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Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

Issues associated with the validation of *in vitro* and *in vivo* methods for assessing endocrine disrupting chemicals

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Abstract: The concepts requiring validation in the subject of endocrine disruption are listed and discussed. The main mechanisms by which endocrine disruption can occur are identified, and the assays required for the detection of adverse endocrine disruption toxicities, associated with these mechanisms, are discussed. The process of assay validation is considered within the context of the criteria established by Balls & Karcher. The validation of structure activity relationships, the need for reference chemicals, and the problems recently encountered when attempting to reproduce endocrine disruption data are also explored. The most important conclusions to derive from this analysis are first, that given the immature state of research into endocrine disruption toxicity, testing strategies and the types of assay employed should be kept under constant review, with the inevitable need for future revision of each being accepted from the outset. Second, that given the current absence of any chemical universally accepted as being devoid of endocrine toxicity, assay specificity will be impossible to assess, and that imposes the need for alternative objective criteria for assessing the value of individual assays.

INTRODUCTION

The word validation implies the need to ensure that an article/assumption is sound, genuine, logical, satisfactory, authoritative, convincing. These words provide a good starting point for this chapter, but they are without meaning in the absence of description of that which is to be validated. At the simplest level (as encountered in the lay press), the perception is that some environmental chemicals may be capable of disturbing the endocrine system of animals leading to adverse reproductive or developmental outcomes, and that this potential to disturb can be assessed using a few standard assays that require only minimal validation before they can be routinely deployed. In fact, the issue in question is highly complex and a range of distinct validation needs are evident. At the most fundamental level there is a need to validate the basic assumption that the reproductive capacity and sexual development of humans and/or wildlife species have already been, or in future could be, compromised by ambient levels of exposure to endocrine disrupting (ED)† chemicals. Certain adverse human and wildlife effects have been associated with chemical exposures, but proof of causation usually remains for study. The validation process required to transform ED associations into ED causations has been discussed (1) within the context of the hypothesis testing criteria of Hill (2). This critical validation need is not pursued further herein because causation has already been assumed by a sufficient number of regulatory authorities to require the development of assays for ED activities and the formulation of testing strategies/legislative testing guidelines.

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† ED is used to represent endocrine disruption and/or endocrine disrupting throughout the text.

Once it is assumed that some environmental chemicals may present a hazard to the endocrine system of one or more organisms, several additional needs present themselves. The most important of these is the need to devise and validate a testing framework that will include assays to cover the several possible mechanisms by which chemically-induced reproductive or sexual development toxicities can be produced. At present these assays fall into four major classes:

- *In vitro* assays that determine the ability of an agent to interact with natural hormone receptors
- *In vivo* assays to measure disturbances to the normal biochemical synthesis and degradation of the natural steroid hormones.
- Assays to determine adverse ED effects in mammals.
- Additional tests using wildlife species, as individually considered appropriate (see further discussion under Testing Strategies later herein).

Validation of the final list of assays will involve the collection of test data to justify the inclusion or exclusion of individual assays, and agreement on the types of organism to be monitored. In addition, the assays selected for routine use will require to be validated in order to establish that they are sensitive to reference endocrine disrupters operating by the appropriate mechanism, and to confirm that they are practical to conduct and give reproducible test data within and between laboratories. This last validation need requires the existence of a library of reference chemicals known to possess the adverse ED activities being predicted, together with agents known to be inactive in these respects.

It is relevant to note at this point that the above approach to validation could have been usefully applied to the study of environmental carcinogens. However, in that endeavour the mistakes listed below were made:

1 The risk posed to humans by exposure to animal carcinogens was assumed and not proven, and has since been seriously questioned.

2 A range of different testing strategies were applied retrospectively, and then only when it was realised that different countries were employing different predictive assays, usually for different purposes.

3 Chemical carcinogenicity was initially assumed to represent a singular property of chemicals. That led to a fruitless search for a single ‘wonder assay’ for all possible types of rodent /human carcinogen.

4 Evaluation of the practicality and reproducibility of assays was left until a late stage, by when many unreliable/unjustified assays had been incorporated into the regulatory guidelines of some countries.

A repetition of these same mistakes in the prediction of endocrine disruptors can only be avoided by adherence to an internationally agreed validation process.

