 pg of 2,3,7,8-T_4CDD by reduction of the assay volume to 250 μL. The stability of the

Ah receptor preparation at -80°C and the potency of the radioligand suggest that the assay could be commercialized. Such a product would help to promote the wider use of this high performance RBA. That prospect is further enhanced by the lack of interference shown by the radioligands decay products, which means that the assay's performance characteristics should remain stable over a reasonable period. The Swedish Dioxin Survey has modified Bradfield and Poland's sensitive assay[66] by shortening the incubation period to 2 hours; the modified assay had an EC_{50} of 22 pg/tube.[67]

Nigel Bunce at the University of Guelph has published some preliminary work that is aimed at adapting the Ah receptor assay for use in the detection of planar HAH compounds in environmental samples.[68] His assay system uses [^{3}H]2,3,7,8-T_4CDD as the radioligand and a hydroxylapatite separation technique[69] in a protocol similar to that of Poland et al..[18] The hydroxylapatite method for the separation of the bound and free phases yields NSB levels that were in the region of 14% of the specifically bound tracer. The assay responds to mixtures of planar HAHs in an additive manner.

1. RBAs conclusions

Although there has been little use of RBAs for the detection of planar HAHs in environmental, biological, or food samples the assays are expected to be susceptible to the same types of error as the IAs. It is likely, for example, that oily matrices will require extensive preparation both to render the analytes accessible to the receptor and to minimize negative interferences. Positive interferences are likely to be a more serious problem for the RBAs because of the receptor's ability to bind a variety of planar molecules such as PAHs. The Ah based RBA would estimate the Ah active molecules in a sample, except for cases where the sample's planar HAH content is known to be restricted to a particular class of compounds. As with the IAs, all positively identified samples should be confirmed by independent means. The ability of the Ah receptor based assays to respond to analyte molecules roughly in proportion to their toxic potency and enzyme inducing potency is an important advantage. Although that relationship is undermined by the cells' ability to detoxify HAH molecules that have substituent free

adjacent carbon atoms. The wider distribution of the Ah receptor would encourage evaluations of the RBAs for HAHs as a supplementary analytical tool. A priority in such studies would be to interface the assay with a suitable extraction and clean-up method.

III. Bioassays

The planar HAHs induce a broad suite of biochemical and toxic responses in susceptible organisms. The full set of toxic effects do not occur in all species, although the various congeners do tend to induce similar subsets of responses in individual species. In addition, the size of the induced effects can vary between species and tissues.[70,71] Full accounts of the toxic effects of planar HAHs are available elsewhere.[64,70,72,73]

A. Dioxins' mode of action

The mechanism(s) by which the toxic HAHs exert their effects have yet to be fully unravelled, however it seems that the toxic HAHs, unlike many PAHs, do not need metabolic transformation to reactive intermediates.[70] Evidence suggests that 2,3,7,8-T_4CDD, at least, does not directly damage[76-review] or bind to DNA;[74] in other words TCDD is non-mutagenic.[75] Current thinking is that the toxic planar HAHs probably share a common mechanism at the molecular level[76] with the chain of molecular events being initiated by binding of the HAH molecule to a cytosolic protein: the Ah receptor.[63,70,77-79] That initial step is followed by transportation of the ligand-receptor complex across the nuclear membrane where the complex interacts with control genes and triggers the activation of structural genes. The products of those genes participate in a variety of cellular processes (Fig. 16) that include the production of oxidative enzymes, and also have an hypothesised role in the expression of toxic symptoms.[75]

Many studies have shown that the toxic potency of the HAH congeners depends on their molecular structure. Planar HAHs that have chlorine atoms in the "2,3,7 and 8" positions bind most readily to the Ah receptor and also tend to be the most toxic congeners.[74] Loss of chlorine or the addition of a chlorine atom in a non-lateral position reduces a congener's affinity for the receptor and also tends to lower its toxicity.[80] In

many ways the relationship between the HAHs and the Ah receptor parallels that of the steroid hormones and their receptor molecules;[70,79] in fact the Ah model (Fig. 16) is based on the known mode of action of steroid hormones.[64,73]

The proposed toxic mechanism has some shortcomings. For example, it does not yet explain how the various toxic symptoms arise, nor does it account for the large inter-species differences that can occur in induced effects even when Ah receptor levels are similar.[64] Neubert et al.[71] are concerned that although the induction of some oxidative enzyme systems are clearly mediated by the Ah receptor, such a relationship has yet to be established for all other toxic effects. The possibility remains that the Ah receptor may not mediate the entire set of toxic effects.[64,81] Rozman[82,83] argues, that the mechanisms responsible for enzyme induction and acute toxicity must be independent of each other, largely on the basis of the wide (two orders of magnitude) sensitivity differences that have been observed between the dose response curves for both processes in the rat. Nebert et al.[75] list some early TCDD induced events, such as elevation of AP-1 transcription factor, in mouse Hepa-1 *wt* cells that are independent of the Ah receptor, and are thought to promote tumours through the induction of cell proliferation. Despite its perceived shortcomings and incompleteness, however, the Ah mediated mechanism remains the most widely accepted hypothesis.[84]

B. Data normalization (TEF-TEQ)

The reams of analytical data that are generated for the planar HAH congeners in environmental and biota samples present difficulties for the regulators, who must devise guidelines for food consumption and industrial emissions - and for those wishing to make toxicological sense of the data. A data normalization system has been devised to facilitate both tasks by relating the toxic potency of mixtures of HAH congeners to that of the most toxic congener: 2,3,7,8-T_4CDD.[71,84,85] The data normalization system is based on several observations and underlying assumptions. Firstly, the individual planar HAHs induce similar biochemical and toxic responses in a variety of organisms and in-vitro test systems; the structure activity relationships (SARs) for many of those induced effects are

similar. Furthermore, the SARs for several toxic effects, such as acute lethality, thymic atrophy, and body weight loss in rats, are generally similar to those that describe the ability of HAH congeners to bind to the Ah receptor.[74,86] It was assumed that mixtures of congeners act additively on target organisms, and that the toxic mechanism is mediated by the Ah receptor.

The toxic potencies of the individual congeners are expressed as a factor of 2,3,7,8-T_4CDD's: the estimated ratio is the congener's toxic equivalency factor (TEF).[85,87,88] Although originally based on data for acute toxicity in the guinea pig,[85] the TEFs have since been updated with a variety of acute, chronic, immunotoxic, enzyme induction, and teratogenic data;[86] particular emphasis is placed on data that are relevant to toxicity in humans (Table 2). The TEFs are used to transform the data for individual or grouped HAH congeners to a toxicity equivalency value (TEQ).

All is not perfect with the TEF/TEQ approach, however. A recent study suggests that current TEFs do not reliably predict the ability of repeated low doses of planar HAHs to induce mixed function oxidase (MFO) activity in mouse tissues.[88] Moreover, the same study showed that the relative potencies of individual PCDD/PCDF/PCB congeners can vary between target tissues. Pharmokinetic variables, such as absorbtion rates and metabolic enzyme systems, are thought to be responsible for the inter tissue differences. Moreover, as Neubert et al.[71] point out, parallel dose-response relationships have yet to be demonstrated between the toxic responses to 2,3,7,8-T_4CDD and the other PCDD/PCDF congeners for many systems. Such relationships must exist if TEFs are to be applicable over a range of concentrations. There is also evidence that antagonistic interactions can occur between individual congeners;[89,902] in violation of a key underlying assumptions. Because the Ah - ligand interaction is competitive, less potent congeners can, if present at high levels, compete successfully for receptor sites[71,80] and thus mask lower levels of more potent congeners. For example the PCB mixture

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[2]Cites relevant studies

Arclor 1254 can reduce 2,3,7,8-T_4CDD's immunotoxicity and its ability to induce MFO enzymes.[89] Safe estimated that the ratios of antagonist/agonist that cause non-additive interactions can overlap with ratios that have been measured in humans and in the environment.[91] A data transformation system that could account for such interactions would need complex correction factors because of the mixture dependency of the interactions. Bioassay derived TEQs, on the other hand, can automatically adjust for all such non-additive interactions.

The need to refine and adapt the TEQ approach to allow the prediction of toxic effects in a broader range of species was emphasised by Williams & Giesy[92] who failed to find a significant relationship between levels of PCBs (expressed as TEQs), in eggs of chinook salmon (*Oncorhynchus tshawytscha*) and mortality of the emerging juveniles: the TEQs in that study had been calculated using mammalian TEF values. The ability of the TEF/TEQ system to make reliable estimates of health hazards should improve as the TEFs are further tested and refined for a variety of organisms.

C. Biological response parameters

The bioassays for planar HAHs address many of those shortcomings by providing an integrated response to the biologically potent HAHs present. That response is directly proportional to the mixture's toxic potency. The bioassay automatically corrects for any antagonistic and synergistic (unlikely) effects, and the results can be normalized with respect to 2,3,7,8-T_4CDD's potency in the bioassay. To be suitable for use in a practical bioassay for the planar HAHs a response parameter should meet several criteria. It should be readily inducible, easy and inexpensive to measure, and should have a good response at low analyte levels. The assay's base-line response should be consistently low, and the assay should respond precisely and in a linear manner to increased doses of the analyte. The key bioassays that have been developed for the detection of toxic HAH compounds are outlined in this section. The response parameters that have been used include toxic symptoms (keratinization, flat cell formation), enzyme induction (AHH and EROD), and biochemical changes (porphyrin chemistry).

D. Enzyme induction bioassays

1. MFO system

Located mainly in the liver, the MFOs are a complex of enzymes that insert oxygen into many xenobiotic chemicals such as pesticides and drugs. Oxidation by MFOs makes those molecules vulnerable to further enzymatic or chemical action, including conjugation to polar groups and molecules, which aids their elimination from the body.[63,78,79,93-95] Paradoxically, the electrophilic character of the oxidized molecules also makes them reactive towards electron rich centres in DNA, and can under certain circumstances promote the carcinogenicity of planar aromatic compounds.[75]
Nebert[81] estimates that each species probably has about 60 cytochrome P-450 genes, with each gene coding for a unique enzyme. The Ah locus helps to regulate the expression of two genes: *CYP1A1 and CYP1A2.* CytP450IA1, a key component of the AHH and EROD oxidative systems, is induced by 3-MC, B(a)P, 2,3,7,8-T_4CDD, and other planar molecules.[96] The cytochrome P4501A1 system oxidizes planar aromatic molecules that contain joined rings[97] (Cytochrome P450 nomenclature is discussed in detail elsewhere).[93,98,99]

Within the main groups of planar HAHs, congeners that have high affinity for the Ah receptor also tend to be strong inducers of AHH and other cytochrome P450 dependant MFOs,[77] although those parameters do not appear to be either linearly related or statistically significant. The absence of a strong relationship has been attributed to several causes: variable receptor levels, metabolic alteration of some congeners - particularly those with adjacent carbon atoms that are chlorine free,[100] interactions involving the receptor-ligand complex, or poor ligand solubility.[101,103] The SARs for the induction of several toxic effects in the rat are similar to those for the induction of MFO activity,[64,70,73,74,76,78,100,103,104] as demonstrated by the data in Fig. 17 which show a linear relationship between the two effects for a set of more than 30 planar HAHs. Those data, however, are plotted on log/log axes which can compress fairly large discrepancies. Where tested, the toxic potency of congeners within the PCDD and PCDF groups also corresponds to their ability to induce AHH.[103,105,106]

PCBs boost a variety of MFO activities that resemble those induced by both phenobarbital (PB) and 3-MC.[74] By assuming a planar configuration the co-planar and weakly co-planar PCBs compete with 2,3,7,8-T_4CDD for binding sites on the Ah receptor and so elicit similar biochemical and toxic responses to the PCDDs.[73,78,104]
Thus, there are sound reasons to use induced MFO activity as a response parameter in screening tests for toxic planar HAHs, with the caveat that other planar aromatic compounds that can bind to the Ah receptor may also cross-react in the assay.[70,79,93,96,107] Taylor et al.'s recent observation that CytP4501A1 induction and acute toxicity are independent of each other in mice, even though both may be mediated by the Ah receptor, suggests there may be limits to the conclusions that can be drawn from the results of MFO induction assays.[108]

2. In-vitro MFO assays

Niwa et al.[109] who studied the kinetics of AHH induction by 2,3,7,8-T_4CDD in a range of tissues and cell types, were the first to propose an enzyme induction assay for the detection of 2,3,7,8-T_4CDD. The original assay could detect low (10^{-14} mole/mL = 10 fmol = 3 pg/mL = 9 pg/plate) levels of 2,3,7,8-T_4CDD mainly because of the low base line activity and strong response of the H-4-11-E rat cells. Bradlaw and co-workers,[110] among others, (Table 3) modified Niwa et al.'s assay to facilitate the routine detection of small quantities (low pg) levels of PCDDs and other cytP4501A1 inducing compounds (Table 3). The assay procedure is outlined in Fig. 18. Stock cultures of the cells were maintained in liquid nitrogen, and working cultures were renewed after 20 cell passages. The cell culture and harvesting techniques have been described in detail by Nebert and Gelboin[111] and in Bradlaw and Casterline's original account of the method.[112,113] The cells were exposed to the test chemicals in 60 x 15 mm tissue culture plates: about 10^6 cells were added to each plate. After a pre-incubation for 24 h, the test chemical, or portions of sample extract (100 μL), were added to the appropriate plates and all plates were re-incubated for a further 72 hours. By that time the cell count had risen to about $3x10^6$ per plate. Once harvested, it was possible to store the cells at -70°C, before

they were homogenized in preparation for the enzyme assay step. The AHH enzyme assay is outlined in Fig. 19. The bioassay (Table 3) had an ED_{50} value of 0.14 pmol of 2,3,7,8-TCDD when iso-octane[3] was used as the solubilization agent. An assay calibration curve that is based on the mean of 17 determinations is presented in Fig. 20. Because the maximum amount of induced enzyme activity can vary from week to week (40 - 120 units/mg protein/min), Bradlaw et al.[113] normalized the data with respect to the maximum induced response of a standard that is run on the same day. The assay is most sensitive to PCDD and PCDF congeners that were substituted in the 2,3,7,8 positions but can also detect a broad range of other congeners. PCBs that were co-planar with respect to 2,3,7,8-T_4CDD are as reactive as the more potent PCDD and PCDF congeners.
That early version of the AHH induction assay was successfully validated for gelatin samples, fractionated extracts of contaminated rice oil that had been implicated in the now famous "Yusho" poisonings,[114] and food extracts.[112] Casterline et al.[115] used the assay to screen eight extracts of fresh water fish that were prepared by fractionation on silica gel. Although the sample preparation was not optimized for the recovery of planar HAHs, there was good agreement between the assay and the conventional data; there were no false negative results.

The MFO induction assay was systematically characterized and evaluated by Tillitt et al.[116] who used EROD activity as the bioassay's end point. A fluorometric method for the estimation of EROD activity is outlined in Fig. 21.[117-119] BSA was included in the enzyme assay mixture to help solubilize the hydrophobic substrate and to improve the assay's linearity. The performance of the EROD induction assay compared well with that of the AHH based bioassay (Table 3). EROD activity can also be measured by a spectrophotometric procedure without loss of performance.[120] That procedure which requires less expensive equipment, exploits the distinct absorbtion peaks of 7-ethoxyresorufin (482 nm) and resorufin (572 nm) in visible light. The fluorometric determination of EROD activity has been miniaturized and adapted for use with 96 well

[3] One pmol of 2,3,7,8-T_4CDD weighs 322 pg.

microtitre plates.[121] The method is 10 - 15 times faster than the tube based assay and allows the simultaneous measurement of 20 samples without loss of precision or sensitivity.

Tillitt et al.'s work demonstrated the EROD based bioassay's precision: the intra-assay CV for the ED_{50} estimate was 3.7%; the CV for 2,3,7,8-T_4CDD in an extract was 5-15%. However, the inter assay CV for the ED_{50} estimate was 33.8%, which led the authors to suggest that a calibration curve should be run with each set of samples. The response data for a variety of planar HAHs compared well with data from other laboratories. That relationship did not hold up, however, when the data were expressed as EC_{50} values; which suggested to the authors that ED_{50} values may be more useful in comparisons of induced EROD activity. Tillitt et al. have used the EROD induction assay to screen extracts of eggs from colonies of fish eating birds in the Great Lakes.[122] The bioassay results were consistent with earlier residue data, with known pollution patterns, and with recorded biological effects in the colonies. An interesting recent study from the same group describes the use of the MFO induction bioassay to study the biomagnification of planar HAHs in a freshwater ecosystem.[123] The study confirmed that food ingestion is the main pathway for contamination of organisms by dioxin like molecules and demonstrated the practical utility of the MFO based bioassay. The H4IIE bioassay was also used to measure TCCD equivalents in tissues of aquatic and terrestrial birds taken from Green Bay, Michigan. The bioassay derived TCDD-EQs were correlated with analytically derived TEQs. However the analytically derived data consistently underestimated the bioassay data, probably, the authors speculated, because of the presence of non-determined planar hydrocarbons.[123] Of special interest was the observation that TEFs derived using the H411E bioassay yielded TEQ estimates that were lower than the bioassay's values (x1/3) whereas TEFs derived from mammalian end points yielded TEQs that were three times higher.

Steve Safe's Texas team have modified the H-4-11-E MFO induction assay so that both EROD and AHH activities can be measured in suspensions of whole cells. That

modification was not only more convenient, but also paved the way for the use of micro-titre plates which, in turn, should facilitate the assay's miniaturisation and automation.[124] Once a stock culture of the cells was confluent the cells were harvested and seeded into 5 mL of medium in 25 cm^2 culture flasks at a cell density of 10^6 cells per flask. After 24 h the cleaned-up samples or standards were added to the flask in DMSO (0.5% final concentration); the final cell pellet was adjusted to 2 mg of protein/mL. The EROD activity of the cell pellet was measured as described in Fig. 21 except that 0.05 mg - 0.1 mg of cell suspension was added to each assay tube (16 mm x 100 mm). The samples were pre-incubated for 2 min. at 37 °C, and 50 μL of ethoxyresorufin (100 μM) in MeOH was then added to each tube, after which the tubes were re-incubated for 15 minutes. AHH activity was measured as described in Fig. 19 with the following modifications. After the 2 minute pre-incubation, 50 μL of B(a)P (2 mM in MeOH) was added to the reaction mixture. After a 15 minute incubation the reaction was terminated by the addition of 4.25 mL of hexane/acetone (3.25:1). The extracted 3OH-B(a)P (Fig. 19) was quantified in a spectrofluorometer (λ excit. = 550 nm, λ emiss. = 585 nm).
Safe et al. used their version of the cyt P4501A1 induction assay to screen extracts of a flyash sample that was heavily contaminated with PCDDs and PCDFs. The data from the in vitro tests was linearly related to data produced by the in vivo induction of AHH in the rat.[125] The AHH and EROD induction assays were also used to screen extracts of 25 fish from the Great Lakes.[126] With the exception of the fish from L. Ontario and L. Erie, the bioassay data differed from the GC-MS values Σ(PCDD + PCDF) by less than a two fold margin. The difference was larger, however, when the GC-MS data were expressed as TEQs. The bioassay data for the L. Ontario and L. Erie fish were much higher (>4 fold) than the GC-MS derived values. The authors speculated that the higher bioassay values could arise from synergistic effects or from the presence of enzyme inducing compounds other than PCDDs/PCDFs.

The Swedish Dioxin Survey has also assessed the performance of the in-vitro EROD induction assay (Table 3).[127] The EROD and GC-MS data (expressed as TEQs) for the PCDD/PCDF fraction of eight herring muscle extracts were in good agreement which

suggests that the HAH congeners had an additive effect on the test cells. Data for herring and Osprey samples that contained PCBs, polychlorinated naphthalenes (PCNs), and polychlorinated diphenylethers (PBDEs) agreed with the estimated TEQs for the co-planar PCBs. However EROD data for white fish, and grey and ringed seals did not compare as well with the GC-MS data, possibly because of interference by yet to be identified planar contaminants, and in the case of the seal extracts, the presence of cytotoxic compounds.

The cytochrome P4501A1 bioassay would be more attractive if the exposure and enzyme assay steps were simplified and automated. Kennedy et al. have taken some important steps in that direction.[128] They initially studied the ability of PCBs to induce EROD and porphyria in primary cultures of hepatocytes from chicken embryos.[129] After a 24 hour incubation of the primary culture in 12 well culture plates the medium was replaced and serial dilutions of extract or standards, prepared in DMSO, were added to the wells. After a further 24 h incubation the induced EROD activity was measured in the harvested cells. The EROD and GC-MS data for a six sample set of wild bird eggs from the Great Lakes and British Columbia were highly correlated (r=0.95) when the data were expressed as PCB-126 equivalents. That result contrasts with the weaker EROD-GC/MS relationship that was reported by Zacharewski et al.[126] for highly contaminated fish samples from the Great Lakes. The EROD test responded to extracts that had no observable effect on porphyrin levels. An improved version of the assay (Fig. 23) uses 48 well culture plates which can be read directly in a fluorescence plate reader.[128,130] This innovative assay is 100 times shorter than previous methods largely because it eliminates two time consuming steps: the harvesting and transfer of treated hepatocyte cells and the manual measurement of the MFO's fluorescent product. After exposure to the test chemicals the medium is removed and the plates are stored at -80 °C, after first being frozen on dry ice to halt residual enzyme activity. A resorufin standard curve is prepared in a separate plate under identical conditions. Protein determinations, however, must also be made in replicate plates, and that determination still requires the time consuming transfer of well contents to vials. Although it should be possible to eliminate the transfer

step by measuring the protein in the microtitre wells as shown by Renzi et al.[131] Some typical dose response curves for the assay are presented in Fig. 24. The decline in the assay's response at high doses is not fully understood but may be caused by either competitive inhibition of the enzyme system by free analyte molecules or the disruption of cellular processes by the excess analyte molecules. The method's excellent sensitivity (DL of 0.16 pg 2,3,7,8-T_4CDD/well; Table 3) was attributed to the small volume of cell culture medium and possibly, the superior response of chicken embryo hepatocytes compared to H-4-II-E cells. Total porphyrin levels can also be measured in the same wells that are used for the EROD determinations. Yao et al.[132] who have also used chicken embryo hepatocytes as the test cells in an MFO induction bioassay (Table 3) observed that some co-planar PCBs (3,3',4,4'-T_4CB and 3,3',4,4',5-P_5CB) were more active than 2,3,7,8-T_4CDD in the assay. On the basis of the activity of these and other PCBs the authors concluded that the chicken hepatocytes were not as selective as the H411E cell line.

Among the other interesting variants of the cytochrome P4501A1 based enzyme induction assay are the use of primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes as the test cells. That assay could detect TCDD,[133,134] and would probably be a more realistic choice for the testing of industrial effluents and sediment samples than rat cell based assays. Westinghouse Bio-analytic Systems Co. used a line of cloned cells from a mutant mouse in an ultra sensitive EROD induction assay.[135] The assay, which only required an incubation period of 18-24 hours, could detect as little as 0.0625 pg of 2,3,7,8-T_4CDD per mL (Table 3) and had a dynamic range that extended from the DL to 1.0 pg/mL. Such performance should make the assay ideal for environmental applications if the cell line is made available. The assay was reportedly used to screen water, sediment, and soil samples for 2,3,7,8-T_4CDD and like compounds.

a. Calculation methods

Hanberg et al. have considered the procedures used to calculate the results of MFO induction experiments.[127] They recommend that a 2,3,7,8-T_4CDD calibration curve

be run with each set of samples and prefer that the TCDD-Equivalents ratio be calculated on the basis of a sample concentration that induces an EROD response that corresponds to the linear section of 2,3,7,8-T_4CDD's concentration-response curve. Usually the comparison will be with the ED_{50} value for 2,3,7,8-T_4CDD. It is critically important that the concentration of sample that is used in such comparisons be from the pre-plateau section of the concentration-response curve. That method seems preferable to those that use the ratio between the sample's ED_{50} and that of T_4CDD to estimate the sample's potency. The accuracy of that ratio depends on the sample and T_4CDD having the same maximum responses - which need not be the case. If the sample's maximum response should be lower, the estimated T_4CDD-EQ would be inflated.

3. In-vivo MFO assays

There are several in-vivo bioassays that can be used to assess the inductive potency of pure chemicals, sample extracts, or effluents. The more popular of those use chick embryos as the test organism. Brunstrom and co-workers have studied the toxicity and MFO inducing abilities of PAHs and PCBs in chick embryos and young chicks.[96,136-138] Solutions of the test chemicals in peanut oil are injected into the air sac of eggs that have been pre-incubated for 7 days. After a further 72 h incubation the liver is removed from the embryo and homogenised in 350-1400 μL of buffer and the EROD activity of the homogenate is measured. The toxicity and EROD inducing abilities of the PAH congeners followed the same rank order. PCDD/PCDF/PCB congeners can also induce EROD in chicken embryos: 2,3,7,8-T_4CDD had an ED_{50} of 10 ng per egg in the assay.[90] The following technique is useful for injecting test solutions into eggs.[139] The eggs are first examined by means of a strong light to determine whether an embryo has formed. A small hole is then drilled into the shell in the centre of that space. The sample is then injected through the hole onto the inner shell membrane. The hole is then sealed with wax and the eggs are re-incubated; 12 - 18 livers from 5 day old embryos are pooled to prepare liver homogenates; single embryos were used from older embryos and chicks. The chicken embryo bioassay has been used to screen fractionated extracts of suspended sediment from the Stockholm archipelago.[140] The sediment fraction that contained

diaromatic compounds was a less potent inducer of EROD activity than the polyaromatic fractions. However the EROD levels induced by samples from the inner archipelago were unexpectedly high. More work is needed to establish whether this effect was caused by synergistic effects or the presence of some yet to be identified contaminants.