MECHANISMS OF ED

The several mechanisms by which endocrine disruption may occur will ultimately dictate the types of assay required for the detection/assessment of endocrine disrupting chemicals (see testing strategies, later herein). The mechanisms currently being considered by the United States EPA Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (3) are represented in Figure 1. These mechanisms include either disturbance of the biochemical production and/or destruction of the natural hormones oestradiol, dihydrotestosterone and triiodothyroxine, or competitive agonist/antagonist activities of xenobiotic chemicals at the naturally-occurring receptors for these hormones [the oestrogen receptor (ER), the androgen receptor (AR) and the thyroid hormone receptor (THR), respectively]. To date, most attention, and most data, have been concerned with ER agonists and AR antagonists, but there is general agreement that these are but two of several potential mechanisms of ED toxicity. There is an essentially endless list of possible ways of altering the biochemical production/destruction/homeostasis of the three natural hormones under consideration, but available evidence only supports this concern for the inhibition of either dihydrotestosterone or oestradiol production (e.g., by inhibition of the cytochrome P450 enzymes 5α-reductase and aromatase, respectively; see Figure 1)

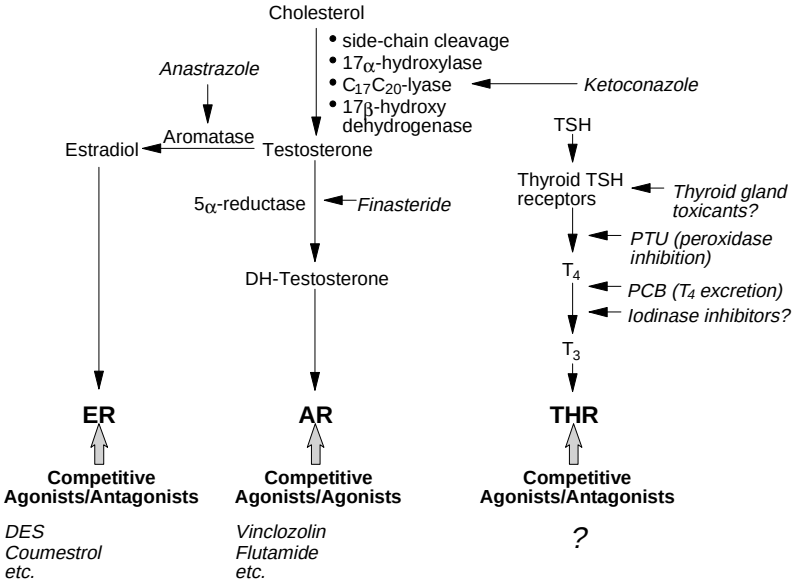

Fig. 1. Biosynthesis of natural hormones affecting the oestrogen receptor (ER), the androgen receptor (AR) and the thyroid hormone receptor (THR) and chemicals known, or predicted, to modify these normal functions. TSH = thyroid stimulating hormone. PTU = 6-propylthiouracil, PCB = polychlorinated biphenyls.

Concerns regarding the disturbance of thyroid gland function are less well defined, but are based on the anti-thyroid (hypo-thyroid) activities of 6-propylthiourea and the PCBs, each activity leading to increased testis size in treated rodents (4,5). These and other possible ways in which disturbance of the normal functioning of the thyroid gland could be induced are shown in Figure 1. It is noticeable, that to date, no ED activities have been associated with THR agonists/antagonists, with hyper-thyroid chemicals or with agents capable of inhibiting iodinase enzymes (responsible for the conversion of T_4 to T_3 in the liver).

The extent to which these many activities of chemicals can be assessed using *in vitro* assays will need to be carefully considered and validated. On the one hand, receptor-mediated effects seem optimal for assessment using appropriate *in vitro* assays (particularly when such assays are made to be metabolically competent); in contrast disturbances in steroidogenesis and thyroid gland function would seem best suited to evaluation in the whole organism. It is critical that the difference between an activity (e.g., ER binding, enzyme inhibition) and an adverse toxicity (e.g., reduced testes size, increased day of vaginal opening) be carefully distinguished when attempting to validate assays, in particular, those conducted *in vitro.*

When considering hormone receptor-mediated ED toxicities there are four main receptors currently under consideration, ERα, ERβ (6,7), AR and THR. In addition, there are two ways in which the normal functioning of these receptors in their natural environment can be modified—competitive receptor agonism and receptor antagonism (Figure 1). These eight points of endocrine disturbance currently suggest the need for eight separate assays. The following points, however, argue against this and indicate the need for a constant review of the assays actually required. First, the assumed discrete nature of ER/AR agonist/antagonist properties of chemicals underlies the EDSTAC proposal to monitor these activities separately for each chemical (3). However, the knowledge-base supporting this quadrupling of testing effort is thin, and is often not supportive. For example, the simple model assumed by the current EDSTAC approach is that some chemicals will act as either pure agonists, or pure antagonists, solely as a function of their chemical structures. An alternative prospect, with very different implications for testing, is that interaction of a chemical with ER is the only useful thing that can be discerned *in vitro*, with the study of agonist/antagonist effects only being approachable in intact organisms. Supporting this proposition, Horwitz (8) has reviewed the range of ER-related responses elicited by tamoxifen in humans and has shown that they are dependent upon the tissue being monitored and the duration of dosing. Similar conclusions apply to the selective oestrogen raloxifene (9–11). Further, Willson *et al.* (12) have

shown that GW5638, an ER antagonist *in vitro*, and in the rat uterus *in vivo*, acts as a full ER agonist in the rat bone and cardiovascular system. Such subtle responses indicate that receptor agonist/antagonist activities are not a function of the chemical structure of an agent, but rather, a function of the receptor response element and/or the tissue under study (i.e., these are toxico-dynamic and pharmaco-dynamic issues). If this is so, the critical need becomes the study of how chemicals interact with ER/AR in a range of different genetic and/or tissue environments (13). Despite these concerns the current concept that receptor agonist/antagonist effects reflect intrinsic properties of chemicals (as opposed to biological environments) is maintained in Table 1 and Figure 2. The further complexity engendered by the recent recognition of two forms of the oestrogen receptor (6), the tissue- and chemical-specific responses of these receptors (7), and the recognition that several oestrogens can also function as anti-androgens (14), will inevitably complicate this matter further; thus the need for constant review (validation) of ED assays and testing strategies.

Table 1. Sentinel chemicals representing different mechanisms of endocrine disruption. These agents are suggested as suitable for the initial validation of ED assays. Documentation of the adverse properties of these chemicals will be necessary before they are adopted (see Table 2).

Agent	Proposed mechanism
Methoxychlor	Synthetic oestrogen
Diethylstilœstrol	Synthetic oestrogen
Genestein	Phyto-oestrogen
Coumestrol	Phyto-oestrogen
ICI 182,164	Anti-oestrogen
Dihydrotestosterone	Androgen
Flutamide	Anti-androgen
Cyproterone acetate	Anti-androgen
Vinclozolin	Anti-androgen
p,p'-DDE	Anti-androgen
Ketoconazole	P450 inhibitor
Diethylstilboestrol	P450c17 inhibitor
Finasteride	5α-reductase inhibitor
Anastrazole	Aromatase inhibitor
PCBs	Anti-thyroid activity
6-Thiouracil	Anti-thyroid activity

THE PROCESS OF ASSAY VALIDATION

The process to be followed when validating a test method has been discussed in detail by Balls & Karcher (15), based on the conclusions of a series of workshops. The advice given is particularly relevant to the validation of endocrine disruption assays, and the essence of that advice is endorsed and reproduced here.