4. Conclusions in-vitro/vivo MFO assays

The inherent tendency of the MFO induction assays to cross react with compounds, such as PAHs, that can bind to the Ah receptor can confound the use of those tests for the detection of planar HAHs and may cause some of the discrepancies that has been noted between MFO estimates and analytical data. Unidentified compounds that are Ah active can generate both false positive results or falsely elevated values when the assays are used to estimate the levels of dioxin like molecules in a sample. If those unidentified compounds happen to be planar HAHs, the elevated assay results may be more accurate than the analytical data. Ah antagonists such as α naphthoflavone can inhibit 2,3,7,8-T_4CDD's ability to induce EROD,[141] whereas "non-PCDD/PCDF/co-planar PCB" agonists can induce EROD. Such agonists include b-naphthoflavone,[134] dibenzo-ah-anthracene, benzo(k)fluoranthrene, indeno[1,2,3-cd]pyrene,[142] benzo(a)anthracene,[63] xanthone, [123] naphthalenes, DPEs, dibenzothiophenes, B(a)P and several other chlorinated and brominated PAHs and related compounds[79,93] many of which are active in MFO induction assays.

The inability of the MFO induction assays to discriminate between dioxins, PCDFs and co-planar PCBs is beneficial since compounds from all three groups are potent toxicants. Recent studies, for instance, have suggested that the toxic co-planar and mono-ortho substituted PCB congeners, when expressed as TEQs, are often present at high levels in biota and sediment samples.[2,143,144] On the other hand, there is concern that the conservative criteria used to estimate the TEFs for PCBs may overestimate the contribution by PCBs to overall TEQ values.[88]

False positive errors are best identified by the use of conventional techniques to confirm all positive results. If the estimated TEQ values consistently disagree with the analytical data a toxicant identification study may be required so as to identify the inducing compound(s).

MFO induction assays that are based on cultured cells are rapid, easily replicated, cost little, and afford the possibility of test standardization. Use of an optimised enrichment procedure for each matrix type will help minimize the effects of interferences on the assay's performance and enhance their role as screening assays for planar HAHs in environmental samples. Although many studies have supported the use of cytP4501A1 induction assays as a complementary analytical and risk assessment technique, there is a need to further probe the relationship between the enzyme inducing and toxic potencies of congeners, on their own or in mixtures, in a broader variety of species. Such data would increase confidence in the ability of cytP4501A1 induction assays to make both health and risk assessments for species other than the rat.[122] Confidence in techniques of this type depends on a solid base of knowledge which serves to define the method's potential, limitations, and practical usefulness.

5. In-situ MFO assays

In the mid-70's Payne and Penrose[146] suggested that AHH levels in fish could reflect exposure to petroleum or other substances that contain PAHs and related compounds. Much subsequent interest has developed in the use of MFO levels in fish and Molluscs as an indicator of whether aquatic ecosystems have been contaminated with PAHs. The MFO parameters can be measured in organisms that have been exposed in the wild, or that have been exposed under controlled conditions either in the laboratory or in-situ. Controlled in-situ exposures are usually made in cages or other water permeable containers. The in-situ MFO tests have turned out to be well suited to the monitoring of aquatic systems where they can help to delimit the zones of influence of point sources of pollution.[95] An organism that is exposed in the field can effectively integrate the effects of pulsed exposures - provided that the interval between the pulses

is not excessive. The in-situ MFO tests have become important research tools because of their ability to signal the onset of contaminant induced changes before gross effects become apparent at the organism or population level. Used this way the tests can provide an early warning that adverse environmental effects may be underway.[107]

An increase in MFO levels in the exposed organism is assumed, with good evidence, to indicate the presence of inducing chemicals in the surrounding milieu. 2,3,7,8-T_4CDD has been shown to induce the production of EROD in rainbow trout,[147] and Van der Weiden et al.[148] have recently shown that the induction of cytochrome P4501A1 activities coincides with the onset of a variety of toxic symptoms in the rainbow trout. The co-planar PCBs on their own or in mixtures are now known to induce MFO formation in a variety of fish.[149-152] However, as with the in-vitro assays the in-situ MFO induction tests also respond to chemicals other than the planar HAHs.[93,98,153] For example b-naphthoflavone and PAHs are well established as inducers of MFO activity in fish.[98] In Payne and Penrose's land mark study, brown trout (*Salmo trutta*) were taken from control and oil contaminated lakes.[146] The livers and gills were cut from the freshly killed fish and the tissue homogenates were prepared using a simplified version of the procedure in Fig. 25. Because cytochrome P450 is reported to be unstable in isolated fish liver,[154] care must be taken in the preparation and storage of tissue, homogenates, and microsome suspensions. Payne and Penrose found that the AHH levels in the fish from the contaminated and control lakes differed. Exposure of control fish to oil contaminated water in the laboratory induced higher AHH levels in both gill and liver tissues of treated fish. Oil residues in the environment were subsequently shown to induce AHH formation in a wide variety of fish.[155,156]

The flow diagram in Fig. 25 summarises some procedures for the preparation of homogenates of liver and gill tissue for use in a MFO assay. Some researchers prefer to prepare the S9 homogenate in the field and to store it in liquid nitrogen for transportation to the laboratory.[157,158] However storage in liquid nitrogen can cause the activity of some liver homogenates to decline,[149] whereas MFO levels remained stable in

chopped liver for three days under similar storage conditions.[159] A protease inhibitor and glycerol can be added to the homogenised liver: the glycerol helps to stabilize the enzyme activity.[158] The EROD assay has been optimised for use with liver microsome preparations.[149] The modified assay mixture contains the following ingredients in 2 Ml Tris buffer (0.1M, with 0.1 M NaCl, pH 8.0): 1 μM ethoxyresorufin and 40 - 200 ug of microsomal protein/mL. After a 2 minute pre-incubation at 30°C, NADPH (250 μM) is added to start the reaction. Very active assay mixtures should be diluted.

There was early interest in the use of mussels as the sentinel organisms for in-situ MFO tests. The anchored habit of the benthic mussels would serve to control the mobility variable. Initial efforts to detect MFO activities in Molluscs met with mixed results.[156,160] However, by 1989 cytochrome P450 related enzyme activity had been detected in more than 20 Mollusc species.[161] Efforts to use Mollusc MFO activity as an indicator of environmental pollution have not always been successful,[161,162] and it has been suggested that for the present fish may be the better choice as a monitoring tool.[163]

Vindimian and Garric found that AHH levels in fish declined with distance from a chemical plant on a polluted French river.[164] A Scandinavian study showed that cytochrome P4501A1 (ELISA estimate) and EROD activity in flat fish were correlated with a coastal contaminant gradient. Fish from another location had elevated cytochrome P4501A1 levels but normal EROD activity, a phenomenon that the authors felt may have been caused by inhibition of enzyme activity by high contaminant levels[157] or by the presence of enzyme inhibitors.[165] EROD activity and immunologically measured cytP1A1 levels in flounders taken from a coastal pollution gradient in Norway were related to PAH and PCDD levels along a pollution gradient.[166,167] Similarly encouraging relationships were found between 2,3,7,8-T_4CDD and EROD/CytP1A1 levels in pike taken from a pollution gradient in Lake Vanern in Sweden. The authors acknowledged, however, that other contaminants may have also acted as inducers.[168] Elevated EROD levels have also been associated with fish that have been exposed to effluents from Kraft pulp mills,[159,165] and effluent from petrochemical plants.[169] In-situ EROD assays have been used to show that

the zone of influence of effluents from bleached kraft mills can extend for more than 20-40 KM.[165]

Some interesting Finnish research has confirmed an earlier hypothesis of Payne and Fancey[170] by demonstrating the ability of unbleached pulp mill effluents to induce EROD activity in both laboratory (cultured fish cells) and field (feral and caged fish) experiments.[171,172] The inducing compounds were probably extracted from the wood stock or formed from precursors during the pulping process - although the inducers could also be present as contaminants in some of the process chemicals. Pesonen and Andersson have pointed out the importance of identifying the responsible components of the unbleached pulp mill effluent and of determining their chemical and toxic properties.[172] In the course of the latter study it was found that dehydroabietic acid, a resin acid, could inhibit the EROD response at environmentally realistic levels, which established a link between resin acids, which are an important component of pulp mill effluents, and the MFO endpoint. It is known that natural plant products, such as turpenoid hydrocarbons,[173] can be readily leached from bark chips (*Abies balsamea*) and spruce needles (*Picea glauca*) in a form that induces MFO activity in fish;[170,173] that observation prompted the original hypothesis that pulp mill effluents may contain high levels of natural MFO inducers.[170] There are many other examples of the use of in-situ MFO assays to detect and delimit pollution by xenobiotics, petroleum, and complex mixtures of organic contaminants.[94,173]

A survey of MFO activity in embryos from Great Lakes herring gull colonies, showed that AHH levels were higher in eggs from 2 of the colonies than in eggs taken from a control colony.[174] The AHH levels were correlated ($r>0.9$) with concentrations of 2,3,7,8-T_4CDD and pentachlorobenzene that had been measured in eggs taken from the same colonies a year earlier. Other potent MFO inducers such as PCDFs, PCDDs, and PCBs were not determined in the egg samples, and may well have been the causative agents. The level of mono-ortho PCBs and EROD activity in eggs from cormorant colonies in the Netherlands were significantly correlated ($r = 0.577$; $P<0.02$).[175]

a. Conclusions in-situ MFO assays

Much further research is needed before changes in the MFO levels of wild or exposed fish can be used to predict perturbations at the organism, community, and ecosystem levels. Van der Weiden et al.[148] stress the importance of caution in the interpretation of elevated cytochrome P4501A1 enzyme activities in fish. In addition to the levels of inducing chemicals, several environmental and physiological variables such as water temperature, contamination by metals, seasonal changes, age, size, sex, and steroid levels can affect CytP4501A1 activities in fish.[94,107,153,176-178] Based on a review of the literature Rattner et al.[95] concluded that the influence of age, sex, and other biological variables on MFO induction needs to be understood if data from such tests are to interpreted with confidence. The inherent variability of the in-situ MFO assays can be exacerbated by the movement of the organism into and out of the surveyed water. The choice of less mobile fish species can help to control that variable. Standardization of the sample collection and assay procedures should also help to reduce the sources of variability and improve the comparability of data from different laboratories.[93]

Although exposure of organisms to low levels of planar HAHs will usually, unless MFO inhibitors are present, result in the induction of elevated MFO levels, the converse is not necessarily true. Supportive analytical data and controlled induction experiments can be used to establish the causes of elevated enzyme levels in organisms from the wild. For sure, the presence of elevated MFO levels in a test organism is worthy of serious investigation, and in any such investigation the planar HAHs would be prime suspects. On balance cytochrome P4501A1 levels and MFO activity in fish and wildlife are potentially useful as early warning indicators of planar HAH contamination in waters affected by industrial and urban outfalls.

6. Immunoassay techniques for cytP4501A1

Immunochemical techniques are ideal for measuring the low levels of cytochrome P4501A1 that can occur in extracts of fish tissues and for rapidly screening large numbers of extracts. For example PABs have been used to detect small quantities

of cytochrome P-450c in cod larvae that had been treated with the water soluble fractions of North sea crude oil.[179,180] Both PABs and MABs that were raised against purified cytochrome P-450E from scup[181] and Atlantic cod[182,183] can selectively bind cytochrome P4501A1 and inhibit EROD and AHH activities in fish liver homogenates. The ABs were also able to bind cytochrome P4501A1 from a broad range of species that included rainbow trout, perch, and flounder.[93,184-186] This important observation means that anti-cod cytP4501A1 ABs can be used in immunoassays of cytP4501A1 from a variety of fish species.

The anti-cod PABs and the anti scup MAB were used together in a non-competitive ELISA for the detection of induced cytochrome P4501A1.[187] The PABs were used as the capture ABs in the ELISA and the MABs were used to sandwich the captured cytochrome. An enzyme labelled anti-mouse AB was then used to measure the amount of MAB in the "sandwich". The immunochemical techniques (ELISA and Western Blotting) were used to demonstrate a dose response relationship for the induction of cytochrome P4501A1 in cod larvae and young fish that were treated with soluble oil components.

Goksoyr et al. later used an indirect version of the non-competitive ELISA to more thoroughly study the effects of the water soluble components of crude oil on cod larvae and young fish (Fig. 26).[188] In this promising assay a liver microsome preparation is used to coat the microtitre plate wells. The remainder of the assay is a fairly standard EIA procedure. The primary incubation period can be for two hours at 37°C or overnight at 4°C. The ELISA technique is robust; the samples are resistant to endogenous proteolysis, boiling, and can be stored at 37°C for one hour without drastically affecting the assay's results.[93,185,188] The ELISA for cytP4501A1 has been evaluated in several recent laboratory and field studies.[93,157,180,183,184,189] Immunological techniques have been used to measure cytP4501A1 induction in fish from PCB and 2,3,7,8-T_4CDD contaminated water.[190,191]

Collier et al. compared the ability of the ELISA technique and the AHH/EROD assays to measure cytochrome P4501A1 levels in a large sample set (1300 analyses) that was composed of samples from 11 fish species.[185] The good agreement between the methods, supports the use of the ELISA as a complementary means of monitoring CytP4501A1 levels in large scale studies. Although as pointed out by Goksoyr and Forlin a statistical analysis of the data suggests that the ELISA in its present form is most useful as a semi-quantitative screening tool.[93] One of the ELISA technique's main advantages is the ability to provide an accurate measurement of CytP4501A1 levels even in cases where excess levels of the inducing chemical, or other interferences, has inhibited MFO activity. For example, the ELISA was able to detect cytochrome P4501A1 induction in flatfish that had been exposed to high levels of PCB contamination but yet gave negative results in the EROD assay.[157] The ELISAs for Cyt P4501A1 share most of the general advantages of IA techniques such as speed, simplicity, and low cost. The ELISA's ability to detect the induction of cyt P4501A1 in small samples could prove useful because of interest in the effects of environmental contaminants on the early life stages of aquatic organisms. Popularity of the technique however will depend on wider distribution of reagent ABs. As was the case for environmental IAs that distribution might be well handled by specialized companies, although exchanges of reagents via the scientific network should also encourage wider use.

E. Toxic response bioassays

1. Porphyria induction

Porphyria, a disorder of porphyrin metabolism in the liver, is typified by elevated porphyrin levels and altered porphyrin profiles, and can be induced by exposure to the HAHs.[192] Porphyria induction is believed to be mediated by the Ah receptor; 2,3,7,8-T_4CDD is a potent inducer of hepatic porphyria. Strik et al.[192] have suggested that humans be screened for urinary porphyrin levels as an inexpensive way of detecting exposure to planar HAHs. Elevated porphyria levels can be readily induced in cultured chicken liver cells.[193]

Kennedy et al.[129] developed a useful bioassay for planar HAHs that is based on the rapid detection of porphyria in chicken embryos cells. The cultured liver cells were exposed in 48 well culture plates. After a 24 hour exposure the total porphyrin content of each well was determined as described in Fig. 23; the described method was adapted from Granick et al.[194] Use of the method to screen egg extracts from several wild bird colonies showed that porphyria induction was a less sensitive response parameter than EROD activity although the data from both bioassays followed the same rank order. The authors suggest that the porphyria assay could complement the EROD assay in some applications, since it appears that planar HAHs that are poor inducers of EROD can be more potent inducers of porphyrin production. The assay was later modified for use with a fluorescence microtitre plate reader which made possible the measurement of porphyrin levels in plates that had previously been used for the determination of EROD.[130] A dose response curve for the modified porphyrin induction assay is shown in Fig. 24.

2. Keratinization and flat cell induction

The exposure of axenic cultures of human epidermal cells to TCDD causes several changes to occur: the cells become more stratified and keratinized, and the number of underlying basal cells decreases.[195] Those are in vitro manifestations of chloracne which is the most obvious symptom of exposure to high levels of toxic PCDDs in humans. Exposure to 2,3,7,8-T_4CDD increases the proportion of basal cells that undergo final differentiation which causes the numbers of basal cells to decline. The cells that begin terminal differentiation cease division, and are shifted to the upper layers where they become keratinized. Knutson and Poland[196] demonstrated that low levels of 2,3,7,8-T_4CDD could induce keratinization in a line of mouse epithelial cells (XB) when the cells were cultured at high density. Keratinization does not occur spontaneously in high density cultures of XB cells. The effect was proportional to the dose of 2,3,7,8-T_4CDD, and was readily observable when the cultures were stained with Rhodanile blue. Similar differentiation also occurs spontaneously when the XB cells are cultured at low density in the absence of 2,3,7,8-T_4CDD. The ability of HAHs and related compounds to induce

keratinization parallels their affinities for the Ah receptor. As little as 16 pg of 2,3,7,8-T_4CDD could induce the keratinization response.

The keratinization assay has been used to screen benzene extracts of 9 soot samples from an office building in which a PCB fire had occurred.[197,198] The GC-MS estimates of the total PCDF levels and the bioassay data were well correlated (r=0.89). The XB phenotype, however, proved to be unstable during repeated sub-culturing, with the result that the working cultures had to be periodically renewed from the master stocks. After about 25 passages of the cells the XB cell line had acquired the ability to grow to higher cell densities than could the low passage cultures.[199] The response of the cells (now called XBF cells) to 2,3,7,8-T_4CDD also changed: they no longer became either terminally differentiated or keratinized, and they were more sensitive to the inhibition of cell proliferation that occurs in confluent cultures. The treated cultures developed a flat "cobblestone" appearance whereas the control cultures had a distinctly fusiform morphology. The XBF cells had also lost their requirement for medium that had been exposed to a layer of feeder cells (3T3 fibroblast feeder cells). The use of conditioned medium, has however been continued in the sensitive epithelial cell, or flat cell, bioassay for PCDDs/PCDFs that shall now be described.

The XBF cell line can be maintained as described by Gierthy and Crane[199] with sub-cultures being prepared as the cells became confluent (7-14 days). The test procedure is outlined in Fig. 27. After 14 days of growth the control cultures have a characteristic fusiform morphology: these cultures consist of multiple layers of spindle shaped cells.[200] The 2,3,7,8-T_4CDD treated cultures become confluent by 7 - 10 days after the initial exposure, and by 14 days develop the typical "cobblestone" like appearance: those cultures are composed of a single layer of flat cells. A typical calibration curve for cell numbers versus dose of 2,3,7,8-T_4CDD had a good response from $< 10^{-12}$ to 10^{-8} M (Fig. 28). Cell numbers are routinely estimated in the bioassay from the intensity of the Giemsa stained wells. The flat cell effect may be observed by phase contrast microscopy.[201]

The epithelial cell bioassay is most specific for PCDDs and PCDFs,[202] and is extremely sensitive to 2,3,7,8-T_4CDD: DL of 10^{-11} M or 3.2 pg/mL; the DL for T_4CDF was 32 pg/mL. PCBs were less potent, by a factor of 10^4 - 10^6, in the assay. Overall the assay seems to be most sensitive to the more toxic PCDD/PCDF congeners, although its response to a broader range of HAHs needs to be investigated.[74] The assay has been used to screen extracts of 10 soot samples from the scene of a PCB fire. The bioassay data agreed with GC-MS estimates of the PCDF contents of the extracts: the log transformed data had a correlation coefficient of 0.82. The bioassay was also used to successfully screen surface wipes of contaminated walls,[203,204] and fish and sediment.[204] The fish samples needed only minimal clean-up: removal of the lipids by acid treatment and subsequent neutralization of the extract. The bioassay accurately predicted the T_4CDD content of the extracts which ranged from 2.7 ppt - 268 ppt. The same low level of clean-up sufficed for the preparation of turtle tissue for screening by the bio-assay.[205] The epithelial cell bioassay is the only screening test for PCDD like compounds that works well with minimally enriched fish samples. Sediment extracts, however, required more extensive clean-up. The apparent ability of the epithelial cell bioassay and the in vitro keratinization assay to selectively detect the more toxic PCDD/PCDF congeners, while ignoring compounds such as B(a)P and 3-MC is an advantage over the other Ah receptor mediated assays.[198,203]

IV. Conclusions

The two main groups of screening assays for dioxin like molecules - the LBAs and the bioassays - include valuable tests that can be used to eliminate negative samples, to rank positive samples, or to confirm the results of conventional analyses. The bioassays have additional value since they also integrate and quantify the biological/toxic potencies of complex mixtures of planar HAHs, which can be helpful in risk assessments and in the formulation of environmental regulations.

Despite efforts to optimize their performances at low analyte levels, it remains to be established whether the LBAs can be used to detect planar HAHs accurately and precisely at the trace levels that are of current environmental concern. With the exception of Bradfield and Poland's[66] receptor binding assay and Ohmicron's EIA for PCBs, the sensitivities of the current LBAs for planar HAHs seem to be inadequate for the detection of trace quantities of analyte. Improvements in the sensitivities of analytical instruments and concerns about the effects of long term exposure to low contaminant doses have driven the desirable DL for the planar HAHs to the ultra-trace (low pg - fg) level for many matrices. A screening strategy could be devised if it were possible to reach consensus as to the concentrations, below which planar HAHs are unlikely to cause chronic biological effects. Samples that contain less than the threshold level could be eliminated from the analytical chain. The main proviso would then become the screening test's performance at the cut-off concentration. Unfortunately, the absence of accepted threshold values, means that the screening tests are, perhaps unrealistically, expected to match the sensitivity of the most sophisticated analytical instruments. For the present the LBAs' ability to perform well at trace levels will largely determine whether they become more widely used. The prospects for many of the LBAs would be improved by a reduction of 1-2 orders of magnitude in their detection limits. However the current LBAs should be able to detect planar HAHs in moderately to highly contaminated samples such as are commonly taken from industrial facilities, waste sites, and from the impact zone of accidental discharges.

As a rule the need for sample clean-up detracts from a screening technique's appeal. There has been little success in reducing the amount of clean-up for high lipid samples without adversely affecting the LBAs' low level performance. Immunoaffinity chromatography, or the use of automated and simplified clean-up systems such as SFE/C, may help solve that problem. The proven ability of the EIAs for PCBs to detect their target analytes in natural water suggests that aqueous samples will probably need less extensive clean-up.

Interest in the MFO bioassays has grown because of their ability to complement traditional analytical methods when used as in-vitro screening assays, and act as early warning indicators and monitors of pollution events when used in-situ. The inconvenience of having to maintain cell lines or test organisms is more than compensated by the ability of the MFO bioassays to provide a biological perspective on conventional analytical data. Similarly, the main attraction of the toxic response bioassays is their ability to express a sample's planar HAH content in terms of toxic potencies. Further research will establish whether the tendency of both types of bioassay to respond to Ah active compounds other than HAHs causes problems with real life samples. It may be possible to exploit that tendency so that analytical efforts are directed towards compounds that have been previously ignored or whose presence was not suspected. Many of the bioassays are sensitive enough to detect planar HAHs at environmentally realistic levels. Normally there should be no need to clean-up water or effluent samples, unless they contain cytotoxic compounds. High lipid matrices, however, would be expected to require extraction and some fractionation.

Several of the screening tests for dioxin like compounds are ready for serious validation using biota and environmental matrices. Much effort has already gone into the validation of the in-situ MFO induction assays. In the interim use of the screening tests as research tools is encouraged, as it will generate useful scientific data while speeding validation. Successful validation studies will not only help to build confidence in the screening tests, and promote their wider use, but will also document and clarify any tendencies towards biased or erroneous results. Once validated a screening test can be added to the array of techniques used to unravel and document the environmental chemistry of the planar HAHs.

REFERENCES

(1) Kuehl, D.W.; Butterworth, B.C.; DeVita, W.M.; Sauer, P. Environmental contamination by polychlorinated dibenzo-p-dioxins and dibenzofurans associated with pulp and paper mill discharges. *Biomed. Environ. Mass. Spec.* **1987**, *14*, 443-447.

(2) Safe, S. Polychlorinated Dibenzo-Para-Dioxins and Related Compounds - Sources, Environmental Distribution and Risk Assessment. *Environ. Carcin. & Ecotoxicol.* **1991**, *9*, 261-302.

(3) Hunter, W.M.; Corrie, J.E.T. *Immunoassays for Clinical Chemistry*, Churchill Livingstone: Edinburgh, **1983** pp.701.

(4) Tijssen, P. *Practice and Theory of Enzyme Immunoassays*, Elsevier: Amsterdam, **1985**, pp. 549.

(5) Voller, A.; Bartlett, A.; Bidwell, D. *Immunoassays for the 80s*, University Park Press: Baltimore, **1981**, pp.508.

(6) Atassi, M.Z.; Van Oss, C.J.; Absolom, D.R. *Molecular Immunology*, Marcel Dekker: New York, **1984**, pp. 725.

(7) Van de Water, C.; Haagsma, N. Sensitive streptavidin-biotin enzyme-linked immunosorbent assay for rapid screening of chloramphenicol residues in swine muscle tissue. *J. Assoc. Off. Anal. Chem.*, **1990**, *73*, 534-540.

(8) Meyer, H.H.D.; Sauerwein, H.; Mutayoba, B.M. Immunoaffinity chromatography and a biotin-strepavidin amplified enzymeimmunoassay for sensitive and specific estimation of estradiol-17b. *J. Steroid Biochem.*, **1990**, *35*, 263-269.

(9) Duchatel, J.P.; Maghuin-Rogister, G. Free and conjugated zeranol residues determined by radioimmunoassay in urine and plasma of calves treated with forplix. *Ann. Rech. Vet.* **1985**, *16*, 93-97.

(10) Rohner, P.; Schallibaum, M.; Nicolet, J. Detection of penicillin G and its benzylpenicilloyl (BPO) - derivatives in cow milk and serum by means of an ELISA. *J. Food Protect.* **1985**, *48*, 59-62.

(11) Degand, G.; Bernes-Duyckaerts, A.; Maghuin-Rogister, G. Determination of clenbuterol in bovine tissues and urine by enzyme immunoassay. *J. Agric. Food Chem.* **1992**, *40*, 70-75.

(12) Sherry, J.P. Environmental chemistry - the immunoassay option. *CRC Crit. Rev. Anal. Chem.* **1992**, *23*, 217-300.

(13) Vanderlaan, M.; Watkins, B.E.; Stanker, L. Environmental monitoring by immunoassays. *Environ. Sci. Technol.* **1988**, *22*, 247-254.

(14) Van Emon, J.; Seiber, J.N.; Hammock, B.D. Immunoassay techniques for pesticide analysis, In *Analytical Methods for Pesticides and Plant Growth Regulators. Vol. XV11, Advanced Analytical Techniques*, Ed. J. Sherma, Acad. Press Inc.: New York, **1989** 217-263.

(15) Newsome, W.H. Potential and advantages of immunochemical methods for analysis of foods. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 919-923.

(16) Albro, P.W.; Chae, K.; Luster, M.; McKinney, J.D.; Chaudhary, S.; Clark, G.; Fawkes, J.; Corbett, J. Radioimmunoassay for 2,3,7,8- tetrachlorodibenzo-p-dioxin. *Environ. Health Perspec.* **1977**, *20*, 247.

(17) Albro, P.W.; Luster, M.I.; Chae, K.; Chaudhary, S.K.; Clark, G.; Lawson, L.D.; Corbett, J.T.; McKinney, J.D. A radioimmunoassay for chlorinated dibenzo-p-dioxins. *Toxicol. Appl. Pharmacol.* **1979**, *50*, 137-146.

(18) Poland, A.; Glover, E.; Kende, A.S. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. *J. Biol. Chem.* **1976**, *251*, 4936-4946.

(19) Bradfield, C.A.; Kende, A.S.; Poland, A. Kinetic and equilibrium Ah receptor-ligand binding: use of [125I] 2-iodo-7,8-dibromodibenzo-p-dioxin. *Molec. Pharmacol.* **1988**, *34*, 229-237.

(20) Sherma, J. Pesticides. *Anal. Chem.* **1989**, *61*, 153R-165R.

(21) Klausner, A. Immunoassays flourish in new markets. *Biotechnol.* **1987**, *5*, 551-556.

(22) Yalow, R.S.; Berson, S.A. Assay of plasma insulin in human subjects by immunological methods. *Nature* **1959**, *184*, 1648-1649.

(23) D.J. McCormick; H.E. Schmitz Radioimmunoassay, In *Molecular Immunology* Eds. M.Z. Atassi, C.J. Van Oss, and D.R. Absolom; Marcel Dekker: New York, **1984**; Chapter 19.

(24) Midgely, A.R.; Niswender, G.D. Radioimmunoassay of steroids. *Acta Endocrin.* **1970**, *147*, 320-331.

(25) Chapman, D.I. Radioimmunoassay. *Chem. in Britain* **1979**, *15*, 439-446.

(26) Clifford, M.N. The history of immunoassays in food analysis, In *Immunoassays in Food Analysis* Eds. B.A. Morris and M.N. Clifford; Elsevier: London; **1985**; pp. 3-19.

(27) Morgan, M.R.A. Mycotoxin immunoassays (with special reference to ELISAs). *Tetrahedron* **1989**, *45*, 2237-2249.

(28) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual.* Cold Spring Harbour Laboratory: NY; **1988**; pp. 726.

(29) Campbell, A.M. *Monoclonal Antibody Technology*, Elsevier: Amsterdam; **1984**; pp. 265.

(30) Chard, T. *An Introduction to Radioimmunoassay and Related Techniques*, Elsevier: Amsterdam; **1987**; pp. 274.

(31) Bolton, A.E. *Radioiodination techniques*, Amersham Corporation: Arlington Heights, Illinois; **1978**; pp.71.

(32) Engvall, E.; Perlmann, P. Enzyme-linked immunoadsorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochem.* **1971**, *8*, ???-871.

(33) Van Weeman, B.K.; Schuurs, A.H.W.M. Immunoassay using antigen-enzyme conjugates. *FEBS Lett.* **1971**, *15*, ???-232.

(34) Vanderlaan, M.; Stanker, L.H.; Watkins, B.E.; Petrovic, P.; Gorbach, S. Improvement and application of an immunoassay for screening environmental samples for dioxin contamination. *Environ. Toxicol. and Chem.* **1988**, *7*, 859-870.

(35) Sherry, J.P.; ApSimon, J.W.; Collier, T.L.; Albro, P.W. Use of dimethyl sulfoxide as solubilization agent in the detection of 2,3,7,8-TCDD by radioimmunoassay. *Chemosphere* **1990**, *20*, 1409-1416.

(36) Sherry J.P.; ApSimon, J.; Collier, T.; Afghan, B.; Albro, P. The use of radioimmunoassay for the detection of PCDDs in fish samples. *Chemosphere* **1989**, *19*, 255-261.

(37) Luster, M.I.; Albro, P.W.; Clark, G.; Chae, K.; Chaudhary, S.K.; Lawson, L.D.; Corbett, J.T.; McKinney, J.D. Production and characterization of antisera specific for chlorinated biphenyl species: initiation of a radioimmunoassay for Aroclors. *Toxicol. Appl. Pharmacol.* **1979**, *50*, 147-155.

(38) Albro, P.W.; Luster, M.I.; Chae, K.; Clark, G.; McKinney, J.D. Radioimmunoassay of chlorinated dibenzo-p-dioxins. *Methods Enzymol.* **1982**, *84*, 619-628.

(39) McKinney, J.; Albro, P.; Luster, M.; Corbett, B.; Schroeder, J.; Lawson, L. Development and reliability of a radioimmunoassay for 2,3,7,8-tetrachlorodibenzo-p-dioxin. In *Chlorinated Dioxins and Related Compounds*, Eds. O. Hutzinger et al.; Pergamon Press: Oxford and New York, **1982**; pp. 67-75.

(40) Sherry, J.P.; Albro, P.W. Radioimmunoassay of chlorinated dioxins: use of [3H]-labelled 2,3,7,8-tetrachlorodibenzo-p-dioxin as radioligand. *Presented at Dioxin '90 Bayreuth, Germany*. September **1990**.

(41) Stanker, L.; Watkins, B.; Vanderlaan, M.; Budde, W.L. Development of an immunoassay for chlorinated dioxins based on a monoclonal antibody and an enzyme linked immunosorbent assay (ELISA). *Chemosphere* **1987**, *16*, 1635-1639.

(42) Stanker, L.H.; Watkins, B.; Rogers, N.; Vanderlaan, M. Monoclonal antibodies for dioxin: antibody characterization and assay development. *Toxicology* **1987**, *45*, 229-243.

(43) Vanderlaan, M.; Van Emon, J.; Watkins, B.; Stanker, L. Monoclonal antibodies for the detection of trace chemicals. In *Pesticide Science and Biotechnology*, Eds. Greenhalgh, R. and T.R. Roberts; Blackwell Scientific: Ottawa, **1987** pp. 597-602.

(44) Luster, M.I.; Albro, P.W.; Chae, K.; Lawson, L.D.; Corbett, J.D.; McKinney, J.D. Radioimmunoassay for quantitation of 2,3,7,8-tetrachlorodibenzofuran. *Anal. Chem.* **1980**, *52*, 1497-1500.

(45) Silberhorn, E.M.; Glauert, H.P.; Robertson, L.W. Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *Critic. Rev. Toxicol.* **1990**, *20*, 439-496.

(46) Abelson, P.H. Excessive fear of PCBs. *Science* **1991**, *253*, 361.

(47) Newsome, W.H.; Shields, J.B. Radioimmunoassay of PCBs in milk and blood. *Intern. J. Environ. Anal. Chem.* **1981**, *10*, 295-304.

(48) Franek, M.; Hruska, K.; Sisak, M.; Diblikova, I. Development of a microcolumn radioimmunoassay for screening of polychlorinated biphenyls in milk and in animal fats. *J. Agric. Food Chem.* **1992**, *40*, 1559-1565.

(49) Chamerlik-Cooper, M.A.; Carlson, R.E.; Harrison, R.O. Determination of PCBs in soil by enzyme immunoassay. *Presented at Second International Symposium, Field Screening Methods for Hazardous Waster Site Investigations, US EPA*: Las Vegas, February, **1991**.

(50) Mapes, J.P.; McKenzie, K.D.; Stewart, T.N.; McClelland, L.R.; Studabaker, W.B.; Manning, W.B.; Friedman, S.B. An on-site immunoassay for detecting PCB in soil. *Bull. Environ. Contam.* **1993**, *50*, 219-225.

(51) Ohmicron Commercial Product Information Kit; supplied as a personal communication. Newtown P.A., U.S.A. **1993**.

(52) Hammock, B.D. Applications of immunochemistry in crop protection and biotechnology. In *Biotechnology for Crop Protection*, Eds. P.A. Hedin; J.J. Menn; R.M. Hollingworth; ACS: Washington, **1988**; pp. 298-305.

(53) Roberts, E.A.; Shear, N.H.; Okey, A.B.; Manchester, D.K. The Ah receptor from rodent to human tissues. *Chemosphere* **1985**, *14*, 661-674.

(54) Hankinson, O. Research on the arylhydrocarbon (dioxin) receptor is primed to take off. *Arch. Biochem. Biophys.* **1993**, *300*, 1-5.

(55) Denison, M.S.; Wilkinson, C.F.; Okey, A.B. Ah receptor for 2,3,7,8-tetrachloro-p-dioxin: comparative studies in mammalian and non-mammalian species. *Chemosphere* **1986**, *15*, 1665-1672.

(56) Roberts, E.A.; Vella, L.M.; Golas, C.L.; Dafoe, L.A.; Okey, A.B. Ah receptor in spleen of rodent and primate species: detection by binding of 2, 3,7,8-tetrachlorodibenzo-p-dioxin. *Can. J. Physiol. Pharmacol.* **1989**, *67*, 594-600.

(57) Lorenzen, A.; Okey, A.B. Detection and characterization of [^{3}H]2,3,7,8-tetrachlorodibenzo-p-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. *Toxicol. Appl. Pharmacol.* **1990**, *106*, 53-62.

(58) Roberts, E.A.; Golas, C.L.; Okey, A.B. Ah receptor mediating induction of aryl hydrocarbon hydroxylase: detection in human lung by binding of 2,3,7,8-[^{3}H]tetrachlorodibenzo-p-dioxin. *Cancer Research* **1986**, *46*, 3739-3743.

(59) Kleman, M.I.; Overvik, E.; Mason, G.G.F.; Gustafsson, J.A. Invitro activation of the dioxin receptor to a DNA-binding form by food-borne heterocyclic amines. *Carcinogenesis* **1992**, *13*, 1619-1624.

(60) Okey, A.B.; Bondy, G.P.; Mason, M.E.; Kahl, G.F.; Eisen, H.J.; Guenthner, T.M.; Nebert, D.W. Regulatory gene product of the Ah locus. *J. Biol. Chem.* **1979**, *254*, 11636-11648.

(61) Poland, A.; Glover, E. Variation in the molecular mass of the Ah receptor among vertebrate species and strains of rats. *Biochem. Biophys. Res. Commun.* **1987**, *146*, 1439-1449.

(62) Nebert, D.W.; Eisen, H.J.; Hankinson, O. The Ah receptor: binding specificity only for foreign chemicals? *Biochem. Pharmacol.* **1984**, *33*, 917-924.

(63) Eisen, H.J.; Hannah, R.R.; Legraverend, C.; Okey, A.B.; Nebert, D. The Ah receptor: controlling factor in the induction of drug-metabolizing enzymes by certain chemical carcinogens and other environmental pollutants. In *Biochemical Actions of Hormones Vol. 10,* Ed. G. Litwack; Academic Press: New York; **1983**; pp. 227-257

(64) Vickers, A.E.M.; Sloop, T.C.; Lucier, G.W. Mechanism of action of toxic halogenated aromatics. *Environ. Health. Perspect.* **1985**, *59*, 121-128.

(65) Hutzinger, O.; Olie, K.; Lustenhouwer, J.W.A.; Okey, A.B.; Bandiera, S.; Safe, S. Polychlorinated dibenzo-p-dioxins and dibenzofurans: a bioanalytical approach. *Chemosphere* **1981**, *10*, 19-25.

(66) Bradfield, C.A.; Poland, A. A competitive binding assay for 2,3,7,8-tetrachlorodibenzo-p-dioxin and related ligands of the Ah receptor. *Molec. Pharmacol.* **1988**, *34*, 682-688.

(67) Hanberg, A.; Waern, F.; Asplund, L.; Haglund, E.; Safe, S. Swedish dioxin survey: determination of 2,3,7,8-TCDD toxic equivalent factors for some polychlorinated biphenyls and naphthalenes using biological tests. *Chemosphere* **1990**, *20*, 1161-1164.

(68) Bunce, N.J.; Logan, R.; Schneider, U.A. Development of a rapid screening assay for PCDDs and PCDFs. *Chemosphere* **1990**, *20*, 1417-1422.

(69) Gasiewicz, T.A.; Neal, R.A. The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-p-dioxin using hydroxylapatite. *Anal. Biochem.* **1982**, **124**, 1-11.

(70) Safe, S. Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. *Ann. Rev. Pharmacol. Toxicol.* **1986**, *26*, 371-399.

(71) Neubert, D.; Golor, G.; Neubert, R. TCDD-toxicity equivalencies for PCDD/PCDF congeners: prerequisites and limitations. *Chemosphere* **1992**, *25*, 65-70.

(72) Safe, S.; Hutzinger, O.; Hill, T.A. *Environmental Toxin Series, 3: Polychlorinated dibenzo-p-dioxins and -furans (PCDDs/PCDFs): Sources and Environmental Impact, Epidemiology, Mechanisms of Action, Health Risks*, Springer Verlag: Berlin, **1990**; pp. 145.

(73) Safe, S. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. *Critic. Rev. Toxicol.* **1984**, *13*, 319-395.

(74) Goldstein, J.A.; Safe, S. *Topics in Environmental Health: Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products*, Eds. R.D. Kimbrough and A.A. Jensen; North Holland: NY; **1989**; Vol. 4, Chapter 9.

(75) Nebert, D.W.; Puga, A.; Vasiliou, V. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. *Immunomod. Drugs* **1993**, *685*, 624-640.

(76) Safe, S.H.; Gasiewicz, T.; Whitlock, J.P., Jr. UAREP-Report on health aspects of polychlorinated dibenzo-p-dioxins (PCDDs) and Polychlorinated dibenzofurans (PCDFs); mechanism of action, In *Polychlorinated Dibenzo-p-Dioxins and -Furans (PCDDs/PCDFs): Environmental Toxin Series 3*, Eds. S. Safe; O. Hutzinger; T.A. Hill; Springer Verlag Berlin: **1990**; 61-91.

(77) Parkinson, A.; Safe, S. Aryl hydrocarbon hydroxylase induction and its relationship to the toxicity of halogenated aryl hydrocarbons. *Toxicol. Environ. Chem. Rev.* **1981**, *4*, 1-45.

(78) De Voogt; P.; Wells, D.E.; Reutergardh, L.; Brinkman, U.A.Th. Biological activity, determination and occurrence of planar, mono- and diorhto PCBs. *Intern, J. Environ. Anal. Chem.* **1990**, *40*, 1-46.

(79) Okey, A.B. Enzyme induction in the cytochrome P-450 system. *Pharmac. Ther.* **1990**, *45*, 241-298.

(80) Safe, S.; Mason, G.; Sawyer, T.; Zacharewski, T.; Harris, M.; Yao, C.; Keys, B.; Farrell, K.; Holcomb, M.; Davis, D.; Safe, L.; Piskorska-Pliszczynnska, J.; Leece, B.; Denomme, M.A.; Hutzinger, O.; Thoma, H.; Chittim, B.; Madge, J.; Development and validation of in vitro induction assays for toxic halogenated aromatic mixtures: a review. *Toxicol. Indust. Health* **1989**, *5*, 757-775.

(81) Nebert, 1989 The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. *CRC Crit. Rev. Toxicol.* **1989**, *20*, 153-174.

(82) Rozman, K.K. Search for the mechanism of toxicity of dioxins - a lesson in toxicology. *Exper. Toxicol. Pathol.* **1992**, *44*, 473-480.

(83) Rozman, K.; Roth, W.L.; Greim, H.; Stahl, B.U.; Doull, J. Relative potency of chlorinated dibenzo-p-dioxins (CDDS) in acute, subchronic and chronic (carcinogenicity) toxicity studies - implications for risk assessment of chemical mixtures. *Toxicol.* **1993**, *77*, 39-50.

(84) Dickson, L.C.; Buzik, S.C. Health risks of dioxins - a review of environmental and toxicological considerations. *Vet.& Human Toxicol.* **1993**, *35*, 68-77.

(85) Eadon, G.; Kaminsky, L.; Silkworth, J.; Aldous, K.; Hilker, D.; Smith, R.; O'Keefe, P.; Gierthy, J.; Hawley, J.; Kim, N.; DeCaprio, A. Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. *Environ. Health Perspect.* **1986**, *70*, 221-227.

(86) Safe, S. Polychlorinated biphenyls (PCBs), Dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors. *CRC Crit. Rev. Toxicol.* **1990**, *21*, 51-88.

(87) Neubert, D.; Golor, G.; Hagenmaier, H. Toxic TCDD-equivalents, problems and applicability for risk assessment, *Presented at Health Effects and Safety Assessment of Dioxins and Furans*, Karlsruhe: Ger. **15-17 Jan. 1990**.

(88) DeVito, M.J.; Maier, W.E.; Diliberto, J.J.; Birnbaum, L.S. Comparative ability of various PCBs, PCDFs, and TCDD to induce cytochrome-P450 1A1 and 1A2 activity following 4 weeks of treatment. *Fund. Appl. Toxicol.* **1993**, *20*, 125-130.

(89) Bannister, R.; Davis, D.; Zacharewski, T.; Tizard, I.; Safe, S. Aroclor 1254 as a 2,3,7,8-tetrachlorodibenzo-p-dioxin antagonist: effects on enzyme induction and immunotoxicity. *Toxicol.* **1987**, *46*, 29-42.

(90) Bosveld, B.A.T.C.; Van den Berg, M.; Theelen, R.M.C. Assessment of the EROD inducing potency of 11 2,3,7,8- substituted PCDD/Fs and 3 coplanar PCBs in the chick embryo. *Chemosphere* **1992**, *258*, 911-916.

(91) Safe, S. Polychlorinated biphenyls - human health effects. *Environ. Sci. Res.* **1988**, *37*, 51-69.

(92) Williams, L.L.; Giesy, J.P. Relationships among concentrations of individual polychlorinated biphenyl (PCB) congeners, 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents (TCDD-EQ), and rearing mortality of chinook salmon (*Oncorhynchus tshawytscha*) eggs from Lake Michigan. *J. Great Lakes Res.* **1992**, *18*, 108-124.

(93) Goksoyr, A.; Forlin, L. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicol.* **1992**, *22*, 287-311.

(94) Payne, J.F. Mixed-function oxygenases in biological monitoring programs: review of potential usage in different phyla of aquatic animals, In *Ecotoxicological Testing for the Marine Environment*, Eds. Personne, G.; Jaspers, E.; Claus, C.; State Univ.: Ghent & Inst. Mar. Sci. Res. **1984**; Vol. 1, 625-655.

(95) Rattner, B.A.; Hoffman, D.J.; Marn, C.M. Use of mixed-function oxygenases to monitor contaminant exposure in wildlife. *Environ. Toxicol. Chem.* **1989**, *8*, 1093-1102.

(96) Brunstrom, B.; Broman, D.; Naf, C. Toxicity and EROD-inducing potency of 24 polycyclic aromatic hydrocarbons (PAHs) in chick embryos. *Arch. Toxicol.* **1991**, *65*, 485-489.

(97) Lewis, D.F.V.; Ioannides, C.; Parke, D.V. Structural requirements for substrates of cytochromes P-450 and P-448. *Chem. Biol. Interact.* **1987**, *64*, 39-60.

(98) Andersson, T.; Forlin, L. Regulation of the cytochrome-P450 enzyme system in fish. *Aquatic Toxicol.* **1992**, *24*, 1-19.

(99) Stegeman, J.J. Nomenclature for hydrocarbon-inducible cytochrome-P450 in fish. *Mar. Environ. Res.* **1992**, *34*, 133-138.

(100) Safe, S. Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. *Chemosphere* **1987**, *16*, 791-802.

(101) Bunce, N.J.; Landers, J.P.; Safe, S. Kinetic models for association of 2,3,7,8-tetrachloro-p-dioxin with the Ah receptor. *Arch. Biochem. Biophys.* **1988**, *267*, 384-397.

(102) Jansing, R.L.; Shain, W. Aryl hydrocarbon hydroxylase induction in adult rat hepatocytes in primary culture by several chlorinated aromatic hydrocarbons including 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fund. Appl. Toxicol. 1985; 5; 713-720.

(103) Bandiera, S.; Sawyer, T.; Romkes, M.; Zmudzka, B.; Safe, L.; Mason, G.; Keys, B.; Safe, S. Polychlorinated dibenzofurans (PCDFs): effects of structure on binding to the 2 ,3,7,8-TCDD cytosolic receptor protein, AHH induction and toxicity. *Toxicology* **1984**, *32*, 131-144.

(104) Safe, S.; Bandiera, S.; Sawyer, T.; Robertson, L.; Safe, L.; Parkinson, A.; Thomas, P.E.; Ryan, D.E.; Reik, L.M.; Levin, W.; Denomme, M.A.; Fujita, T. PCBs: structure-function relationships and mechanism of action. *Environ. Health Perspect.* **1985**, *60*, 47-56.

(105) Mason, G.; Sawyer, T.; Keys, B.; Bandiera, S.; Romkes, M.; Piskorska-Pliszczynska, J; Zmudzka, B.; Safe, S. Polychlorinated dibenzofurans (PCDFs): correlation between in vivo and in vitro structure-activity relationships. *Toxicol.* **1985**, *37*, 1-12.

(106) Safe, S. Polychlorinated dibenzofurans: environmental impact, toxicology, and risk assessment. *Hazard Assess. Chem.* **1990**, *7*, 283-327.

(107) Haux, C.; Forlin, L. Biochemical methods for detecting effect of contaminants in fish. *Ambio* **1988**, *17*, 376-380.

(108) Taylor, M.J.; Lucier, G.W.; Mahler, J.F.; Thompson, M.; Lockhart, A.C.; Clark, G.C. Inhibition of acute TCDD toxicity by treatment with anti-tumor necrosis factor antibody or dexamethasone. *Toxicol. Appl. Pharmacol.* **1992**, *117*, 126-132.

(109) Niwa, A.; Kumaki, K.; Nebert, D.W. Induction of aryl hydrocarbon hydroxylase activity in various cell cultures by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Mol. Pharmacol.* **1975**, *11*, 399-408.

(110) Bradlaw, J.A.; Garthoff L.H.; Hurley, N.E.; Firestone, D. Aryl hydrocarbon hydroxylase activity of twenty three halogenated dibenzo-p-dioxins. *Toxicol. Appl. Pharmacol.* **1976**, *37*, 119.

(111) Nebert, D.W.; Gelboin, H.V. Substrate inducible microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. *J. Biol. Chem.* **1968**, *243*, 6242-6247.

(112) Bradlaw, J.A.; Casterline, J.L. Jr. Induction of enzyme activity in cell culture: a rapid screen for detection of planar polychlorinated organic compounds. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 904-916.

(113) Bradlaw, J.A.; Garthoff, L.H.; Hurley, N.E.; Firestone, D. Comparative induction of aryl hydrocarbon hydroxylase activity in vitro by analogues of dibenzo-p-dioxin. *Fd. Cosmet. Toxicol.* **1980**, *18*, 627-635.

(114) Trotter, W.J.; Young, S.J.; Casterline, J.L.; Bradlaw, J.A.; Kamops, L.R. Induction of aryl hydrocarbon hydroxylase activity in cell cultures by Aroclors, residues from Yusho oil samples, and polychlorinated biphenyl residues from fish samples. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 838-841.

(115) Casterline, J.J.; Bradlaw, J.A.; Puma, B.J.; Ku, Y. Screening of fresh water fish extracts for enzyme inducing substances by an aryl hydrocarbon hydroxylase induction bioassay technique. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 1136-1139.

(116) Tillitt, D.E.; Giesy, J.P.; Ankley, G.T. Characterization of the H411E rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ. Sci. Technol.* **1991**, *25*, 87-92.

(117) Prough, R.A.; Burke, M.D.; Mayer, R.T. Direct fluorometric methods for measuring mixed function oxidase activity. *Methods Enzymol.* **1978**, *52*, 372-377.

(118) Burke, M.D.; Mayer, R.T. Ethoxyresorufin: direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Disp.* **1974**, *2*, 583-588.

(119) Pohl, R.J.; Fouts, J.R. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem.* **1980**, *107*, 150-155.

(120) Klotz, A.V.; Stegeman, J.J.; Walsh, C. An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. *Anal. Biochem.* **1984**, *140*, 138-145.

(121) Eggens, M.L.; Galgani, F. Ethoxyresorufin-o-deethylase (EROD) activity in flatfish - fast determination with a fluorescence plate-reader. *Mar. Environ. Res.* **1992**, *33*, 213-221.

(122) Tillitt, D.E.; Ankley, G.T.; Verbrugge, D.A.; Giesy, J.P.; Ludwug, J.P.; Kubiak, T.J. H411E rat hepatoma cell bioassay-derived 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in colonial fish eating waterbird eggs from the Great Lakes. *Arch. Environ. Contam. Toxicol.* **1991**, *21*, 91-101.

(123) Jones, P.D.; Giesy, J.P.; Newsted, J.L.; Verbrugge, D.A.; Beaver, D.L.; Ankley, G.T.; Tillitt, D.E.; Lodge, K.B.; Niemi, G.J. 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tissues of birds at Green Bay, Wisconsin, USA. *Arch. Environ. Contam.* **1993**, *24*, 345-354.

(124) Zacharewski, T.; Harris, M.; Safe, S.; Thoma, H.; Hutzinger, O. Applications of the in vitro aryl hydrocarbon induction assay for determining "2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents": pyrolysed brominated flame retardants. *Toxicol.* **1988**, *51*, 177-189.

(125) Safe, S.; Mason, G.; Farrell, K.; Keys, B.; Piskorsa-Pilszezynska, J.; Madge, J.A.; Chittim, B. Validation of in vitro bioassays for 2,3,7,8-TCDD equivalents. *Chemosphere* **1987**, *16*, 1723-1728.

(126) Zacharewski, T.; Safe, L.; Safe, S.; Chittim, B.; DeVault, D., Wiberg, K.; Berqvist, P.-A.; Rappe, C. Comparative analysis of polychlorinated dibenzo-p-dioxin and dibenzofuran congeners in Great Lakes fish extracts by gas chromatography-mass spectrometry and in vitro enzyme induction activities. *Environ. Sci. Technol.* **1989**, *23*, 730-735.

(127) Hanberg, A.; Stahlberg, M.; Georgellis, A.; de Witt, C.; Ahlborg, U.G. Swedish Dioxin Survey: evaluation of the H-4-11E bioassay for screening environmental samples for dioxin-like enzyme induction. *Pharmacol. & Toxicol.* **1991**, *69*, 442-449.