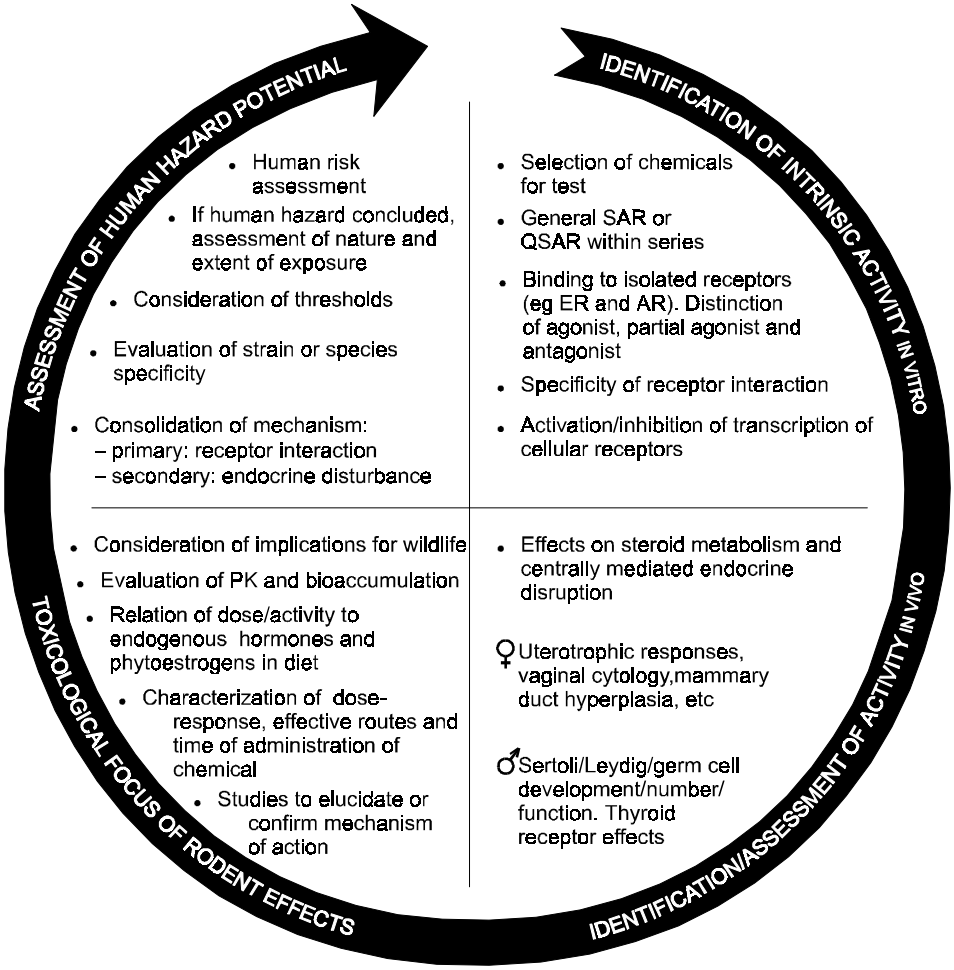

Fig. 2. Framework for the development of a sequential approach to the evaluation of chemicals for endocrine disrupting potential in humans. SAR, structure activity relationships; QSAR, quantitative SAR; ER and AR, estrogen and androgen receptors; PK, pharmacokinetics. Reproduced with permission (31).

Five main stages in the evolution of new test methods were identified: test development (in laboratory of origin), prevalidation (involving an informal interlaboratory study), validation (involving a formal interlaboratory study), independent assessment (of study and proposals) and progression toward regulatory acceptance.

Test development

The following criteria were suggested to be met before a method is considered ready to enter the validation process:

1 A description of the basis of the method coupled with a clear specification of endpoint, endpoint measurement, derivation and expression of results, and their interpretation and application.

2 A definition of its scientific purpose.

3 The case for its relevance and its proposed practical application.

4 An explanation of the need for it in relation to type and extent of effects, levels of assessment, and availability of other methods.

5 The availability of an optimised protocol with a standard operating procedures, including use of adequate control groups.

6 A clear statement about limitations.

7 Evidence of intralaboratory reproducibility and of interlaboratory transferability..

Prevalidation

The proposed prevalidation scheme involves collaboration between established and competent laboratories, as follows: Phase I: protocol refinement; Phase II: protocol transfer, and Phase III: protocol performance.

Validation/independent assessment

The criteria for evaluating a validation study were suggested to include consideration of the following:

1 Clarity of defined goals.

2 Quality of overall design.

3 Independence of management.

4 Independence of selection, coding and distribution of test materials.

5 Independence of data collection and analysis.

6 Number and properties of test materials studied.

7 Quality of interpretation of results.

8 Performance of methods in relation to goals of the study.

9 Reporting of outcome in the peer-review literature.

10 Availability of raw data.

11 Independence of assessment of outcome.

These are stringent requirements that cannot easily be circumvented without prejudicing attempts to rapidly develop reliable assays for endocrine disruption.