(128) Kennedy, S.W.; Lorenzen, A.; James, C.A. A rapid and sensitive cell culture bioassay for measuring ethoxyresorufin-o-deethylase (EROD) activity in cultured hepatocytes exposed to halogenated aromatic hydrocarbons extracted from wild bird eggs. *Chemosphere* **1993**, *27*, 367-373.

(129) Kennedy, S.W.; Lorenzen, A.; James, C.A.; Norstrom, R.J. Ethoxyresorufin-o-deethylase (EROD) and porphyria induction in chicken embryo hepatocyte cultures - a new bioassay of PCB, PCDD, and related chemical contamination in wildlife. *Chemosphere* **1992**, *25*, 193-196.

(130) Kennedy, S.W.; Lorenzen, A.; James, C.A.; Collins, B.T. Ethoxyresorufin-o-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multi-well plate reader. *Anal. Biochem.* **1993**, *211*, 102-112.

(131) Renzi, D.; Valtolina, M.; Forster, R. The evaluation of a multi-endpoint cytotoxicity assay system. *Atla Altern.to Lab. Animals* **1993**, *21*, 89-96.

(132) Yao, C.; Panigraphy, B.; Safe, S. Utilization of cultured chick embryo hepatocytes as in vitro bioassays for polychlorinated biphenyls (PCBs): quantitative structure activity relationships. *Chemosphere* **1990**, *21*, 1007-1016.

(133) Pesonen, M.; Andersson, T. Characterization and induction of xenobiotic metabolizing enzyme activities in a primary culture of rainbow trout hepatocytes. *Xenobiotica* **1991**, *21*, 461-471.

(134) Pesonen, M.; Goksoyr, A.; Andersson, T. Expression of cytochrome P-450 1A1 in a primary culture of rainbow trout hepatocytes exposed to beta-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch. Biochem. Biophys.* **1992**, *292*, 228-233.

(135) Schuman, R.F.; Hunter, K.W. Ultra sensitive bioassay for dioxin, In *Electric Power Institute Report EPRI-EA-EL-5612*; pp. 7-11 - 7-14. **1988**.

(136) Brunstrom, B. Activities in chick embryos of 7-ethoxyresorufin O-deethylase and aryl hydrocarbon (benzo[a]pyrene) hydroxylase and their induction by 3,3',4,4'-tetrachloro biphenyl in early embryos. *Xenobiotica* **1986**, *16*, 865-872.

(137) Brunstrom, B.; Andersson, L. Toxicity and 7-ethoxyresorufin O-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch. Toxicol.* **1988**, *62*, 263-266.

(138) Brunstrom, B. Embryolethality and induction of 7-ethoxyresorufin O-deethylase in chick embryos by polychlorinated biphenyls and polycyclic aromatic hydrocarbons having Ah receptor affinity. *Chem.- Biol. Interact.* **1991**, *81*, 69-77.

(139) Kaminsky, L.S.; DeCaprio, A.P.; Gierthy, J.F.; Silkworth, J.B.; Tumasonis, C. The role of environmental matrices and experimental vehicles in chlorinated dibenzodioxin and dibenzofuran toxicity. *Chemosphere* **1985**, *14*, 685-695.

(140) Brunstrom, B.; Broman, D.; Dencker, L.; Naf, C.; Vejlens, E.; Zebuhr, Y. Extracts from settling particulate matter collected in the Stockholm archipelago waters -embryolethality, immunotoxicity, and EROD-inducing potency of fractions containing aliphatics monoaromatics, diaromatics, or polyaromatics. *Environ. Toxicol. Chem.* **1992**, *11*, 1441-1449.

(141) Merchant, M.; Morrison, V.; Santostefano, M.; Safe, S. Mechanism of action of aryl hydrocarbon receptor antagonists - inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced cyp1a1 gene expression. *Arch. Biochem. Biophys.* **1992**, *298*, 389-394.

(142) Brunstrom, B. Toxicity and EROD-inducing potency of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in avian embryos. *Comp. Biochem. Physiol.* **1991**, *100C*, 241-243.

(143) Huckins, J.N.; Schwartz, T.R.; Petty, J.D.; Smith, L.M. Determination, fate, and potential significance of PCBs in fish and sediment samples with emphasis on selected AHH-inducing congeners. *Chemosphere* **1988**, *17*, 1995-2016.

(144) Smith, L.M.; Schwartz, T.R.; Feltz, K.; Kubiak, T.J. Determination and occurrence of AHH-active polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran in Lake Michigan sediment and biota. The question of their relative toxicological significance. *Chemosphere* **1990**, *21*, 1063-1085.

(145) Safe, S. Risk assessment of PCDDs and PCDFs based on in vitro and in vivo bioassays. *Chemosphere* **1989**, *19*, 609-613.

(146) Payne, J.F.; Penrose, W.R. Induction of aryl hydrocarbon (benzo(a)pyrene) hydroxylase in fish by petroleum. *Bull. Environ. Cont. Toxicol.* **1975**, *14*, 112-116.

(147) Vodicnik, M.J.; Elcombe, C.R.; Lech, J.J. The effect of various types of inducing agents on hepatic microsomal monooxygenase activity in rainbow trout. *Toxicol. Appl. Pharmacol.* **1981**, *59*, 364-374.

(148) Van Der Weiden, M.E.J.; Vanderkolk, J.; Bleumink, R.; Seinen, W.; Van den Berg, M. Concurrence of P450-1A1 induction and toxic effects after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the rainbow trout (*Oncorhynchus-mykiss*). *Aquatic Toxicol.* **1992**, *24*, 123-142.

(149) Tyle, H.; Egsmose, M.; Harrit, N. Mixed-function oxygenase in juvenile rainbow trout exposed to hexachlorobenzene or 3,3',4,4'-tetrachlorobiphenyl. *Comp. Biochem. Physiol.* **1991**, *100C*, 161-164.

(150) Elcombe, C.R.; Franklin, R.B.; Lech, J.J. Induction of hepatic microsomal enzymes in rainbow trout, In *Pesticide and Xenobiotic Metabolism in Aquatic Organisms* Eds.: M.A.Q. Khan; J.J. Lech; J.J. Menn, ACS Symposium Series #99, ACS: Washington; **1979**; 319-337.

(151) Addison, R.F.; Zinck, M.E.; Willis, D.E. Induction of hepatic mixed-function oxidase (MFO) enzymes in trout (*Salvelinus fontinalis*) by feeding Aroclor 1254 or 3-methylcholanthrene. *Comp. Biochem. Physiol.* **1978**, *61C*, 323-325.

(152) Gooch, J.W.; Elskus, A.A.; Kloepper-Sams, P.J.; Hahn, M.E.; Stegeman, J.J. Effects of ortho- and non-ortho substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stentomus chrysops*). *Toxicol. Appl. Pharmacol.* **1989**, *98*, 422-433.

(153) Lindstrom-Seppa, P.; Koivusaari, U.; Hanninen, O.; Pyysalo, H. Cytochrome P-450 and monooxygenase activities in the biomonitoring of aquatic environment. *Pharmazie* **1985**, *40*, 232-234.

(154) Ahokas, J.T. Cytochrome P-450 in fish liver microsomes and carcinogen activation, In *Pesticide and Xenobiotic Metabolism in Aquatic Organisms,* Eds.: M.A.Q. Khan; J.J. Lech; J.J. Menn; ACS Symp. Series. #99; ACS: Washington; **1979**; 279-296.

(155) Payne, J.F. Field evaluation of benzopyrene hydroxylase induction as a monitor for marine pollution. *Science* **1976**, *191*, 945-946.

(156) Payne, J.F.; Fancey, L.L. Effect of long-term exposure to petroleum on mixed-function oxygenases in fish. Further support for the use of the enzyme system in biological monitoring. *Chemosphere* **1982**, *11*, 207-214.

(157) Goksyor, A.; Husoy, A.-M.; Larsen, H.E.; Klungsoyr, J.; Wilhelmsen, S.; Maage, A.; Brevik, E.M.; Andersson, T.; Celander, M.; Pesonen, M. Environmental contaminants and biochemical responses in flatfish from the Hvaler Archipelago in Norway. *Arch. Environ. Contam. Toxicol.* **1991**, *21*, 486-496.

(158) Narbonne, J.F.; Garrigues, P.; Ribera, D.; Raoux, C.; Mathieu, A.; Lemaire, P.; Salaun, J.P.; Lafaurie, M. Mixed-function oxygenase enzymes as tools for pollution monitoring: field studies on the French coast of the Mediterranian sea. *Comp. Biochem. Physiol.* **1991**, *100C*, 37-42.

(159) Andersson, T.; Celander, M.; Goksoyr, A.; Pesonen, M.; Vaillant, C.; Forlin. L. Cytochrome P-450 and induction: monitoring of aquatic environment, In *Cytochrome P-450, Biochemistry & Biophysics.* Ed. I. Schuster; Taylor and Francis: ?? **1988**; 679-684.

(160) Livingstone, D.R., Farrar, S.V. Responses of the mixed function oxidase system of some bivalve and gastropod molluscs to exposure to polynuclear aromatic and other hydrocarbons. *Mar. Environ. Res.* **1985**, *17*, 101-105.

(161) Livingstone, D.R.; Kirchin, M.A.; Wiseman, A. Cytochrome P-450 and oxidative metabolism in molluscs. *Xenobiotica* **1989**, *19*, 1041-1062.

(162) Yawetz, A.; Manelis, R.; Fishelson, L. The effects of Aroclor-1254 and petrochemical pollutants on cytochrome-P450 from the digestive gland microsomes of 4 species of Mediterranean molluscs. *Comp. Biochem. Physiol.* **1992**, *103*, 607-614.

(163) Stegeman, J.J.; Kloepper-Sams, P.J. Cytochrome P-450 isozymes and monoxygenase activity in aquatic animals. *Environ. Health. Perspect.* **1987**, *71*, 87-95.

(164) Vindimian, E.; Garric, J. Freshwater fish cytochrome P-450-dependent enzymatic activities: a chemical pollution indicator. *Ecotox. Environ. Safety* **1989**, *18*, 277-285.

(165) Balk, L.; Forlin, L.; Soderstrom, M.; Larsson, A. Indications of regional and large-scale biological effects caused by bleached pulp mill effluents. *Chemosphere* **1993**, *27*, 631-650.

(166) Stegeman, J.J.; Woodin, B.R.; Goksoyr, A. Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flounder *Platichtys flesus. Mar. Ecol. Prog. Ser.* **1988**, *46*, 55-60.

(167) Addison, R.F.; Edwards, A.J. Hepatic microsomal mono-oxygenase activity in flounder *Platichthys flesus* from polluted sites in Langesundfjord and from mesocosms experimentally dosed with diesel oil and copper. *Marine Ecol. Prog. Ser.* **1988**, *46*, 51-54.

(168) Forlin, L.; Balk, L.; Celander, M.; Bergek, S.; Hjelt, M.; Rappe, C.; de Wit, C.; Jansson, B. Biotransformation enzyme activities and PCDD/PCDF levels in pike caught in a Swedish lake. *Mar. Environ. Res.* **1992**, *34*, 169-173.

(169) Nikunen, E. Toxic impact of effluents from petrochemical industry. *Ecotoxicol. Environ. Safety* **1985**, *9*, 84-91.

(170) Payne, J.F.; Fancey, L.L. Mixed function oxidase enzyme induction as a biological monitor and indicator in aquatic toxicology: further studies, In *ICES Council Meeting*, **1981**, *E-57* pp.16.

(171) Lindstrom-Seppa, P.; Huuskonen, S.; Pesonen, M.; Muona, P.; Hanninen, O. Unbleached pulp mill effluents affect cytochrome-P450 monooxygenase enzyme activities. *Mar. Environ. Res.* **1992**, *34*, 157-161.

(172) Pesonen, M.; Andersson, T. Toxic effects of bleached and unbleached paper mill effluents in primary cultures of rainbow trout hepatocytes. *Ecotoxicol. Environ. Safety* **1992**, *24*, 63-71.

(173) Payne, J.F.; Fancey, L.L.; Rahimtula, A.D.; Porter, E.L. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Physiol.* **1987**, *86*, 233-245.

(174) Ellenton, J.A.; Brownlee, L.J.; Hollebone, B.R. Aryl hydrocarbon hydroxylase levels in herring gull embryos from different locations on the Great Lakes. *Environ. Toxicol. Chem.* **1985**, *4*, 615-622.

(175) van den Berg, M.; Craane, B.L.H.J.; Sinnige, T.; Lutke-Schipholt, I.J.; Spenkelink, B.; Brouwer, A. The use of biochemical parameters in comparative toxicological studies with the cormorant (*Phalacrocorax carbo*) in the Netherlands. *Chemosphere* **1992**, *25*; 1265-1270.

(176) Forlin, L.; Andersson, T.; Hansson, T.; Koivusaari, U. Influence of biological and environmental factors on hepatic steroid and xenobiotic metabolism in fish: Interaction with PCB and b-naphthoflavone. *Mar. Environ. Res.* **1984**, *14*, 47-58.

(177) Snegaroff, J.; Bach, J. The effects of temperature on the basal activity of cytochrome P-450 in rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **1990**, *95B*, 515-519.

(178) Larsen, H.E.; Celander, M.; Goksoyr, A. The cytochrome-P450 system of Atlantic salmon (*Salmo salar*).2. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. *Fish Physiol. Biochem.* **1992**, *10*, 291-301.

(179) Goksyor, A.; Solberg, T.S. Cytochromes P-450 in fish larvae: immunochemical detection of responses to oil pollution. *Sarsia* **1987**, *72*, 405-407.

(180) Goksoyr, A.; Solberg, T.S.; Serigstad, B. Immunochemical detection of cytochrome P450IA1 induction in cod larvae and juveniles exposed to a water soluble fraction of North Sea crude oil. *Marine Poll. Bull.* **1991**, *22*, 122-127.

(181) Park, S.S.; Fujino, T.; West, D.; Guengerich, P.; Gelboin, H.V. ?? *Cancer Res.* **1982**, *42*, 1798-1808.

(182) Goksoyr, A. Purification of hepatic microsomal cytochromes P-450 from beta-naphthoflavone-treated Atlantic cod (*Gadus morhua*), a marine teleost fish. *Biochim. Biophys. Acta* **1985**, *840*, 409-417.

(183) Goksoyr, A.; Husoy, A.M. The cytochrome-P450 1A1 response in fish - application of immunodetection in environmental monitoring and toxicological testing. *Mar. Environ. Res.* **1992**, *34*, 147-150.

(184) Skaare, J.U.; Gram Jensen, E.; Goksoyr, A.; Egaas, E. Response of xenobiotic metabolizing enzymes of rainbow trout (*Oncorhynchus mykiss*) to the mono-ortho substituted polychlorinated PCB congener 2,3',4,4',5-pentachlorobiphenyl, PCB-118, detected by enzyme activities and immunochemical methods. *Arch. Environ. Contam. Toxicol.* **1991**, *20*, 349-352.

(185) Collier, T.K.; Connor, S.D.; Eberhart, B.T.L.; Anulacion, B.F.; Goksoyr, A., Varanasi, U. Using cytochrome-P450 to monitor the aquatic environment - initial results from regional and national surveys. *Mar. Environ. Res.* **1992**, *34*, 195-199.

(186) Stegeman, J.J.; Woodin, B.R.; Park, S.S.; Kloepper-Sams, P.R.; Gelboin, H.V. Microsomal cytochrome P-450 function in fish evaluated with polyclonal and monoclonal antibodies to cytochrome P-450E from scup (*Stenotomus chrysops*). *Mar. Environ. Res.* **1985**, *17*, 83-86.

(187) Goksyor, A.; Serigstad, B.; Solberg, T.S.; Stegeman, J.J. Response of cod (*Gadus morhua*) larvae and juveniles to oil exposure detected with anti-cod cytochrome P-450c IgG and anti-scup cytochrome P-450E MAb 1-12-3. *Mar. Environ. Res.* **1988**, *24*, 31-36.

(188) Goksyor, A. A semi-quantitative cytochrome P-450IA1 ELISA: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. *Sci. Tot. Environ.* **1991**, *101*, 255-262.

(189) Goksyor, A.; Larsen, H.E.; Husoy, A.-M. Application of a cytochrome P-450 IA1-ELISA in environmental monitoring and toxicological testing of fish. *Comp. Biochem. Physiol.* **1991**, *100C*, 157-160.

(190) Haasch, M.L.; Quardokus, E.M.; Sutherland, L.A.; Goodrich, M.S.; Prince, R.; Cooper, K.R.; Lech, J.J. CyP1A1 protein and messenger RNA in teleosts as an environmental bioindicator - laboratory and environmental studies. *Mar. Environ. Res.* **1992**, *34*, 139-145.

(191) Haasch, M.L.; Prince, R.; Wejksnora, P.J.; Cooper, K.R.; Lech, J.J. Caged and wild fish - induction of hepatic cytochrome p-450 (cyp1a1) as an environmental biomonitor. *Environ. Toxicol. Chem.* **1993**, *12*, 885-895.

(192) Strik, J.J.T.W.A.; Debets, F.M.H.; Koss, G. Chemical porphyria, In *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products*; Ed. R.D. Kimbrough; Elsevier: Amsterdam; **1980**, 191-239.

(193) Sinclair, P.; Granick, S. Uroporphyrin formation induced by chlorinated hydrocarbons (lindane, polychlorinated biphenyls, tetrachlorodibenzo-p-dioxin). Requirements for endogenous iron, protein synthesis and drug metabolizing activity. *Biochem. Biophys. Res. Commun.* **1974**, *61*, 124-133.

(194) Granick, S.; Sinclair, P.; Sassa, S.; Grieninger, G. Effects by heme, insulin, and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and spectro fluorometric assay for porphyrin composition. *J. Biol. Chem.* **1975**, *250*, 9215-9225.

(195) Osborne, R.; Greenlee, W.F. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) enhances terminal differentiation of cultured human epidermal cells. *Toxicol. Appl. Pharmacol.* **1985**, *77*, 434-443.

(196) Knutson, J.C.; Poland, A. Keratinization of mouse teratoma cell line XB produced by 2,3,7,8-tetrachlorodibenzo-p-dioxin: an invitro model of toxicity. *Cell* **1980**, *22*, 27-36.

(197) O'Keefe, P.W.; Silkworth, J.B.; Gierthy, J.F.; Smith, R.M.; DeCaprio, A.P.; Turner, J.N.; Eadon, G.; Hilker, D.R.; Aldous, K.M.; Kaminsky, L.S.; Collins, D.N. Chemical and biological investigations of a transformer accident at Binghamton, N.Y.. *Environ. Health Perspect.* **1985**, *60*, 201-209.

(198) Gierthy, J.F.; Crane, D.; Frenkel, G.D. Application of an in vitro keratinization assay to extracts of soot from a fire in a polychlorinated biphenyl containing transformer. *Fund. Appl. Toxicol.* **1984**, *4*, 1036-1041.

(199) Gierthy, J.F.; Crane, D. Reversible inhibition of in vitro epithelial cell proliferation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Appl. Pharmacol.* **1984**, *74*, 91-98.

(200) Gierthy, J.F.; Lincoln, D.W. Detection of 2,3,7,8-tetrachlorodibenzofuran activity in dielectric fluids by an epithelial cell culture bioassay, In *Electric Power Research Institute Report EPRI-EA-EL-5612*, **1988**, pp.7-48 - 7-51.

(201) Gierthy, J.F.; Swami, K.; Narang, R.; Narang, A.; Eadon, G. Detection of dioxin-like activity in dielectric fluids by an epithelial cell culture bioassay. *Chemosphere* **1993**, *26*, 1225-1235.

(202) Gierthy, J.F.; Crane, D. In vitro bioassay for dioxinlike activity based on alterations in epithelial cell proliferation and morphology. *Fund. Appl. Toxicol.* **1985**, *5*, 754-759.

(203) Gierthy, J.F.; Crane, D. Development and application of an in vitro bioassay for dioxin like compounds, In *Chlorinated Dioxins and Dibenzofurans in Perspective*, Eds. C. Rappe; G. Chaudhary; L. Keith; Lewis Publishers Inc.: Michigan; **1986**; 269-284.

(204) Gierthy, J.F.; Lincoln, D.W.; O'Keefe, P.; Smith, R.; Meyer, C.; Hilker, D.; Aldous, K. Detection of dioxin like activity in fish, sediment, and surface wipes using an in vitro bioassay. *Chemosphere* **1989**, *18*, 793-800.

(205) Ryan, J.J.; Lau, B.P.Y.; Hardy, J.A.; O'Keefe, P.; Gierthy, J.F. 2,3,7,8-tetrachlorodibenzo-p-dioxin and related dioxins and furans in snapping turtle (*Chelydra serpentina*) tissues from the upper St. Lawrence river. *Chemosphere* **1986**, *15*, 537-548.

(206) Safe, S. Development, validation and limitations of toxic equivalency factors. *Chemosphere* **1992**, *25*, 61-64.

Table 1: Performance characteristics of ligand binding assays for planar HAHs

Analyte	Format	Binder	Working Range	DL	I_{50}	Ref.
PCDDs 2,3,7,8-T_4CDD	RIA[^{125}I]	PABs	F_{12}Cutscum: 50 pg - 75 ng F_{12} Triton: 20 pg - 2 ng GC_5 Cutscum: 200 pg - 20 ng	25pg	20ng 1ng 4ng	17
PCDDs 2,3,7,8-T_4CDD	RIA[^{125}I]	PABs	GC_5 DMSO: 20 pg - 2 ng	27pg		35
PCDDs 2,3,7,8-T_4CDD	RIA[^{3}H]	PABs	GC_5 DMSO: a: 20 pg - 2 ng b: 2.5 pg - 200 pg	21pg 3.9 pg	350pg 42pg	40
PCDDs 2,3,7,8-T_4CDD	EIA	MABs	40 pg - 10 ng	100pg	1ng	13, 34
PCDFs 2,3,7,8-T_4CDF	RIA[^{125}I]	PABs	20 pg - 4 ng	20pg	250pg	44
PCBs	RIA[^{125}I]	PABs	Aroclor 1254: 30 pg - 400 ng+	1ng[1]		37
PCBs	RIA[^{125}I]	PABs	Aroclor 1260: 100 pg - 3 ng	100pg	400pg	47
PCBs	EIA	PABs	Aroclor 1248	8.4ng		49
PCBs	EIA	PABs	Aroclor 1254: 5 ppm - 50 ppm	5ppm soil		50
PCBs	RIA[^{125}I]	PABs	Aroclor 1260: 125 pg - 5 ng[2]	100pg		48

Analyte	Format	Binder	Working Range	DL	I_{50}	Ref.
PCBs	EIA	PABs	Aroclor 1254: 0.2 - 10 ppb (water)	200 ppt		51
PCBs	EIA	PABs	Aroclor 1248 0.5 - 5 ppm soil 0.1 - 10 μg/L water (SPE)	0.5 ppm (soil) 5.4 ng (water)		Harrison, pers. commun., 1993
Planar HAHs 2,3,7,8-T_4CDD	RBA[^{3}H]	Ah	5×10^{-10}- 10^{-8} (3)		10^{-9}	18
Planar HAHs 2,3,7,8-T_4CDD	RBA[^{3}H]	Ah	10^{-10}- 10^{-7}M		2×10^{-10}M	60
Planar HAHs 2,3,7,8-T_4CDD	RBA[^{3}H]	Ah	0.1 - 10 ng	100pg	3.2ng	65
Planar HAHs 2,3,7,8-T_4CDD	RBA[^{125}I]	Ah	3.2 pg/mL - 64 pg/mL	3.2pg/mL 0.8pg/0.25 mL	40pM	66
Planar HAHs 2,3,7,8-T_4CDD	RBA[^{3}H]	Ah	$-\log_{10}8.5$ - $-\log_{10}10$ $molL^{-1}$		1.1×10^{-9} $molL^{-1}$	68

1: 3,4,3',4'-T_4CBP and 2,6,2',6'-T_4CBP.
2: ng/tube.
3: units not provided - presumably M.

Table 2: TEF systems

Congener	UBA/BGA 1985[1]	US-EPA 1985[1]	Ahlborg 1989[1]	Eadon 1982[1]	NordicNato 1989[2]	Safe 1992[20]
2378-T_4CDD	1	1	1	1	1	1
2378-T_4CDF	0.1	0.1	0.1	0.33	0.1	0.1
12378-P_5CDD	0.1	0.2	0.5	1	0.5	0.5
12378-P_5CDF	0.1	0.2	0.01	0.33	0.05	0.1
23478-P_5CDF	0.1	0.1	0.5	0.33	0.5	0.5
2378-s-H_6CDD	0.1	0.04	0.1	0.03	0.1	0.1
2378-s-H_6CDF	0.1	0.04	0.1	0.01	0.1	0.1
2378-s-H_7CDD	0.01	0.001	0.01	0	0.01	0.01
2378-s-H_7CDF	0.01	0.001	0.01	0	0.01	0.1
non-2378-s-T_4CDD/F	0.01		0			
non-2378-s-P_5CDD/F	0.01					
non-2378-s-H_6CDD/F	0.01		0			
non-2378-s-H_7CDD/F	0.001		0			
O_8CDD	0.001	0	0.001	0	0.001	0.001
O_8CDF	0.001	0	0.001	0	0.001	0.001

s = substituted
1: cited by Neubert et al.[87]
2: cited by Safe[145]

Table 3. Performance of cytochrome P4501A1 based enzyme induction bioassays.

Cells	Enzyme	Linear Range	ED_{50} (TCDD)	DL (TCDD)	Ref.
Rat H-4-11-E	AHH	25 - 500 pg	1.54 pmol/pl.(DMSO) 1.08 pmol/pl.(DMSO) 0.14 pmol/pl. (isooct.)	25 pg/pl. (DMSO)[1] 10 pg/pl. (isooct)[1]	110, 112, 113, 115,
Rat 1° hepatocyte	AHH	$<10^{-11}$ - 10^{-8}M		$\leq 10^{-11}$M (DMSO)	102
Mouse	EROD	0.063 - 1 pg/mL	1.6×10^{-12}M (DMSO) (0.5 pg/mL)	0.063 pg/mL	135
Rat H-4-11-E	EROD	log 1.5 - log 2.4 pg/pl.	0.17 pmol/pl.	10 pg/pl.	116, 122
Rat H-4-11-E	AHH EROD	2×10^{-10} - 10^{-8}M	10^{-10}M	>20 pg/plate	124 - 126
Rat H-4-11-E	EROD	20 pg/pl. - 200 pg/pl.	45.8 ± 15.5 pg/pl.	10 pg/pl.[2]	67, 127
Chicken	EROD	10^{-3} nM - 10^{-1} nM	15 pM	0.16 pg/well	128, 130
Trout 1° liver	EROD	2.5 - 100 pM		4 pM	133, 134
Chicken embryo hepatocytes	AHH EROD	$\log_{10}7$ - $\log_{10}9$ M EROD	7.9×10^{-11} M EROD 2.4×10^{-11} M AHH		132

1: upper confidence limit of analyte free control.
2: Mean of blanks = 3 x S.D.

FIGURES

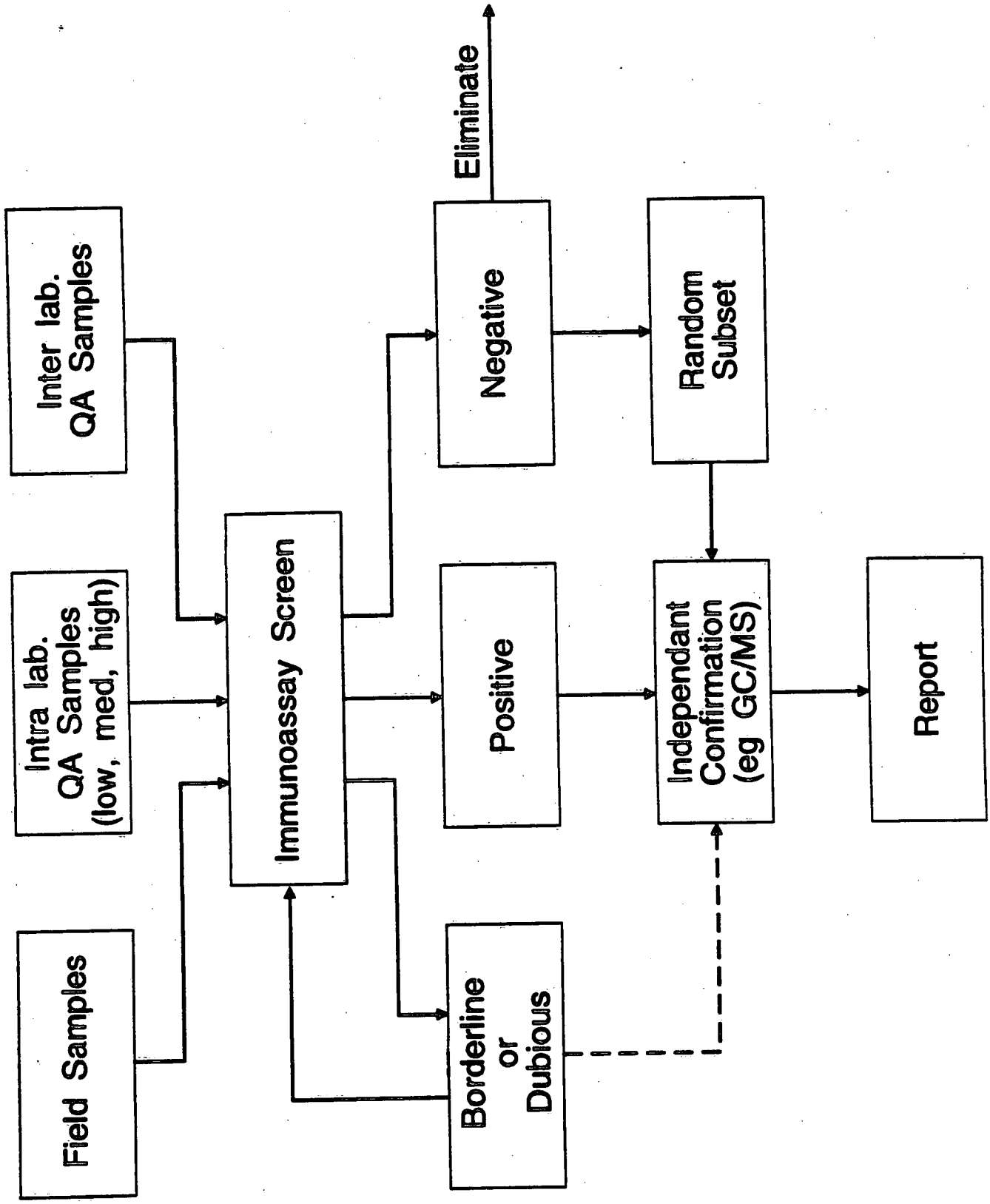

Inter lab.
QA Samples
Intra lab.
QA Samples
(low, med, high)
Field Samples
Immunoassay Screen
Negative
Eliminate
Positive
Borderline
or
Dubious
Random
Subset
Independant
Confirmation
(eg GC/MS)
Report

FIGURE 1.

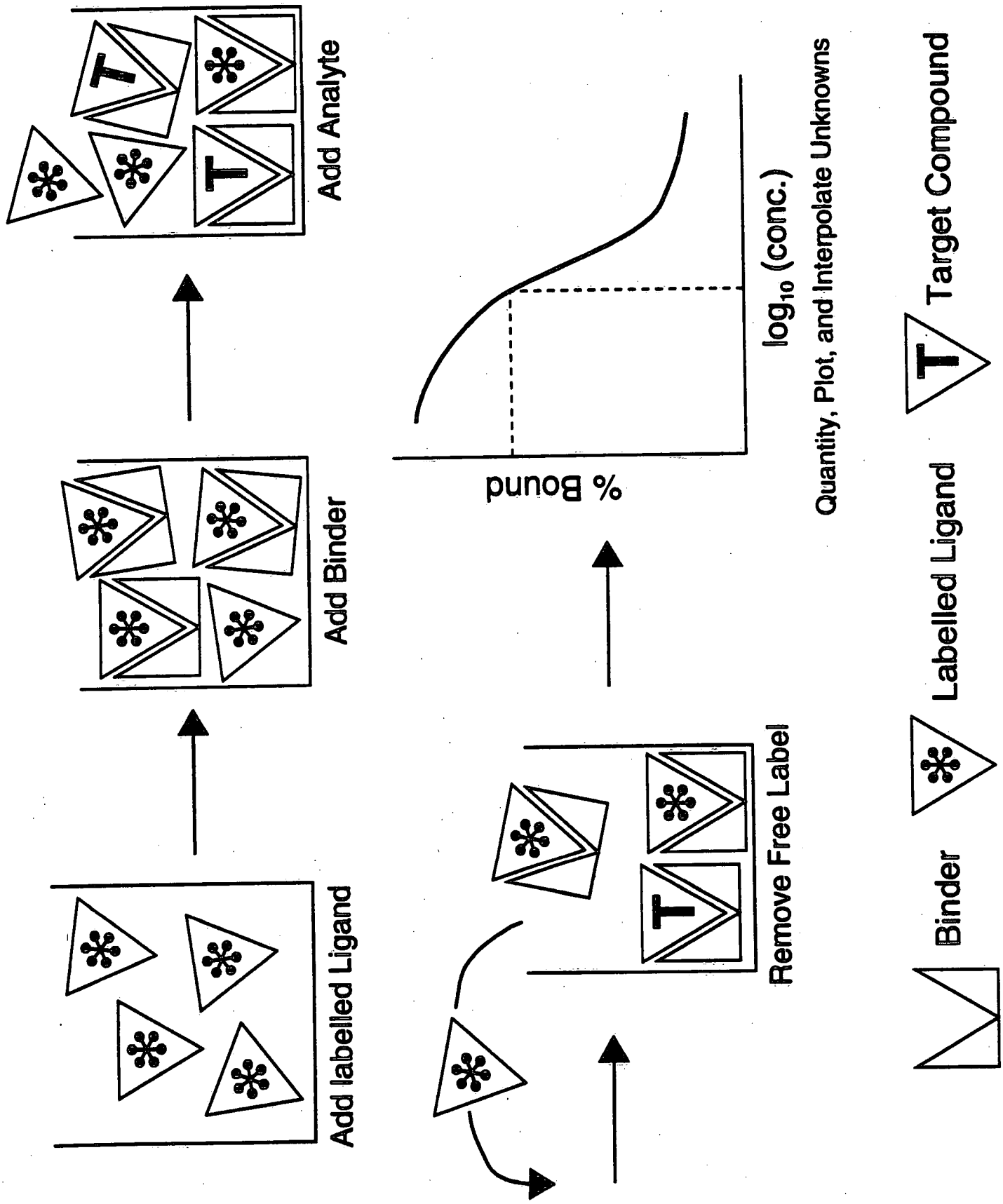

Add labelled Ligand
Add Binder
Add Analyte
Remove Free Label
% Bound
log10 (conc.)
Quantity, Plot, and Interpolate Unknowns
Binder
Labelled Ligand
Target Compound

FIGURE 2.

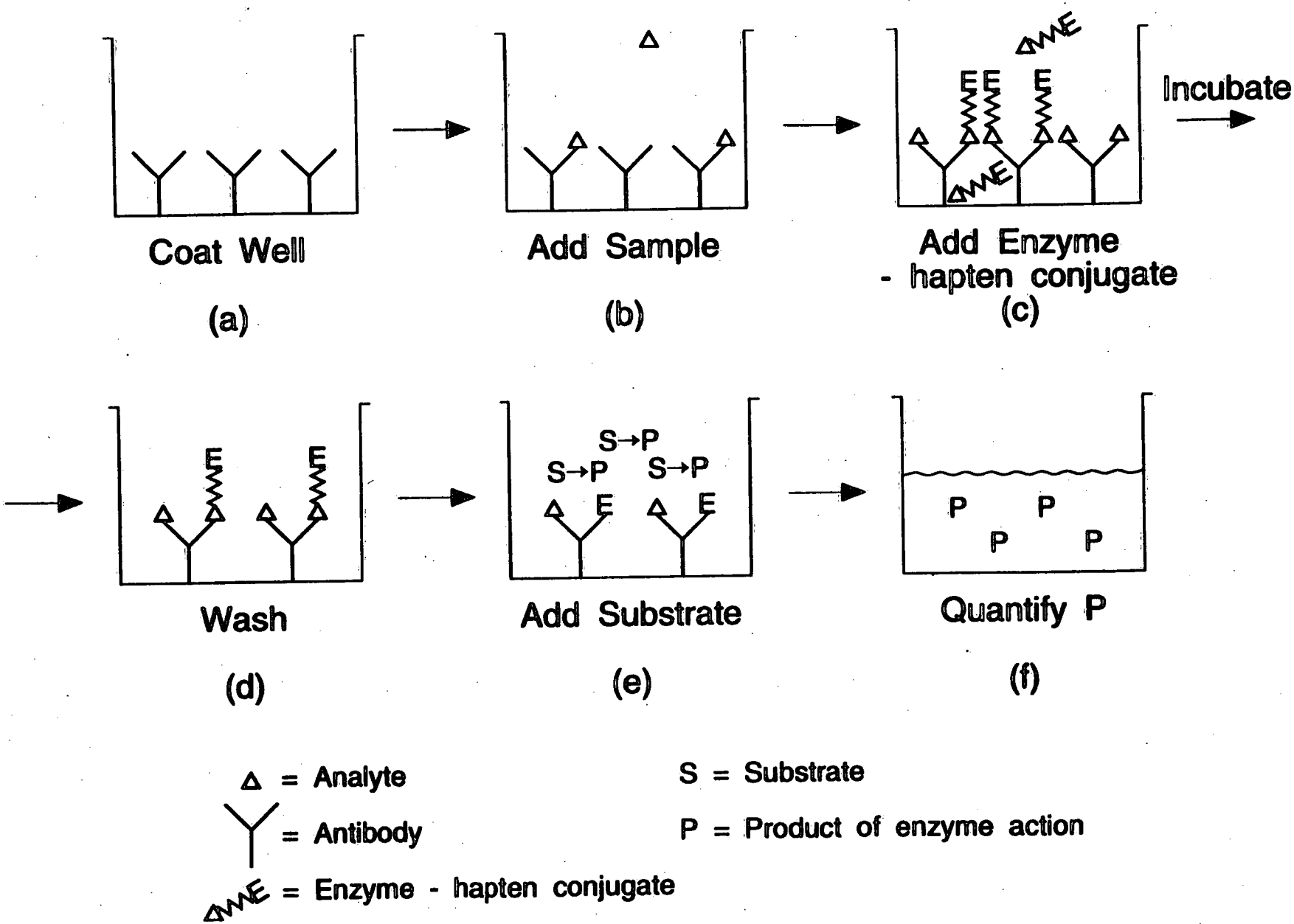

Coat Well
(a)
Add Sample
(b)
Add Enzyme
- hapten conjugate
(c)
Incubate
Wash
(d)
S→P
Add Substrate
(e)
Quantify P
(f)
Δ = Analyte
= Antibody
= Enzyme - hapten conjugate
S = Substrate
P = Product of enzyme action

FIGURE 3.

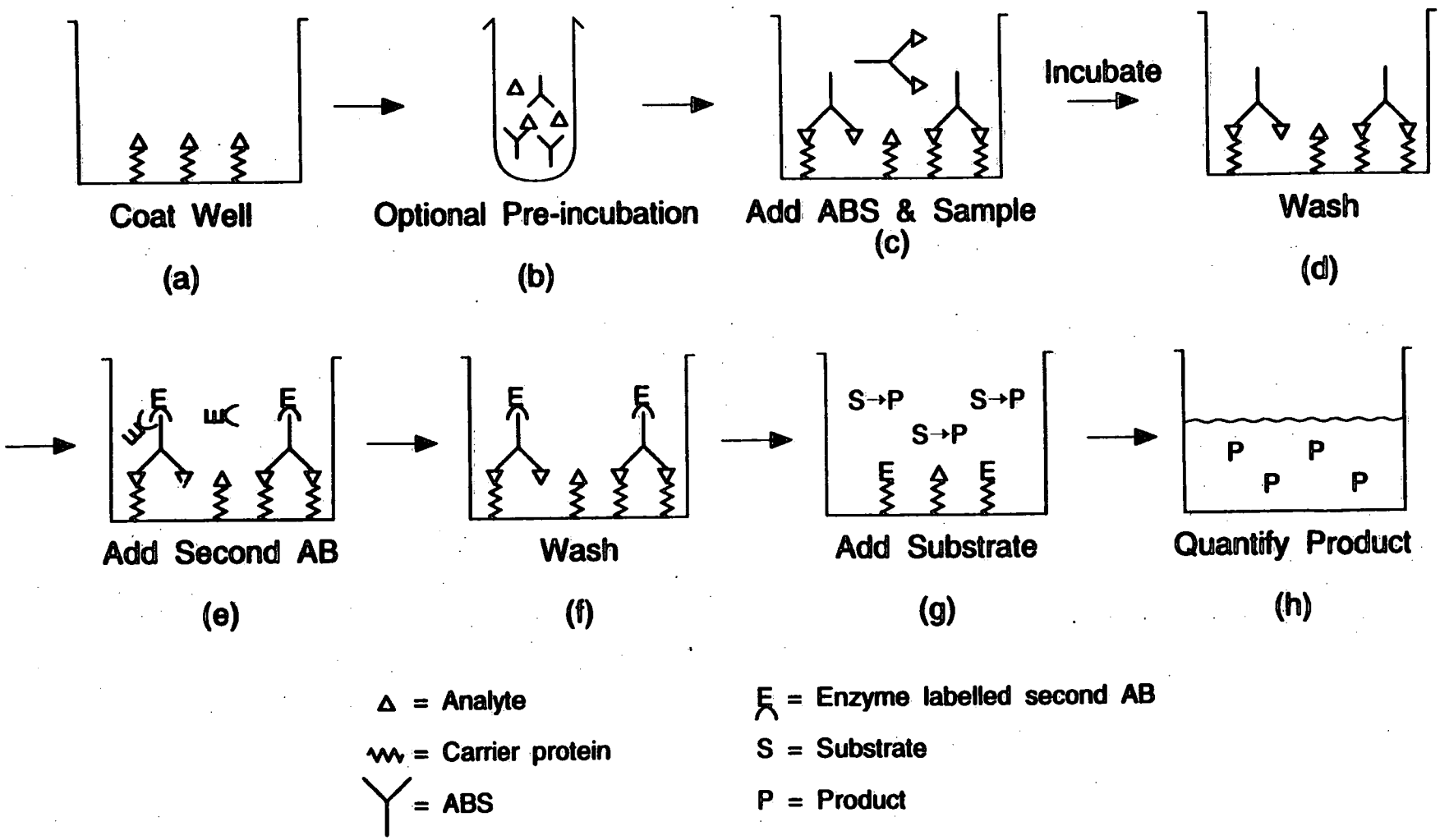
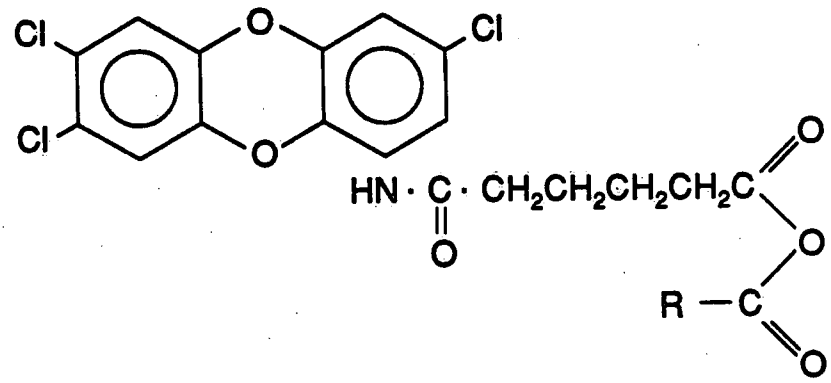
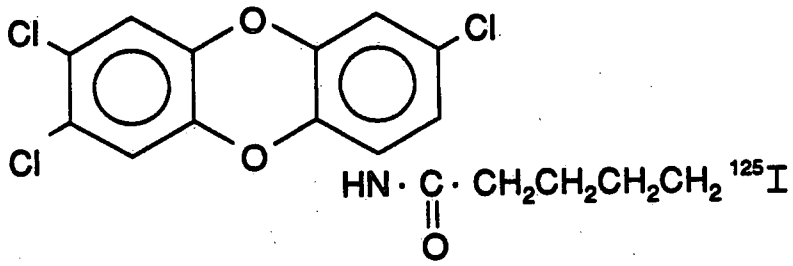

Coat Well
(a)
Optional Pre-incubation
(b)
Add ABS & Sample
(c)
Incubate
Wash
(d)
E
Add Second AB
(e)
Wash
(f)
S→P
Add Substrate
(g)
P
Quantify Product
(h)
Δ = Analyte
= Carrier protein
= ABS
E = Enzyme labelled second AB
S = Substrate
P = Product

FIGURE 4.



Adipamide derivative of 1-amino T_3CDD has been reacted with R-COCl (isobutyl chloroformate) to yield the mixed anhydride.



^{125}I — valeramido - T_3CDD

FIGURE 5.

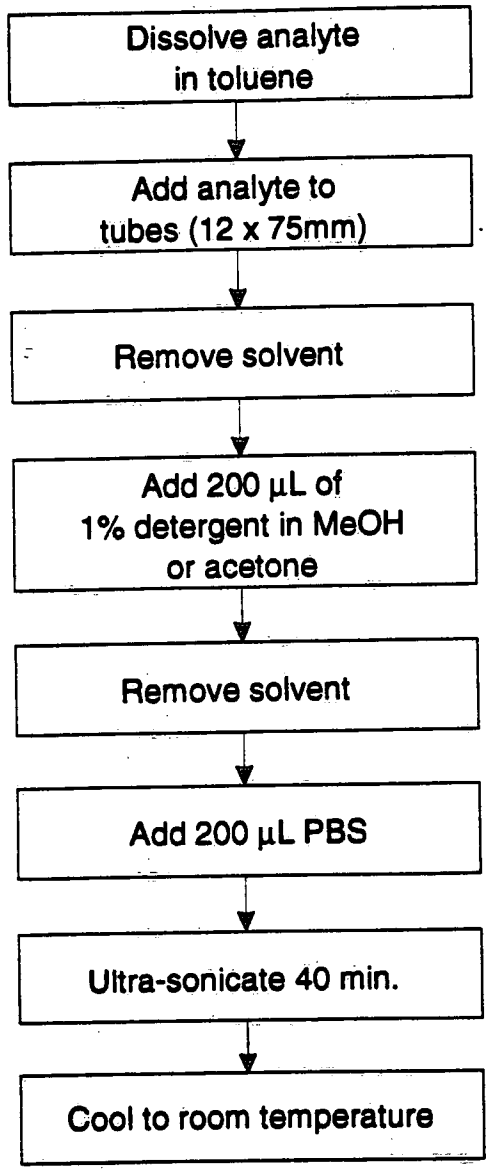

Dissolve analyte in toluene
Add analyte to tubes (12 x 75mm)
Remove solvent
Add 200 µL of 1% detergent in MeOH or acetone
Remove solvent
Add 200 µL PBS
Ultra-sonicate 40 min.
Cool to room temperature

FIGURE 6.

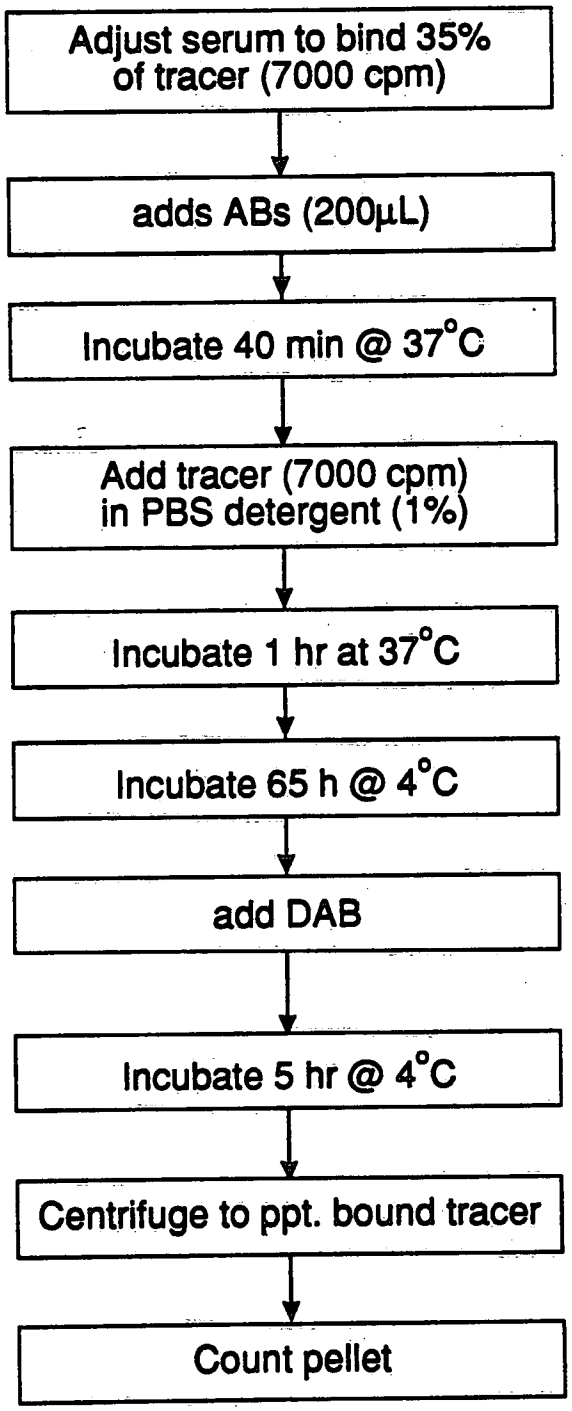

Adjust serum to bind 35% of tracer (7000 cpm)
adds ABs (200µL)
Incubate 40 min @ 37°C
Add tracer (7000 cpm) in PBS detergent (1%)
Incubate 1 hr at 37°C
Incubate 65 h @ 4°C
add DAB
Incubate 5 hr @ 4°C
Centrifuge to ppt. bound tracer
Count pellet

FIGURE 7.

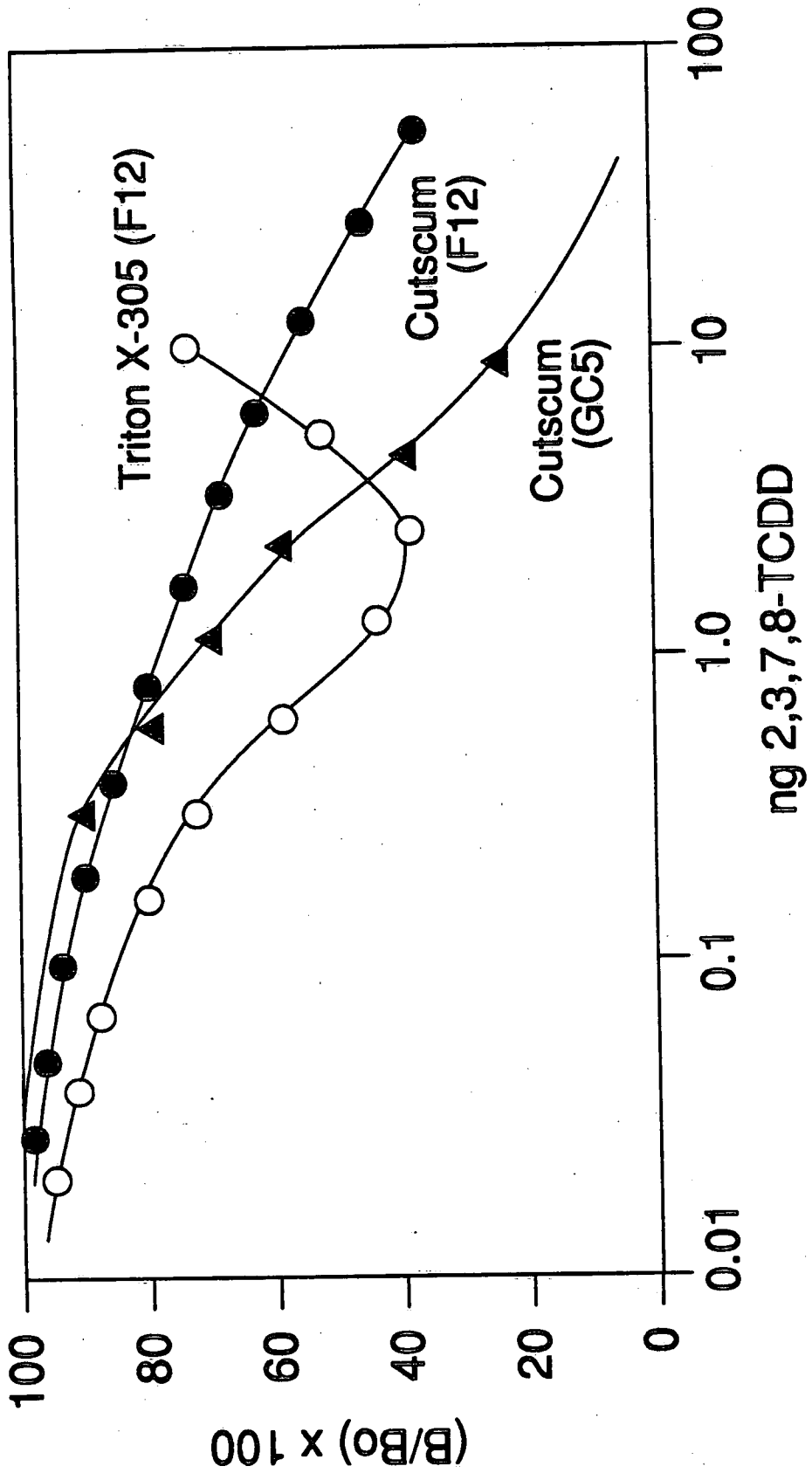

Triton X-305 (F12)
Cutscum (F12)
Cutscum (GC5)
(B/Bo) x 100
100
80
60
40
20
0
0.01
0.1
1.0
10
100
ng 2,3,7,8-TCDD

FIGURE 8.