TEST ORGANISMS

The need to study both humans and wildlife species has been recognised from the outset (16,17). The study of human effects is best conducted in humans; however, as in other branches of toxicology, laboratory species such as the rat and the mouse will be employed as human surrogates. In contrast, the extent to which rodent data can be used to anticipate possible ED effects in all wildlife species has yet to be resolved. The limited data available indicate that ED hazards to wild mammals can be predicted using the approach adopted for human hazard assessment, but invertebrate, avian and fish may not always be covered by this approach. Recent reviews (18,19) have considered this topic in detail, including suggestions as to which sentinel wildlife species would be most appropriate for use. Perhaps the most important point raised in those reviews was the current paucity of knowledge of the working of the endocrine system of most wildlife species, in particular, of invertebrates (19). In practice, this means that assessment of non-mammalian ED effects will be extremely difficult in the immediate future, and in this area, the concept of validation will be concerned more with the selection and study of sentinel species than with the sensitivity and performance of assays based on those species.

STRUCTURE ACTIVITY RELATIONSHIPS (SAR)

Different SAR will be required for each mechanism of ED; there will be no general SAR of ED. The only qualification to this is that there is growing evidence for overlap between receptors; thus SAR for ER may have some implications for the SAR of AR, etc. The validation of SAR in ED will therefore require a clear definition of the mechanism of ED involved, and specific clarification of the level of biological data being considered. For example, the SAR of ER binding *in vitro* will be different to the

SAR of activity in a rodent uterotrophic assay, despite the facts that the latter assay monitors ER activity and the former assay is predictive of activity in the latter assay. SAR for the oestrogen receptor is discussed below to illustrate the potential complexity of what is often regarded as the simplest of predictive assays. SAR for ER interactions is currently anchored to the chemical structure of oestradiol (E_2) itself (20), and the manner of its interaction with the ER. Thus, the molecular shape of E_2 enables the oestrogenic activity of the 'look-alike' hydroxylated polychlorinated biphenyls to be estimated (21). However, Connor *et al*. (21) concluded as follows: '*structure-oestrogenicity/anti-oestrogenicity relationships for the eight compounds studied were complex and response-specific. The structure-ER binding relationships were different in the rat and the mouse, and no dose-dependent oestrogenic activities were observed in the rat or mouse uterus. Several compounds exhibited anti-oestrogenic activity and two inhibited progesterone receptor (PR) binding in the mouse uterus'*. This complex picture is further confused by our recent finding that estratriene (E_2 with both of its -OH groups removed) is a potent oestrogen both *in vitro* and *in vivo* (Ashby & Sumpter, unpublished, 1997). This complexity indicates that computerised structural databases based on the chemical structure of the sex hormones may alert to obvious structural analogues, but at present they will be unable to venture into the area of real need, the prediction of activity for structurally remote analogs of E_2 (e.g., kepone and dieldrin), or of testosterone (e.g., vinclozolin) (22,23).

REFERENCE CHEMICALS

Assessment of assay sensitivity and specificity requires access to established ED agents, and agents agreed to be devoid of ED activities. The composition of these two groups of chemicals has yet to be agreed upon.

Reference ED agents. The tabulation of known ED active chemicals is an easier task, but one that is not without its own problems. To the extent that a set of genuinely distinct mechanisms of ED toxicity exist [e.g., interaction with the oestrogen receptor (ER)] it should be possible to select representative chemicals for each mechanism for validation of the appropriate assay. The problem immediately encountered with this approach, using the same example, is that although many chemicals are known to interact with oestrogen receptors *in vitro*, the number of these that have produced sentinel ER-mediated toxicities in animals or wildlife species is limited. For example, despite the fact that many studies have established the capability of bisphenol-A to produce *effects* associated with its interaction with ER *in vitro*, it is difficult to identify *confirmed* toxicities causally aligned with this property; a critical distinction. An analogy derived from carcinogen screening experience is that some *in vitro* assays that found non-carcinogens positive tended to be justified by reference to similar activities found for the chemical in question in other *in vitro* assays—a dangerous cyclic process. This problem was discussed by Gina Solomon during a recent US EDSTAC meeting, as reported by Endocrine/Oestrogen Letter (3): '*if you require that something has to have an adverse effect before labelling it as an endocrine disrupter, then you are shifting the level of scientific proof needed to a very high level. It is not hard to show that something mimics oestrogen, but to show that it also causes an adverse effect is surprisingly tricky'*. That dilemma is probably the most serious being faced at present.