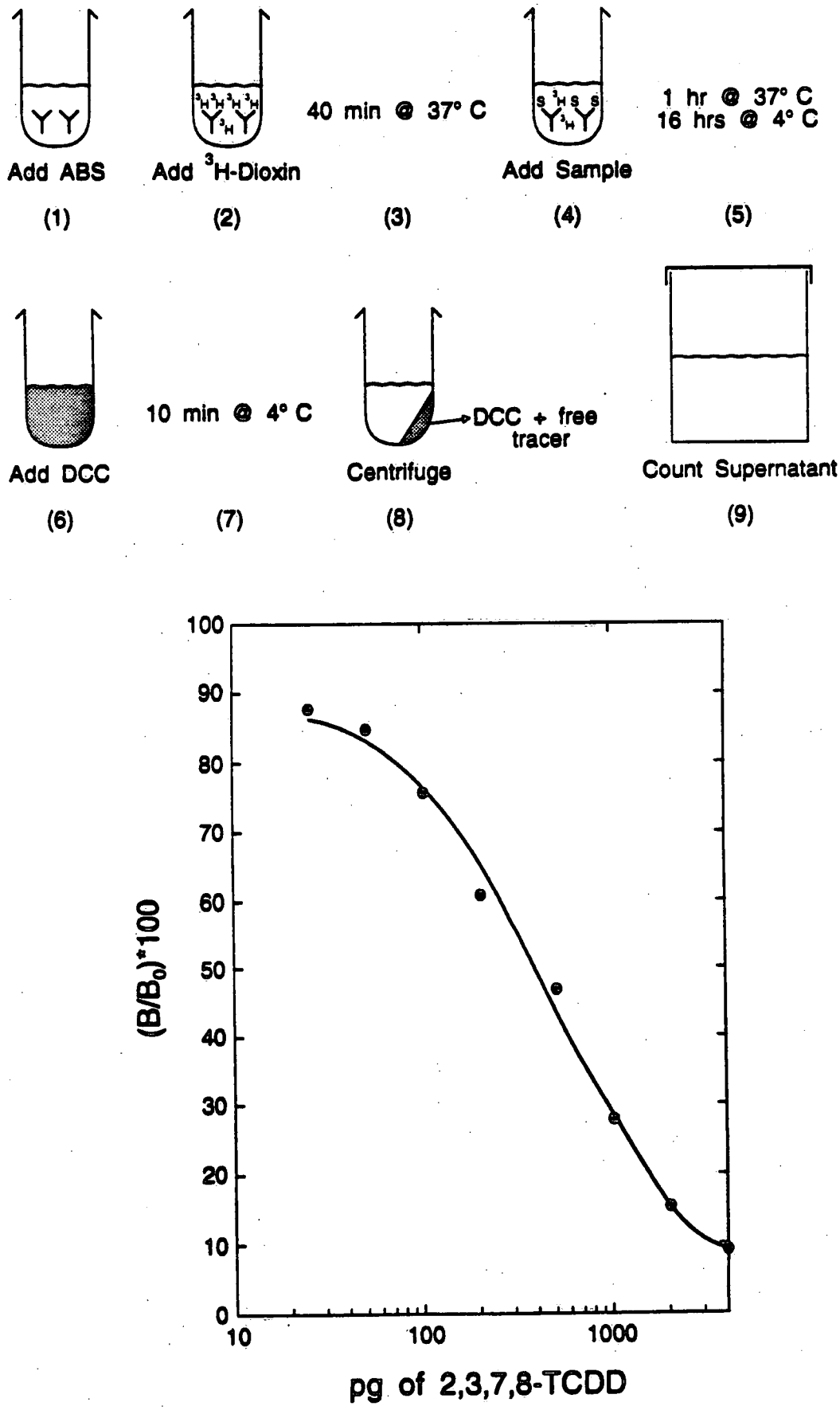

Add ABS
(1)
Add ^{3}H-Dioxin
(2)
40 min @ 37° C
(3)
Add Sample
(4)
1 hr @ 37° C
16 hrs @ 4° C
(5)
Add DCC
(6)
10 min @ 4° C
(7)
Centrifuge
DCC + free tracer
(8)
Count Supernatant
(9)
100
90
80
70
60
50
40
30
20
10
0
10
100
1000
(B/B$_0$)*100
pg of 2,3,7,8-TCDD

FIGURE 9.

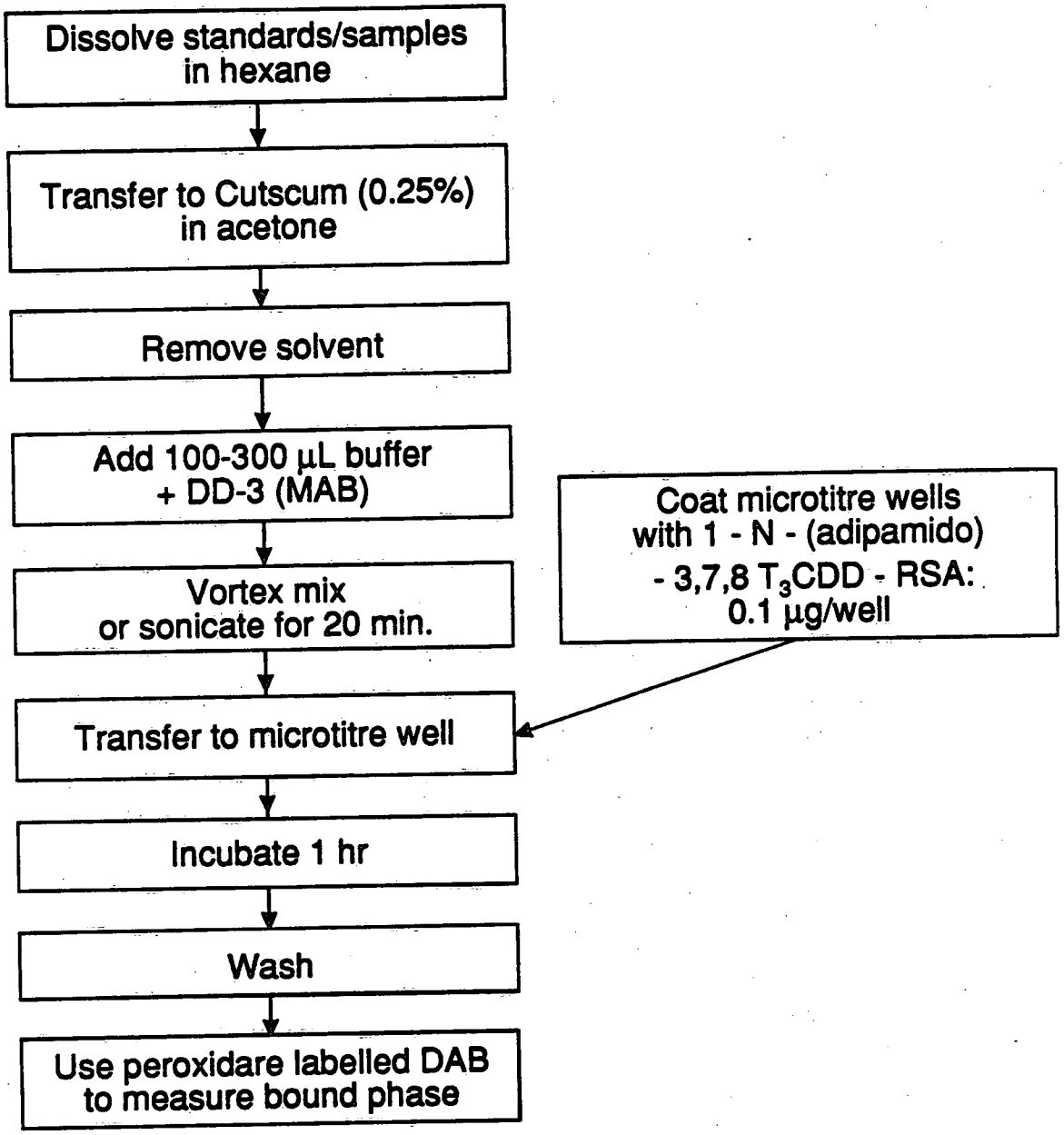

Dissolve standards/samples in hexane
Transfer to Cutscum (0.25%) in acetone
Remove solvent
Add 100-300 µL buffer + DD-3 (MAB)
Vortex mix or sonicate for 20 min.
Transfer to microtitre well
Incubate 1 hr
Wash
Use peroxidare labelled DAB to measure bound phase
Coat microtitre wells with 1 - N - (adipamido) - 3,7,8 T_3CDD - RSA: 0.1 µg/well

FIGURE 10.

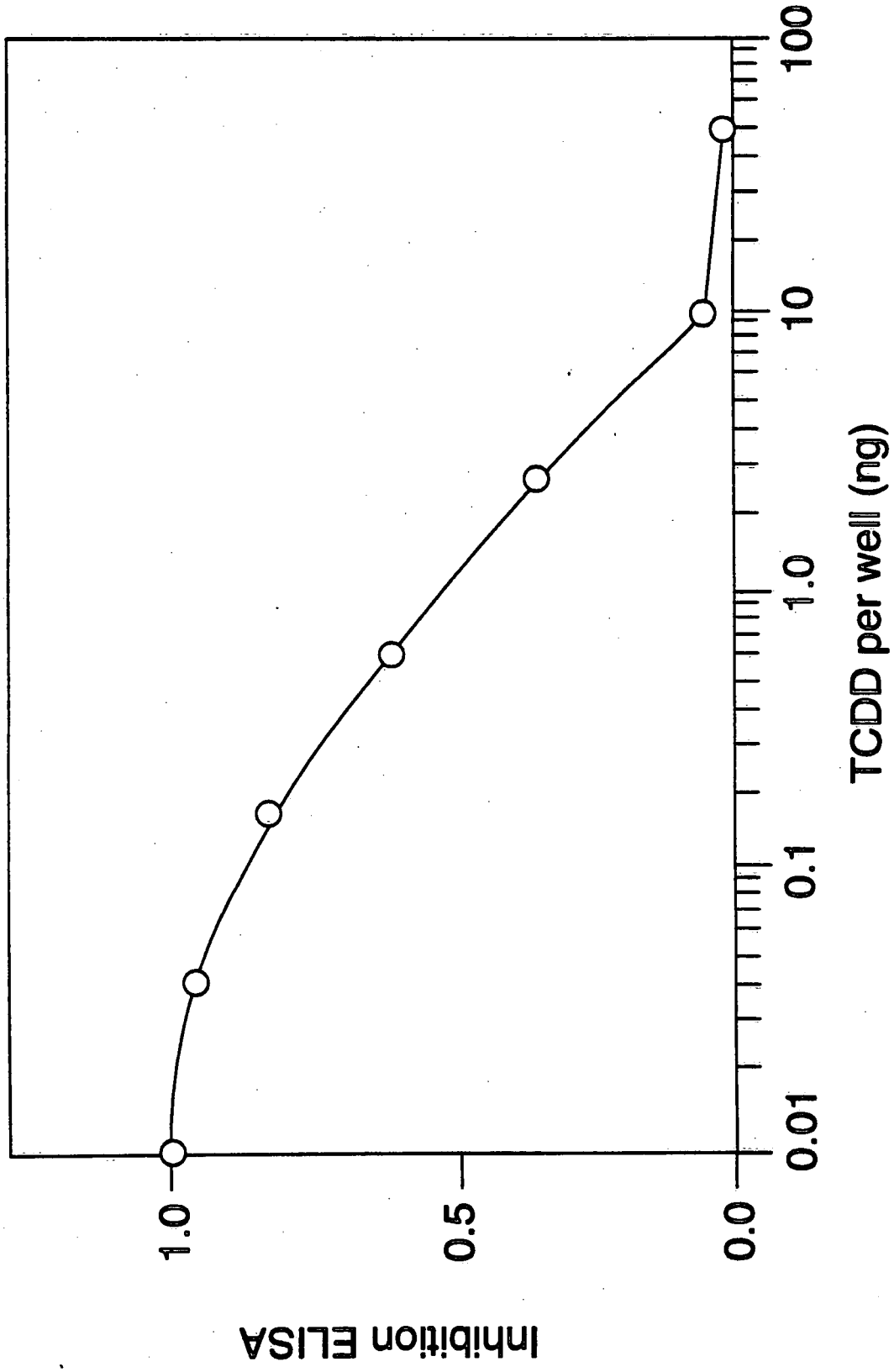


FIGURE 11.

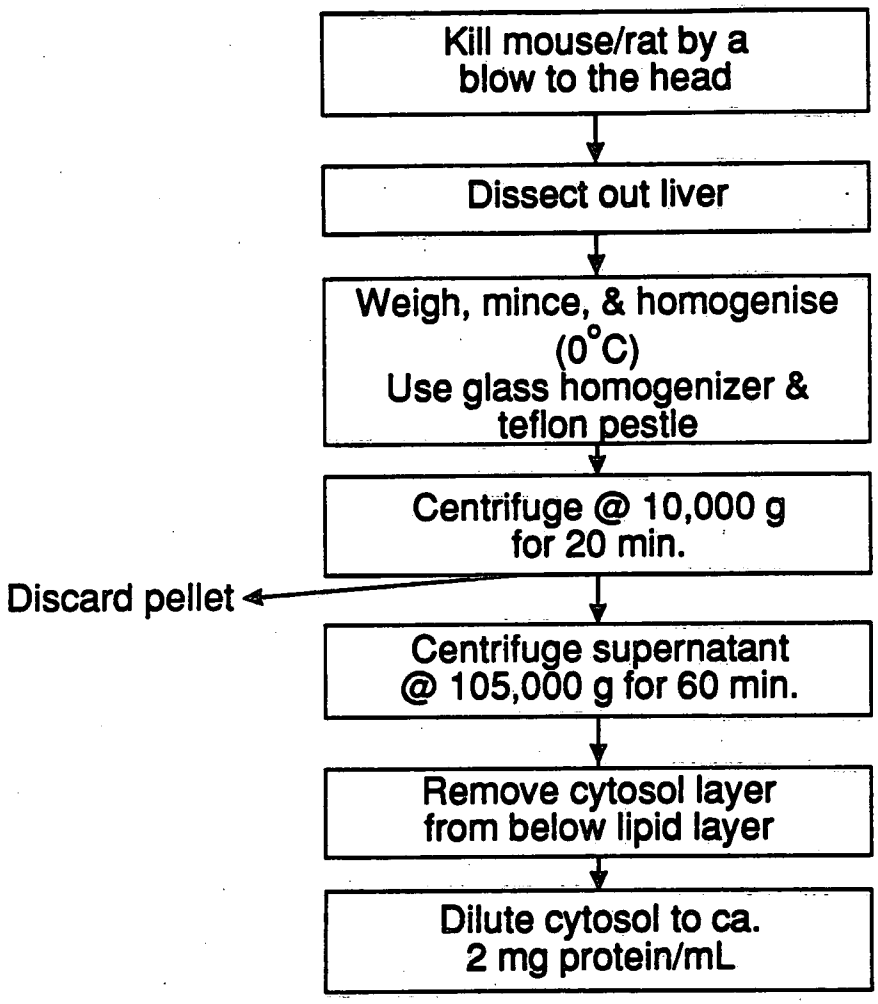

Kill mouse/rat by a blow to the head
Dissect out liver
Weigh, mince, & homogenise (0°C)
Use glass homogenizer & teflon pestle
Centrifuge @ 10,000 g for 20 min.
Discard pellet
Centrifuge supernatant @ 105,000 g for 60 min.
Remove cytosol layer from below lipid layer
Dilute cytosol to ca. 2 mg protein/mL

FIGURE 12.

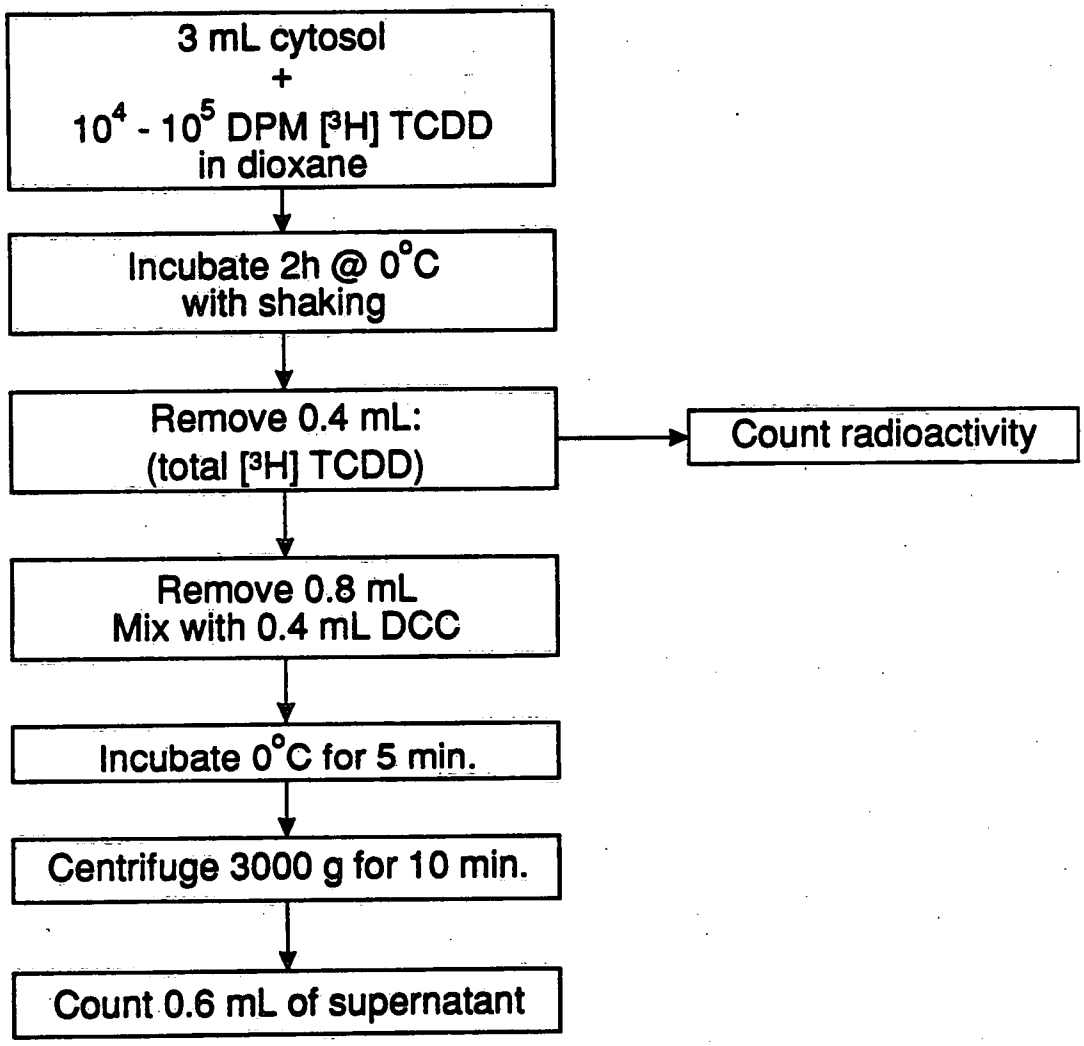

3 mL cytosol
+
10^4 - 10^5 DPM [^{3}H] TCDD
in dioxane
Incubate 2h @ 0°C
with shaking
Remove 0.4 mL:
(total [^{3}H] TCDD)
Count radioactivity
Remove 0.8 mL
Mix with 0.4 mL DCC
Incubate 0°C for 5 min.
Centrifuge 3000 g for 10 min.
Count 0.6 mL of supernatant

FIGURE 13.

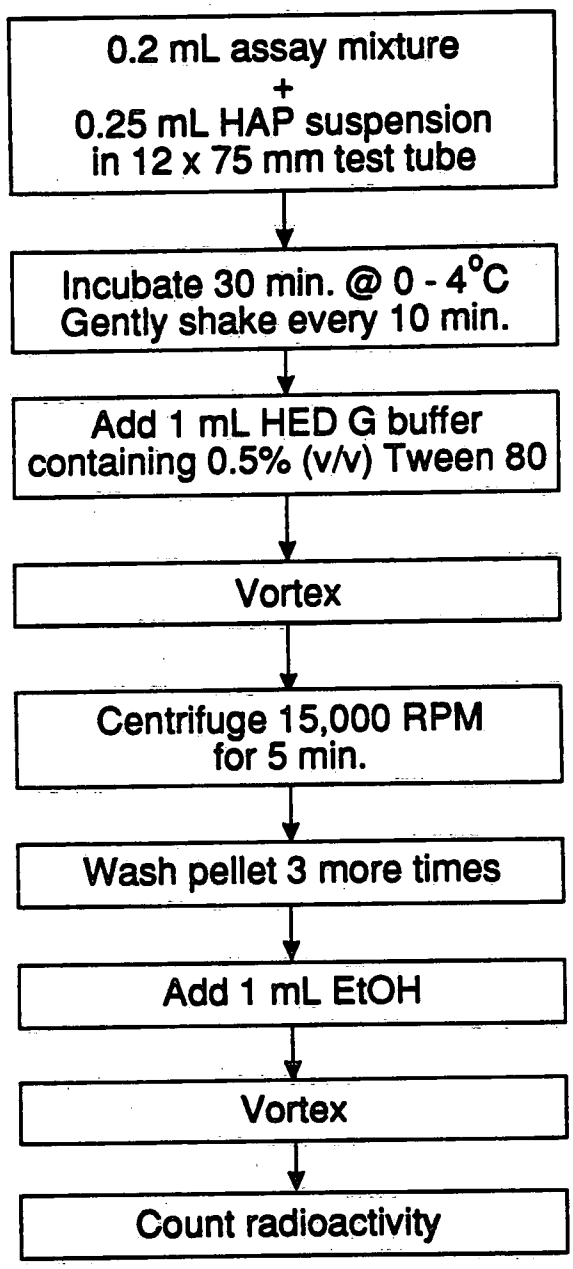

0.2 mL assay mixture
+
0.25 mL HAP suspension
in 12 x 75 mm test tube
Incubate 30 min. @ 0 - 4°C
Gently shake every 10 min.
Add 1 mL HED G buffer
containing 0.5% (v/v) Tween 80
Vortex
Centrifuge 15,000 RPM
for 5 min.
Wash pellet 3 more times
Add 1 mL EtOH
Vortex
Count radioactivity

FIGURE 14.

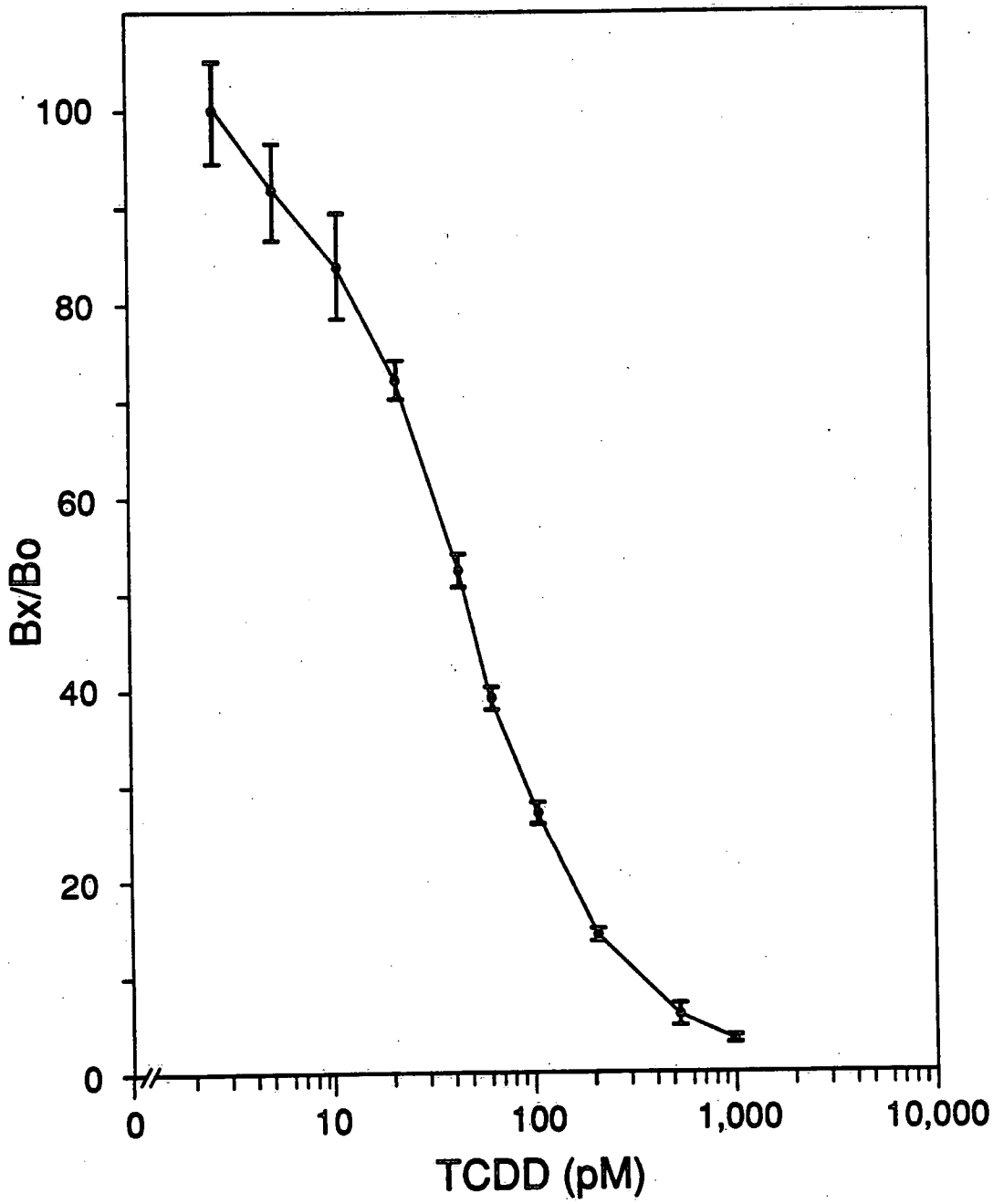


FIGURE 15.

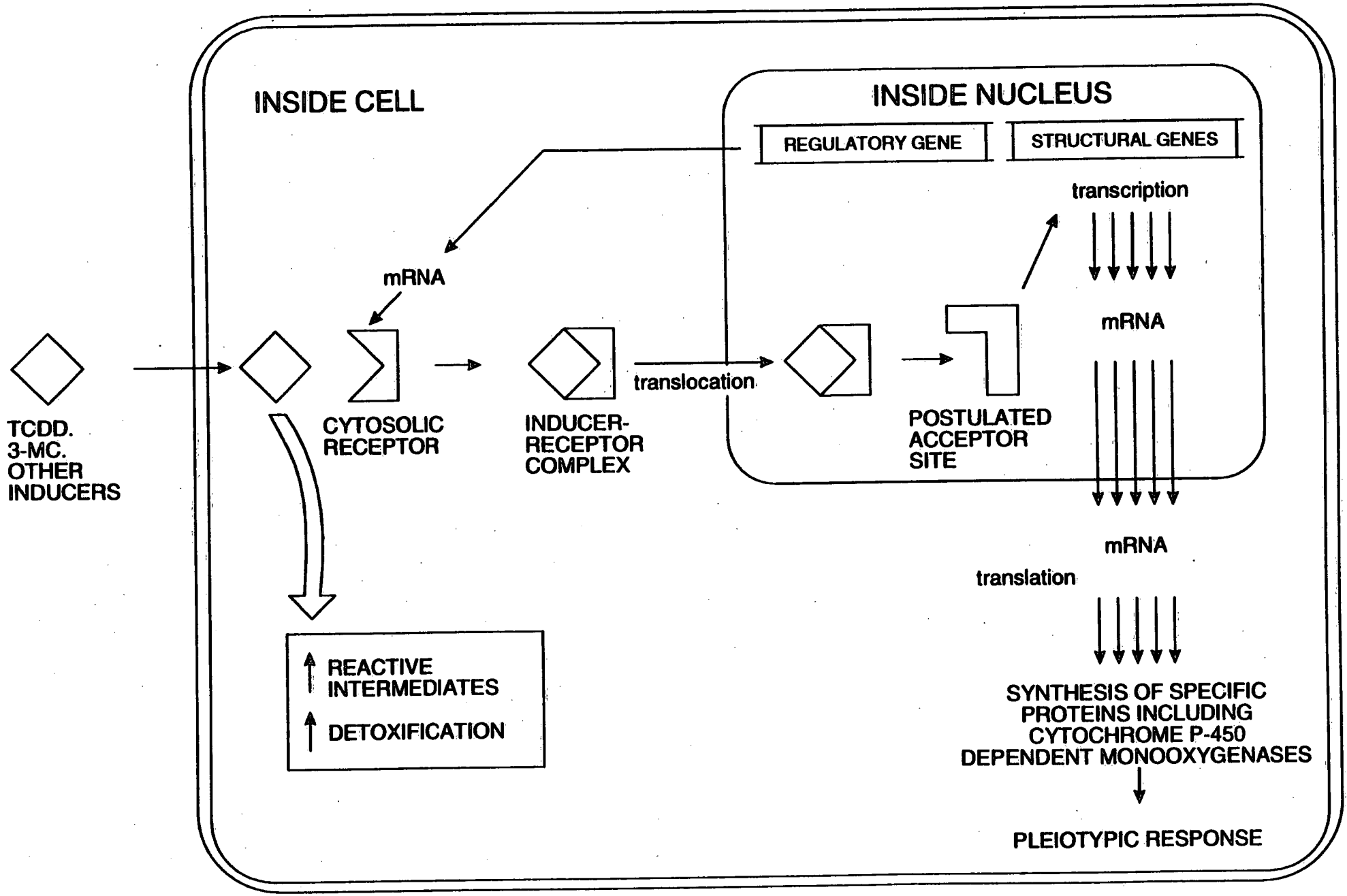
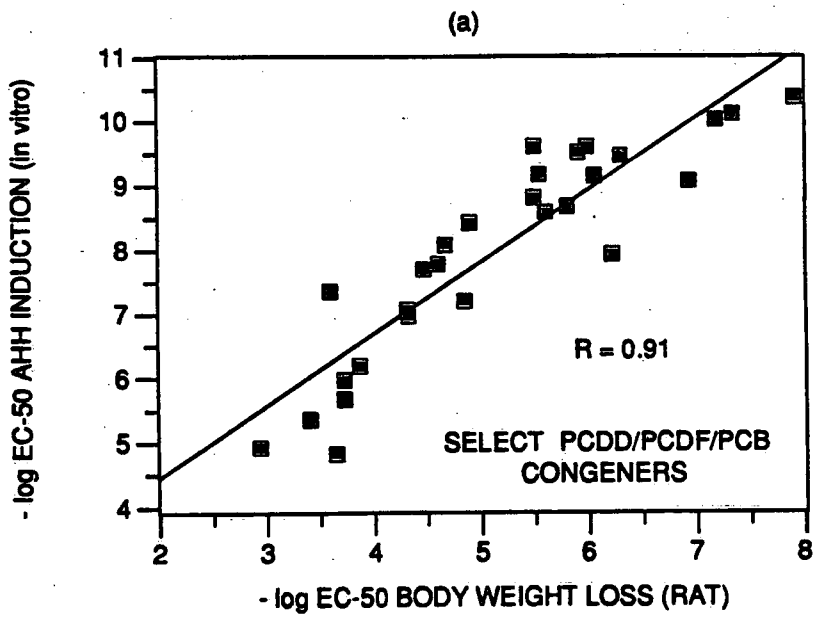
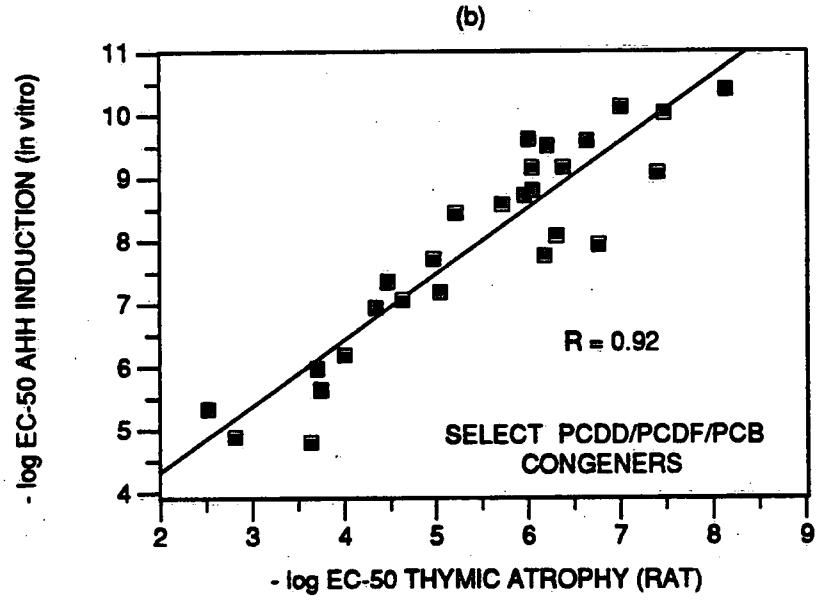

INSIDE CELL
INSIDE NUCLEUS
REGULATORY GENE
STRUCTURAL GENES
transcription
mRNA
mRNA
translocation
TCDD.
3-MC.
OTHER
INDUCERS
CYTOSOLIC
RECEPTOR
INDUCER-
RECEPTOR
COMPLEX
POSTULATED
ACCEPTOR
SITE
mRNA
translation
REACTIVE
INTERMEDIATES
DETOXIFICATION
SYNTHESIS OF SPECIFIC
PROTEINS INCLUDING
CYTOCHROME P-450
DEPENDENT MONOOXYGENASES
PLEIOTYPIC RESPONSE

FIGURE 16.


(a)
- log EC-50 AHH INDUCTION (in vitro)
- log EC-50 BODY WEIGHT LOSS (RAT)
R = 0.91
SELECT PCDD/PCDF/PCB
CONGENERS


(b)
- log EC-50 AHH INDUCTION (in vitro)
- log EC-50 THYMIC ATROPHY (RAT)
R = 0.92
SELECT PCDD/PCDF/PCB
CONGENERS

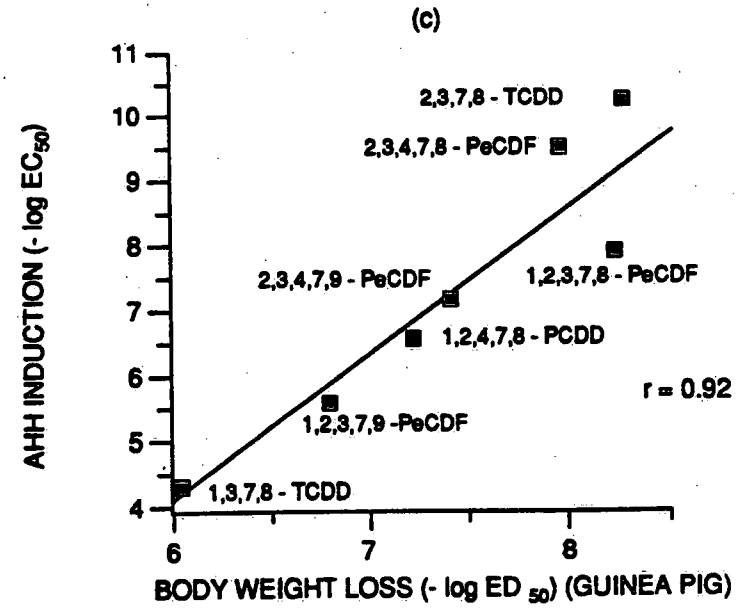

(c)
AHH INDUCTION (- log EC50)
BODY WEIGHT LOSS (- log ED 50) (GUINEA PIG)
2,3,7,8 - TCDD
2,3,4,7,8 - PeCDF
2,3,4,7,9 - PeCDF
1,2,3,7,8 - PeCDF
1,2,4,7,8 - PCDD
1,2,3,7,9 -PeCDF
1,3,7,8 - TCDD
r = 0.92

FIGURE 17.

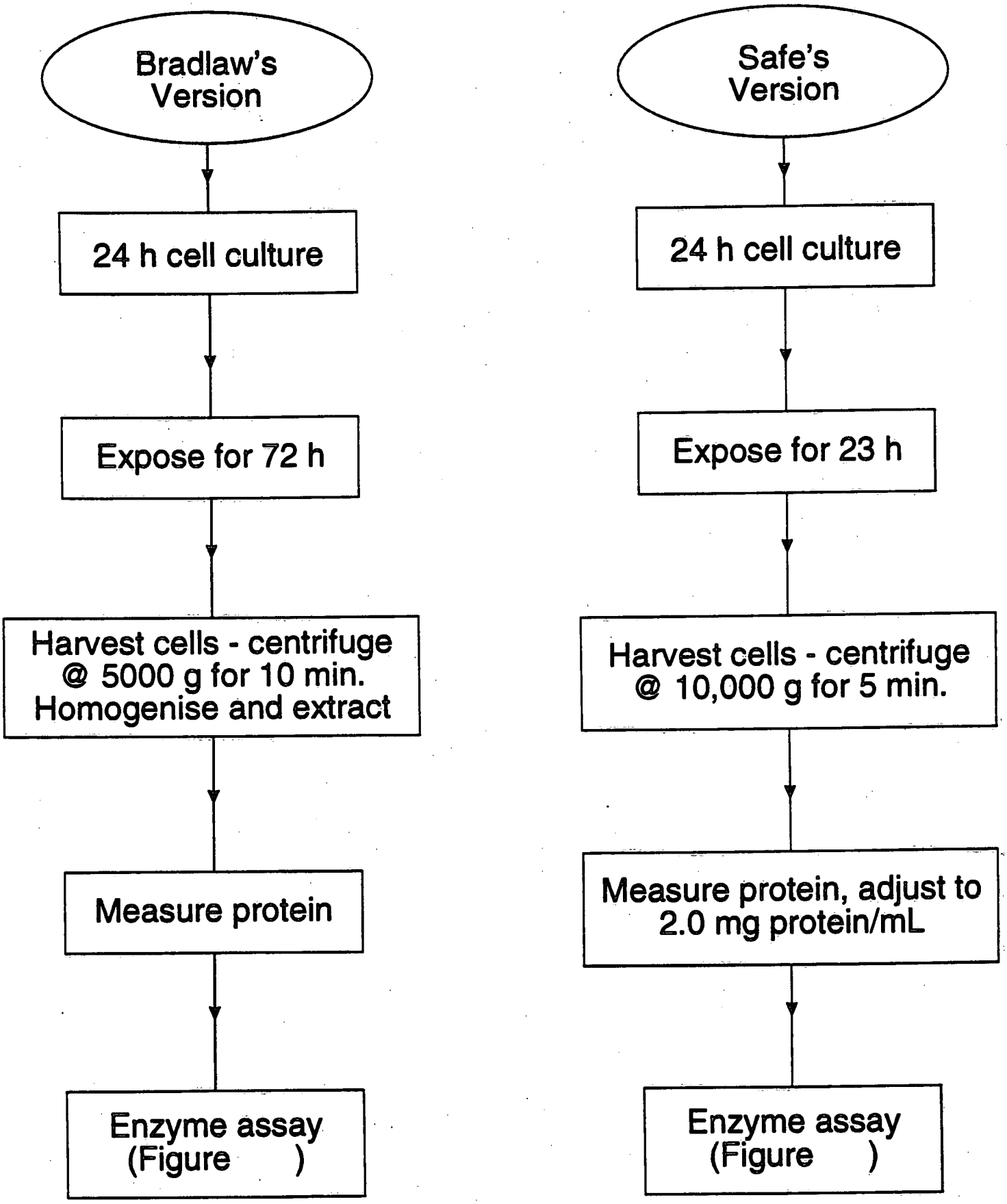

Bradlaw's Version
24 h cell culture
Expose for 72 h
Harvest cells - centrifuge @ 5000 g for 10 min. Homogenise and extract
Measure protein
Enzyme assay (Figure)
Safe's Version
24 h cell culture
Expose for 23 h
Harvest cells - centrifuge @ 10,000 g for 5 min.
Measure protein, adjust to 2.0 mg protein/mL
Enzyme assay (Figure)

FIGURE 18.

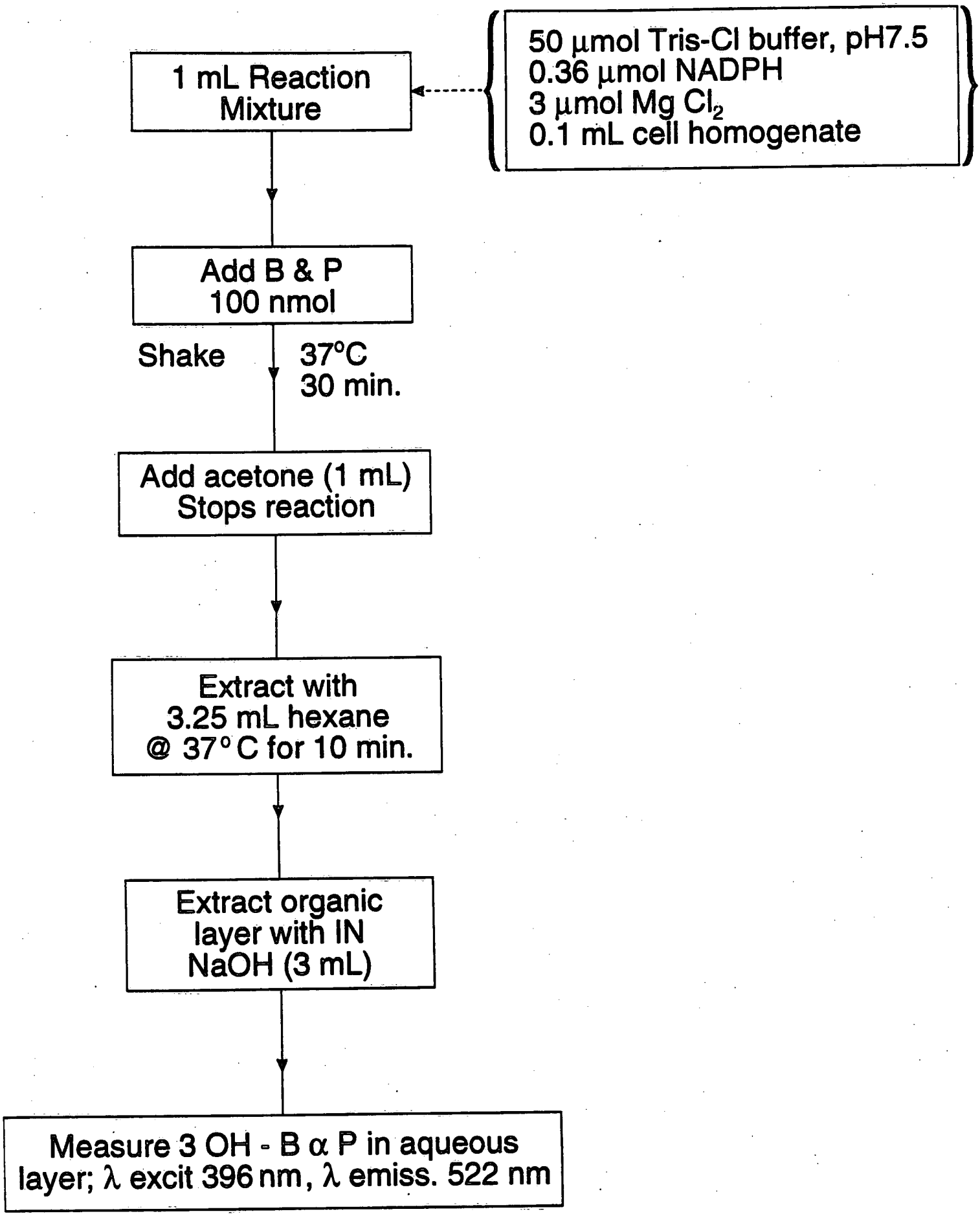

1 mL Reaction Mixture
50 µmol Tris-Cl buffer, pH7.5
0.36 µmol NADPH
3 µmol Mg Cl2
0.1 mL cell homogenate
Add B & P 100 nmol
Shake
37°C 30 min.
Add acetone (1 mL) Stops reaction
Extract with 3.25 mL hexane @ 37° C for 10 min.
Extract organic layer with IN NaOH (3 mL)
Measure 3 OH - B α P in aqueous layer; λ excit 396 nm, λ emiss. 522 nm

FIGURE 19.

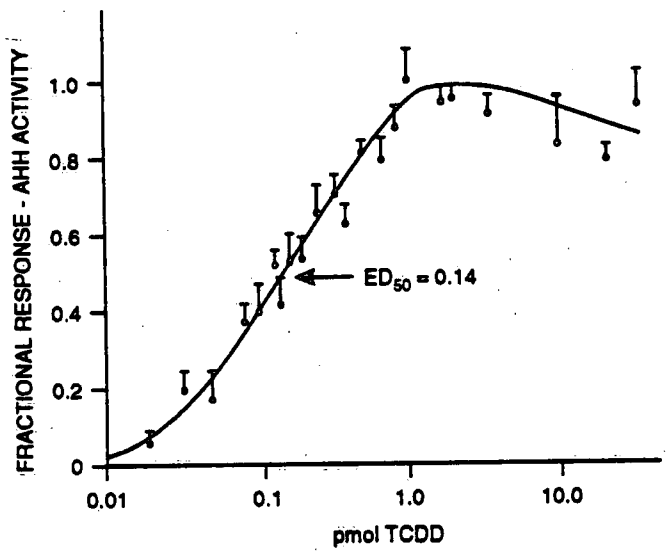
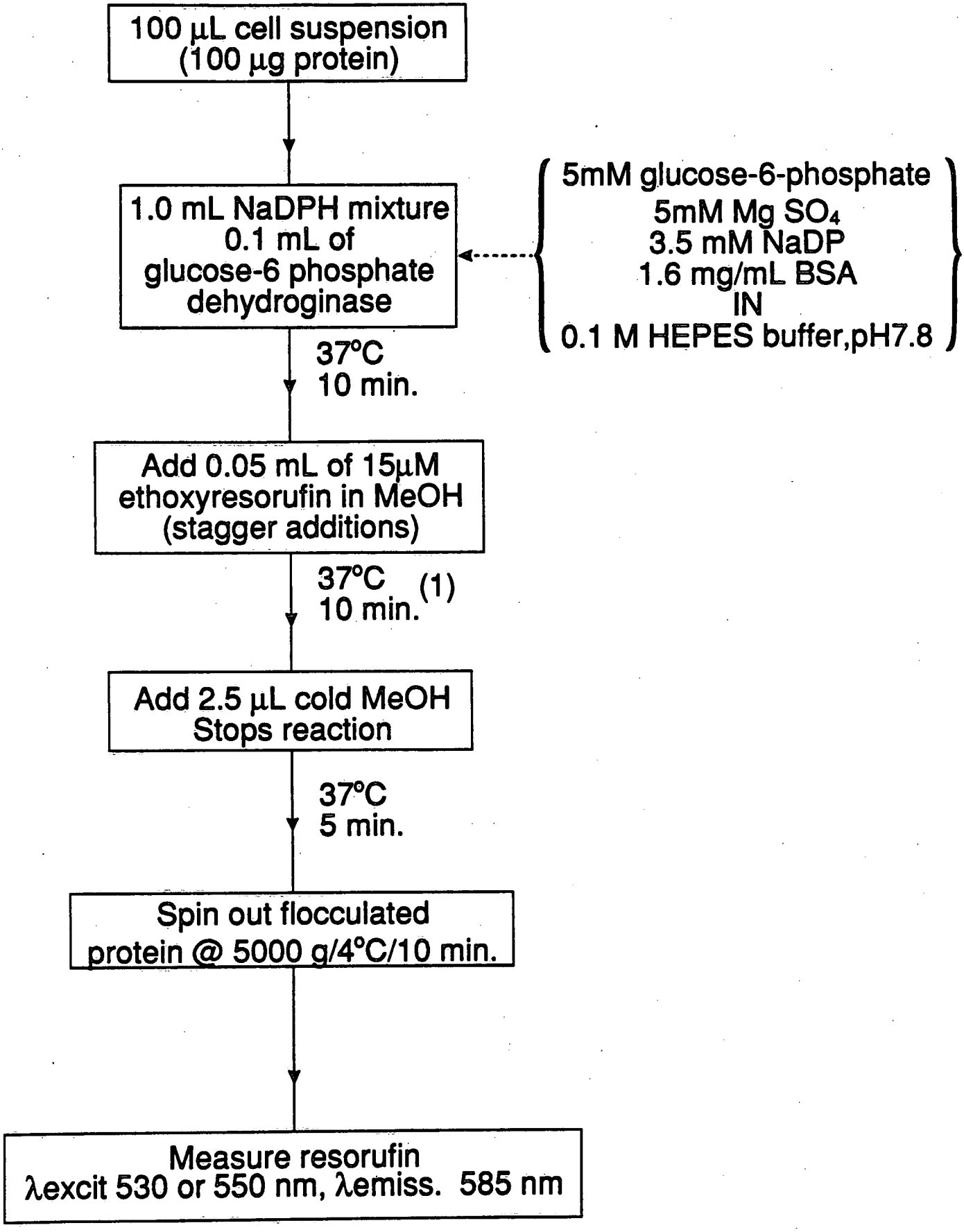
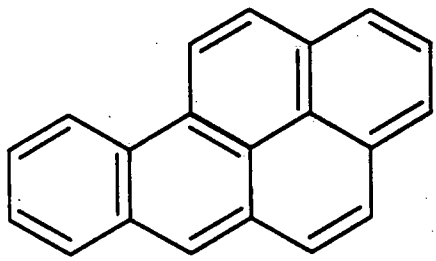

FRACTIONAL RESPONSE - AHH ACTIVITY
1.0
0.8
0.6
0.4
0.2
0
ED50 = 0.14
0.01
0.1
1.0
10.0
pmol TCDD

FIGURE 20.



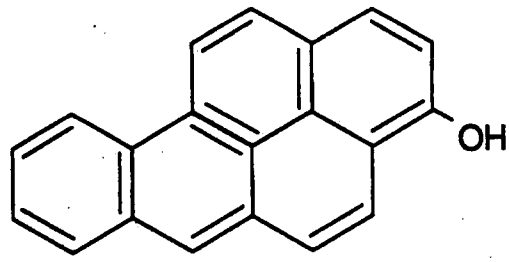
(1) Response must be linear with time

FIGURE 21.



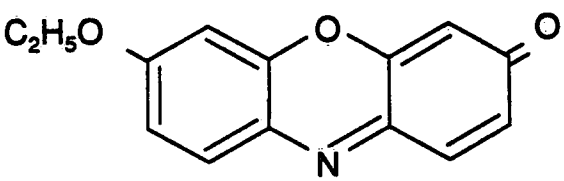
Benzo (α) pyrene

AHH



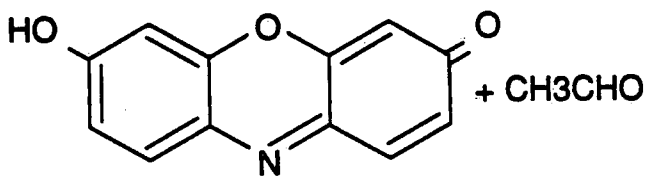
3 - hydroxybenzo[α]pyrene

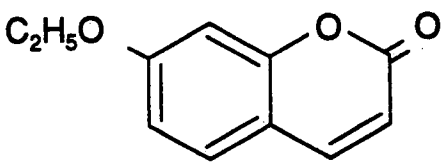
Other hydroxy derivatives & phenolic compounds



Ethoxy resorufin[1]

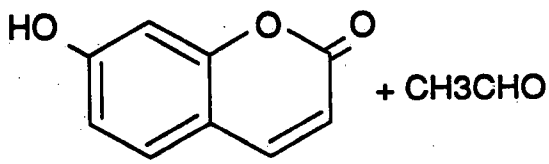
EROD





Ethoxycoumarin[2]

ECOD



[1] available from Sigma
[2] available from Aldrich

FIGURE 22.

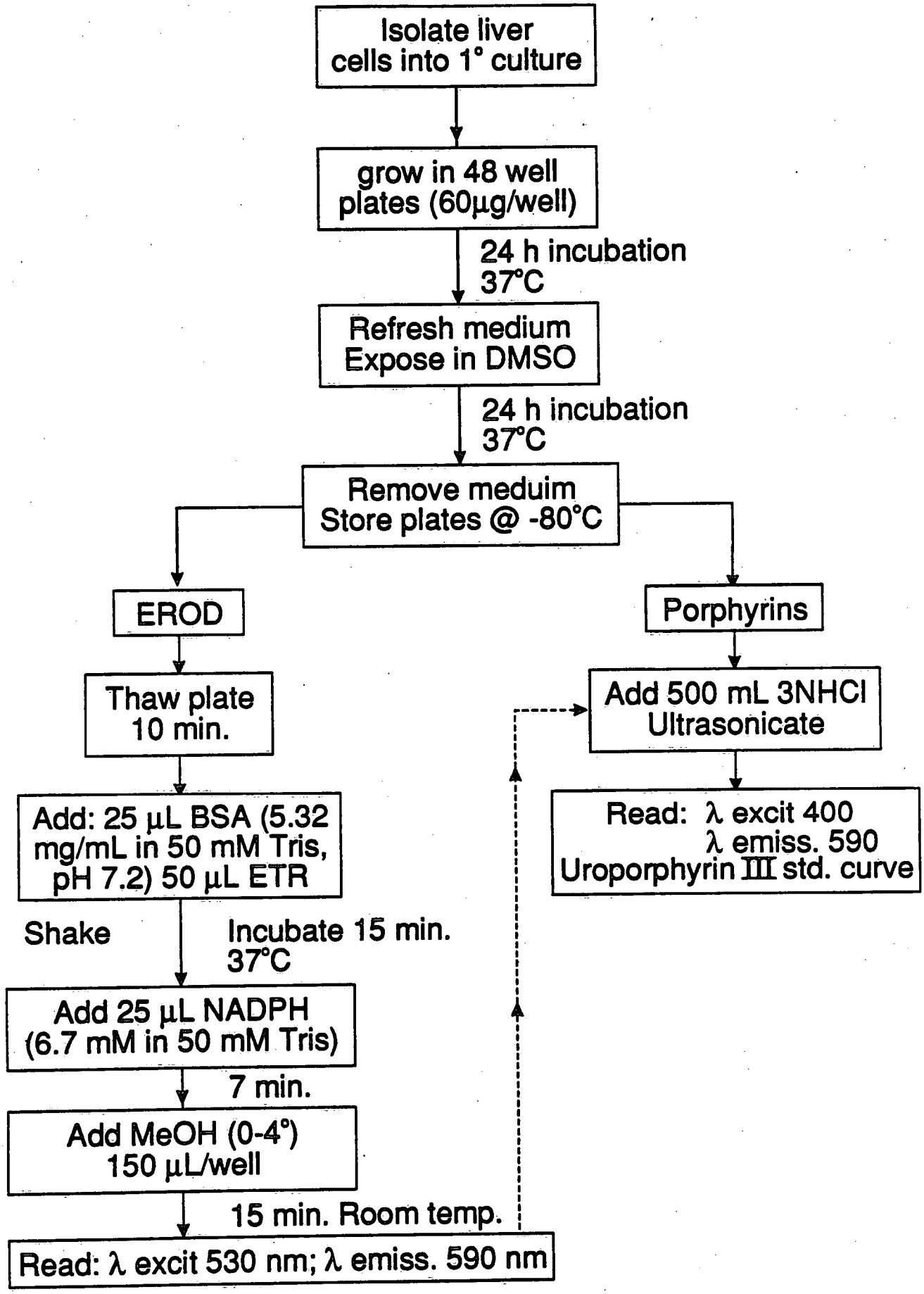
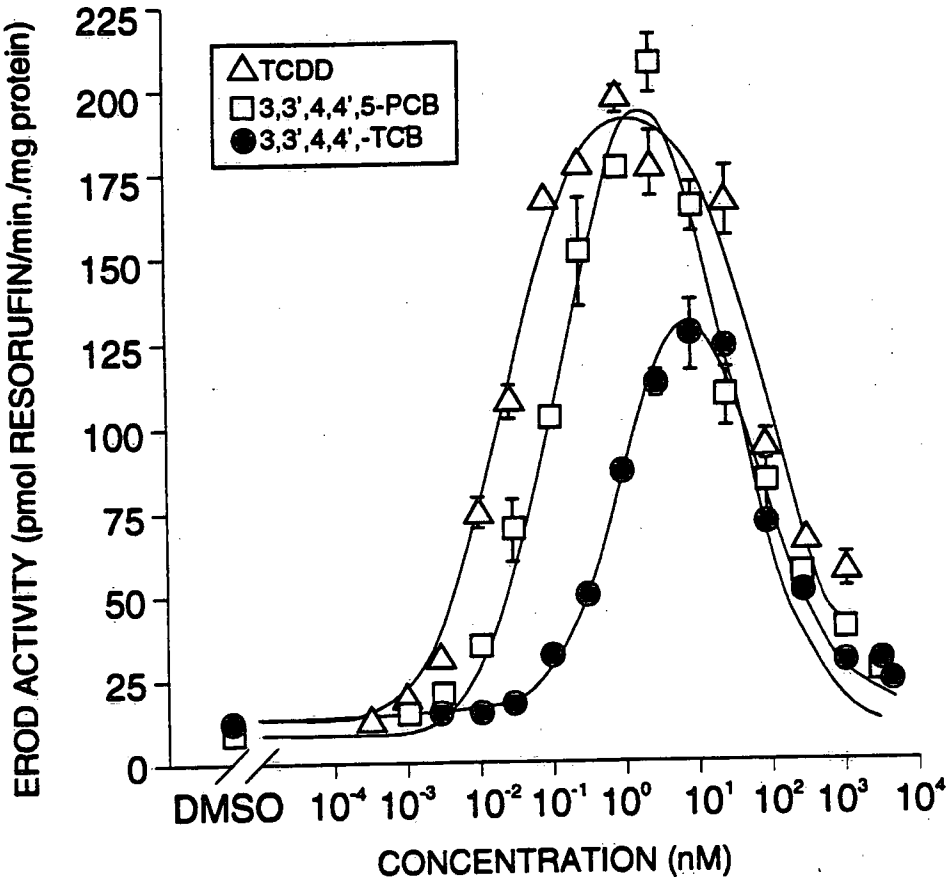

Isolate liver cells into 1° culture
grow in 48 well plates (60µg/well)
24 h incubation 37°C
Refresh medium Expose in DMSO
24 h incubation 37°C
Remove meduim Store plates @ -80°C
EROD
Thaw plate 10 min.
Add: 25 µL BSA (5.32 mg/mL in 50 mM Tris, pH 7.2) 50 µL ETR
Shake
Incubate 15 min. 37°C
Add 25 µL NADPH (6.7 mM in 50 mM Tris)
7 min.
Add MeOH (0-4°) 150 µL/well
15 min. Room temp.
Read: λ excit 530 nm; λ emiss. 590 nm
Porphyrins
Add 500 mL 3NHCl Ultrasonicate
Read: λ excit 400 λ emiss. 590 Uroporphyrin III std. curve

FIGURE 23.


EROD ACTIVITY (pmol RESORUFIN/min./mg protein)
225
200
175
150
125
100
75
50
25
0
TCDD
3,3',4,4',5-PCB
3,3',4,4',-TCB
DMSO
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
CONCENTRATION (nM)

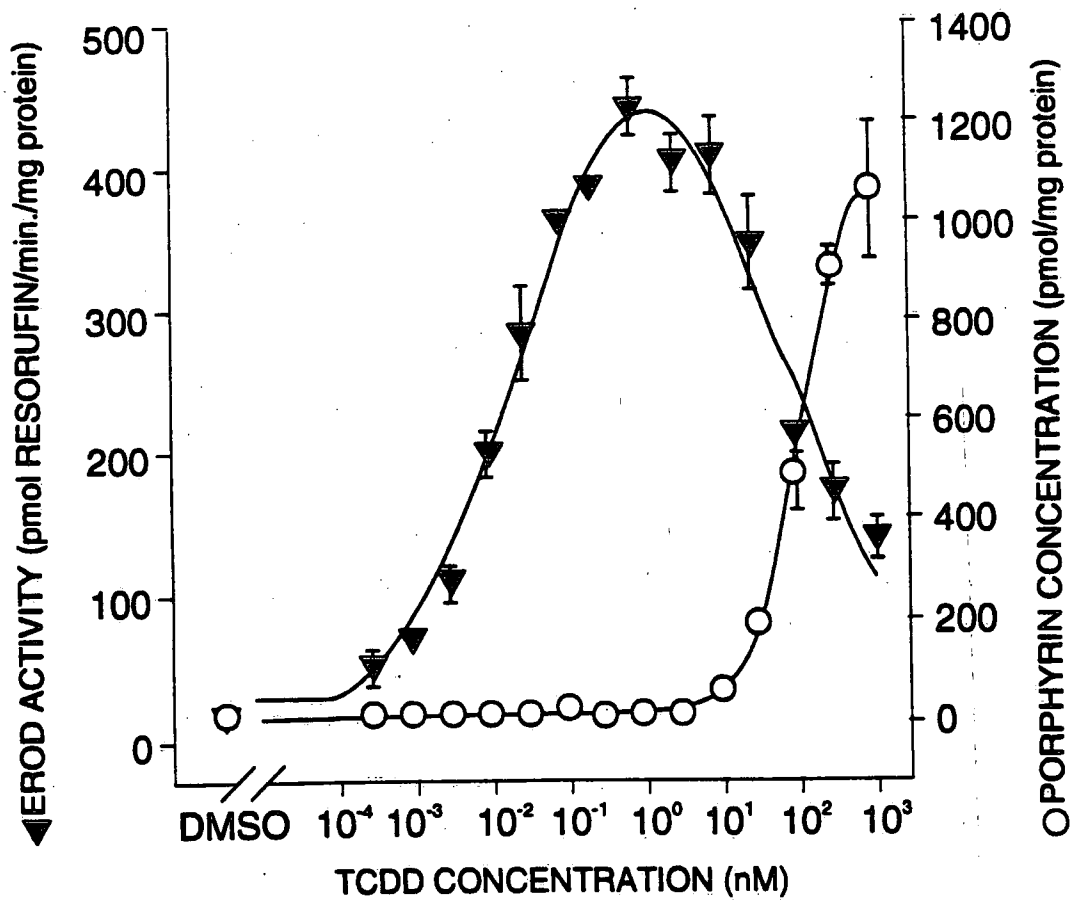
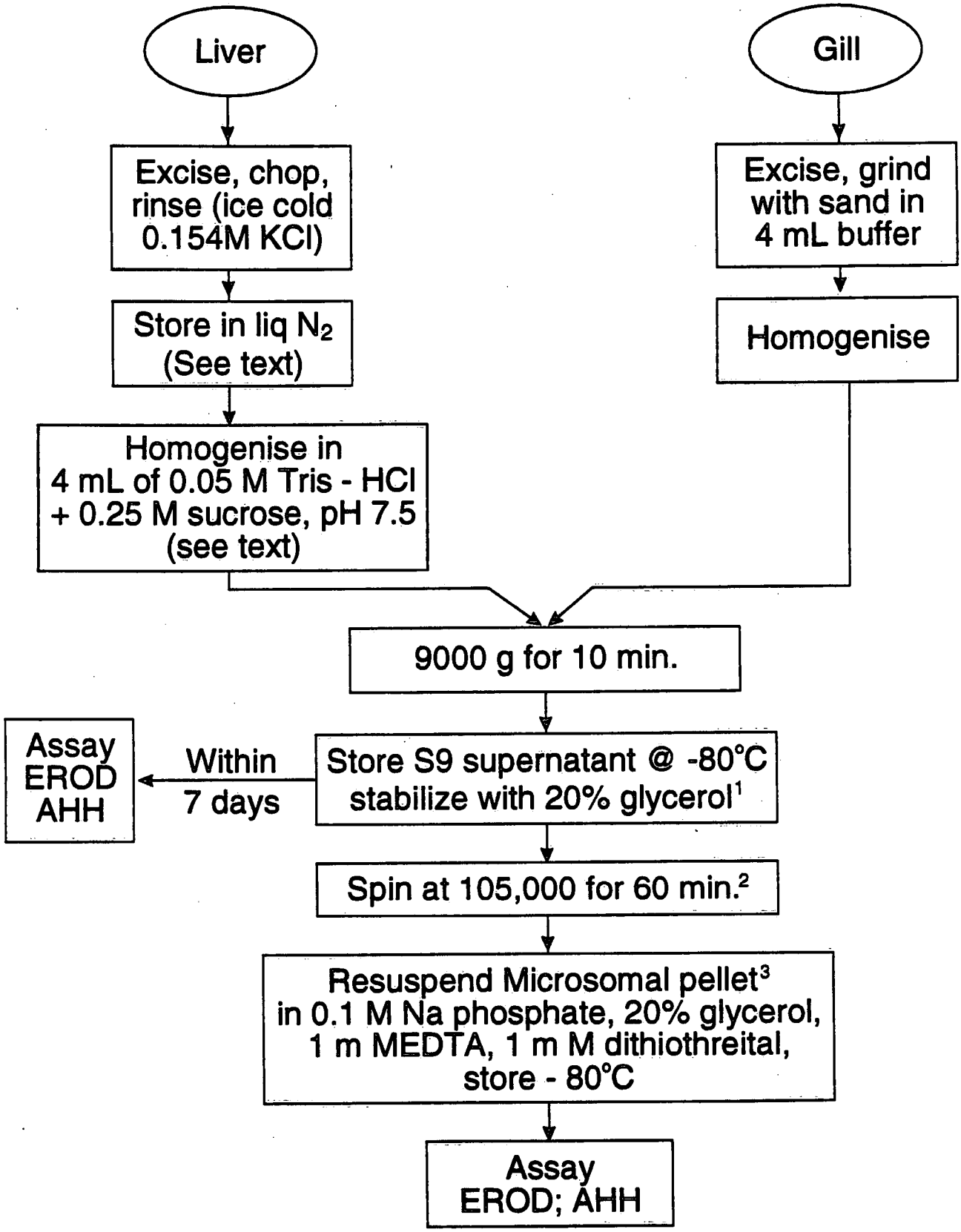

EROD ACTIVITY (pmol RESORUFIN/min./mg protein)
500
400
300
200
100
0
1400
1200
1000
800
600
400
200
0
PORPHYRIN CONCENTRATION (pmol/mg protein)
DMSO
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
TCDD CONCENTRATION (nM)

FIGURE 24.



All steps at 0 - 4°C

FIGURE 25.

ELISA assay overview

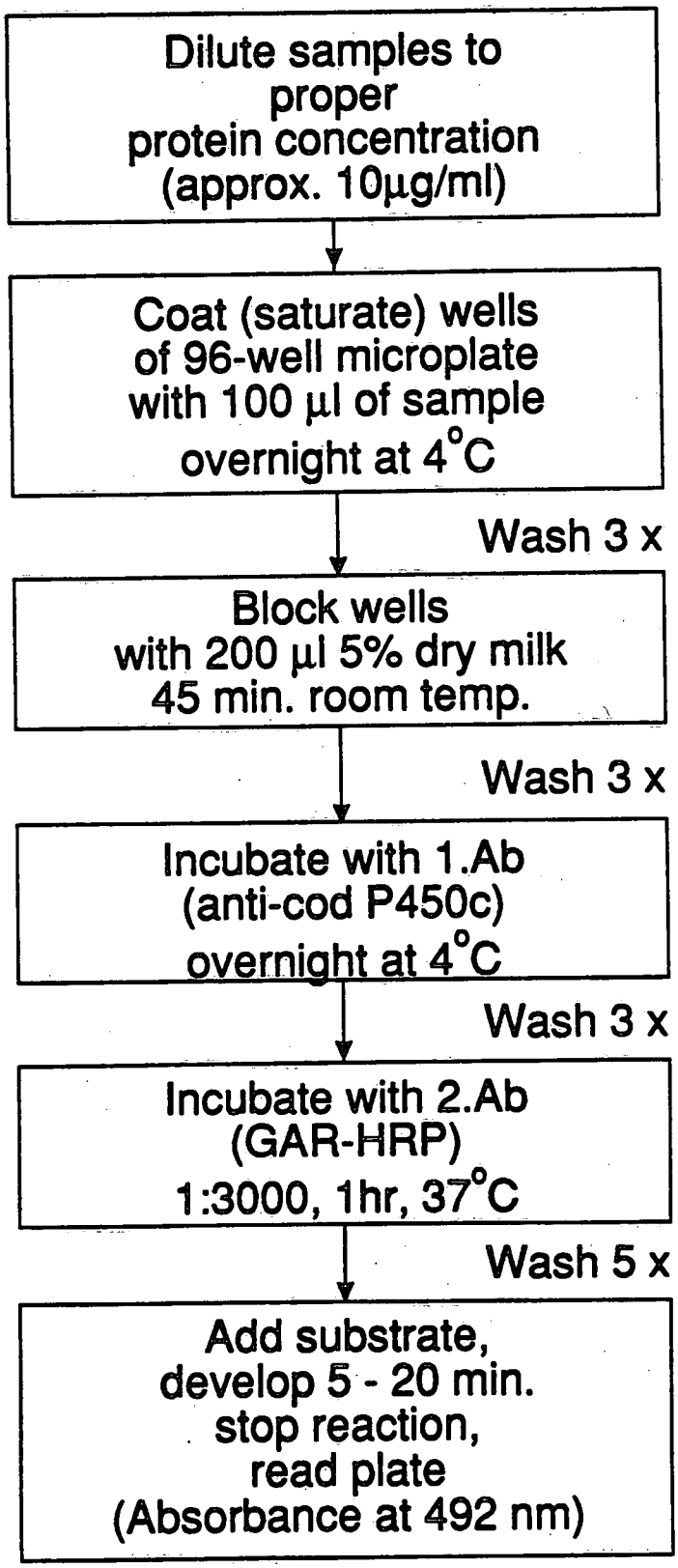
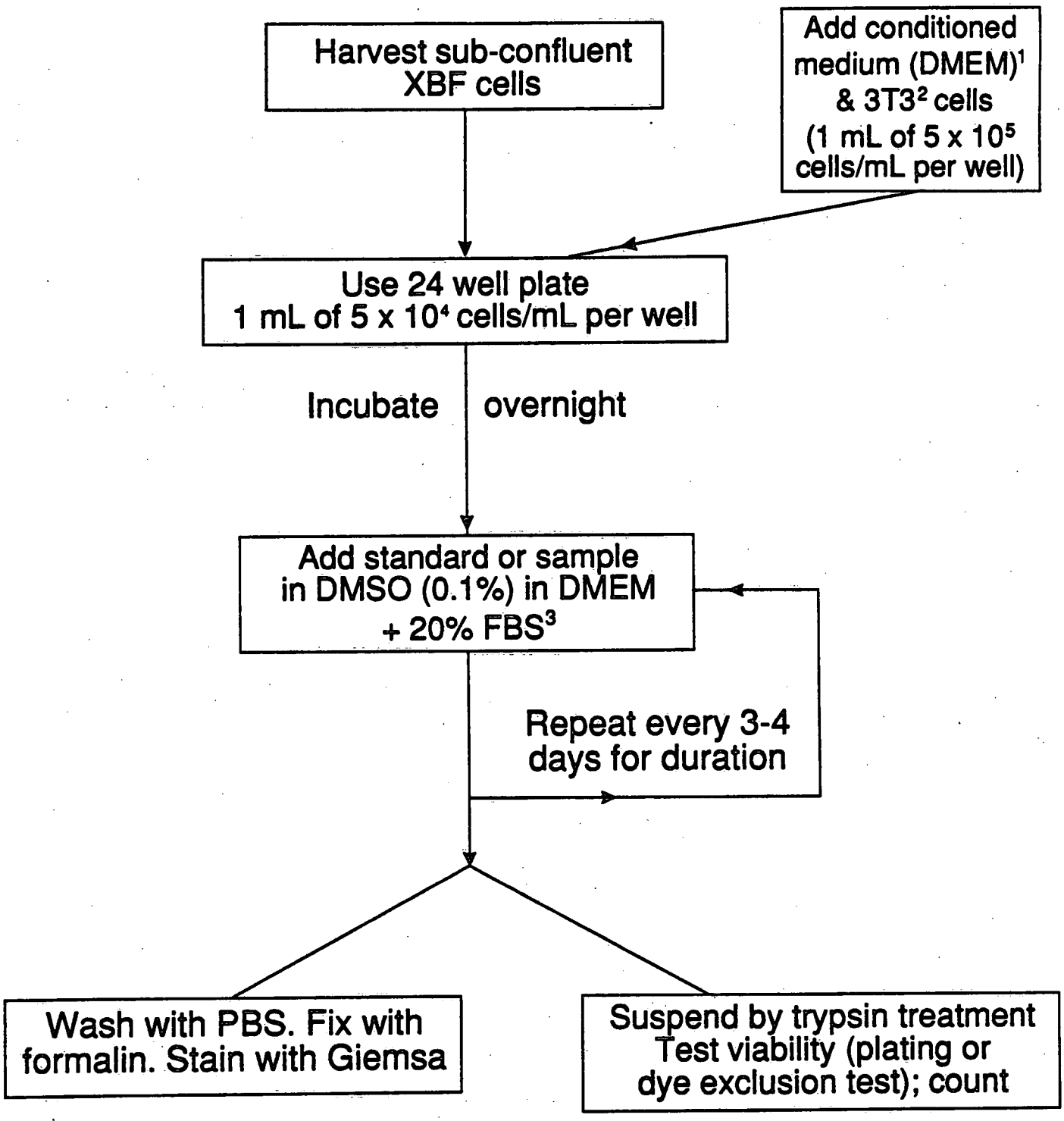


FIGURE 26.



[1] DMEM = Dulbecco's modified eagle medium
[2] 3T3 cells are irradiated with 6000 rads from a cesium source
[3] FBS = fetal bovine serum

FIGURE 27.

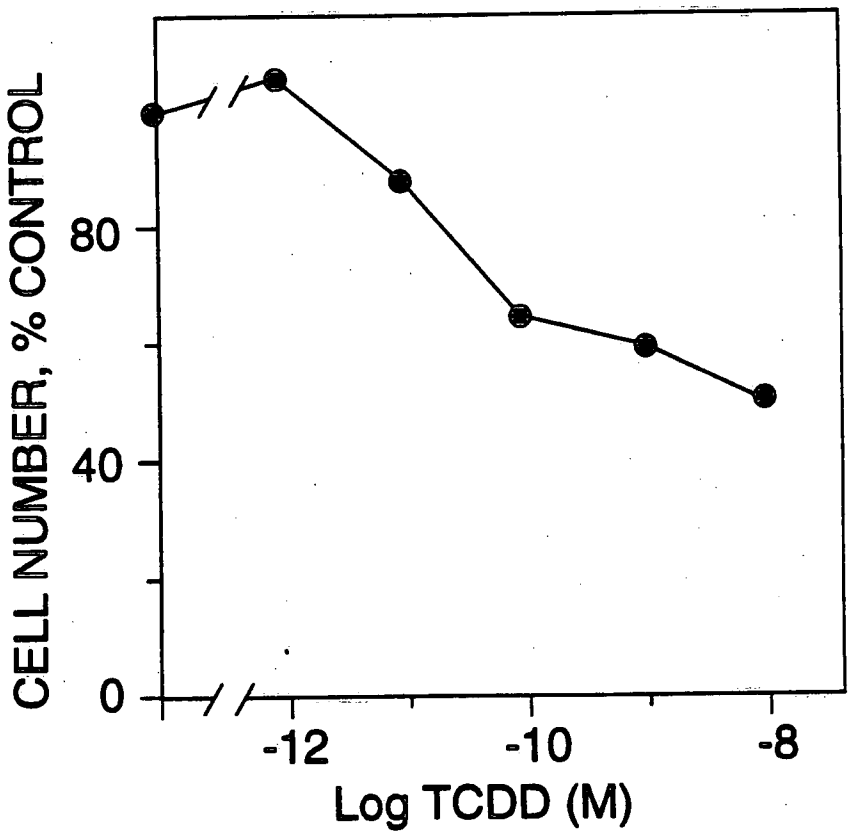


FIGURE 28.

NATIONAL WATER RESEARCH INSTITUTE
P.O. BOX 5050, BURLINGTON, ONTARIO L7R 4A6

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INSTITUT NATIONAL DE RECHERCHE SUR LES EAUX
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