An illustration of the difficulties discussed above is provided by the data shown in Table 2. This Table is based on illustrative slides shown at a recent meeting on the development of ED assays. The reference ED chemicals shown in Table 2 were tentatively suggested as suitable for the validation of assays for oestrogens and antiandrogens. Four things are of interest about this list of chemicals. First, they provide a good starting point. Second, they only cover ER- and AR-mediated ED toxicities. Third, there is clearly a strong element of repetition of chemical types in the list, a situation that requires to be justified in order to conserve resources. Fourth, and of greatest importance, only the chemical names and the broad classification of ER- or AR-mediated effects were provided. The remaining empty columns in Table 2 must be completed before the calibrant sentinel toxicities in question can be defined in detail (a task not attempted here and which will reveal that some of these chemicals have not been established as producing adverse toxic effects, despite their ability to interact with hormone receptors in model systems). Completion of Table 2 will also enable the many hidden traps of validation to be recognised.

For example, if an agent has been defined as giving adverse ED effects in the mouse following dosing for 28 days *via* subcutaneous injection, it would be unwise to over-interpret a negative predictive assay response obtained following a single oral administration of the chemical to rats. Like must be compared to like, and this is only possible when the calibrant toxicity data have been adequately analysed and presented.

Table 2. Agents suggested to be of use when validating ED assays (adapted from slides presented at a recent conference). The many empty boxes in this Table require to be completed before these chemicals can be regarded as, or used as, calibrant chemicals for ED assays.

Chemical	Calibrant adverse ED effect	Condition of observation of adverse effects				Probable mechanism of effect	Assay required to detect effect, and response expected
		Species/ strain	Duration of dosing	Dose level	Route		
Oestradiol 17β						ER mediated (stated)	
Estrone							
Tamoxifen; hydroxy tamoxifen							
Nafoxidine							
ICI 182,164							
Chlordecone							
Methoxychlor							
o,p'DDT; p,p'DDT							
Bisphenol A							
Coumestrol							
Genistein							
β Sitosterol							
Equol							
Zearalenone; zeranol							
DES							
Benzyl butylphthalate							
Chlordane; endosulphan							
Lindane							
Octyl phenol; nonyl phenol							
Testosterone						AR mediated (stated)	
Dihydrotestosterone							
Corticosterone							
Progesterone							
Aldosterone							
Levonogestrel							
Flutamide; hydroxyflutamide							
Norgestrel							
Norethridone							
Cyproterone acetate							
Vinclozolin							
Procymidone							
p,p′-DDE							

An initial list of calibrant ED chemicals is suggested in Table 1, based on the mechanisms of ED toxicity illustrated in Figure 1. These chemicals have adequate evidence of producing adverse ED-mediated toxicities *in vivo*, but those data still require to be documented, as described above. The agents shown in Table 1 do not include those chemicals that may be capable of affecting uniquely invertebrate endocrine systems. The number of chemicals capable of disturbing thyroid gland function is currently limited to the two agents shown in Table 1 [6-thiouracil and polychlorinated biphenyls, both of which increase testis weight in rats (4,5)].

Agents devoid of ED activities. At present there are few, if any, agents generally agreed to have no ED activities, and this is due to the current absence of chemicals classified as negative in a rodent multi-generation study conducted according to a protocol that includes the recently recommended additional markers of sexual development. As a consequence, assessment of the specificity of ED assays will be difficult in the immediate future. Nonetheless, the need for such data when assessing newly derived assays cannot be over-emphasised. In the absence of such agents, the response given to a chemical by a new ED assay will not be predictive of activity, but definitive of activity (no false positive predictions having been shown for the assay). For example, it is often stated at conferences that the MCF7 assay (the E-screen) has given no false positive responses to date, a meaningless statement in the absence of named chemicals inactive as endocrine disrupters.

LISTS OF ENDOCRINE DISRUPTING AGENTS

To legitimately be on a list of ED agents it is necessary that the chemical has been shown to disrupt an endocrine system, and that implies the availability of test data from an organism with an intact endocrine system. In the absence of such data, potential ED agents may be added to lists of oestrogens, androgens or progestins, for example, based on their ability to bind to the appropriate isolated receptor. The latter lists will be of little toxicological value, but they will be valuable for prioritising agents for further study. The illusory value of lists of chemicals has recently been documented by Scialli (24) in an article of immediate relevance to endocrine disrupters—'*Identifying Teratogens: The Tyranny of Lists*'.

REPRODUCIBILITY OF ENDOCRINE DISRUPTION DATA

The many problems encountered when attempting to confirm independently reported ED effects has been commented on earlier (25), and this can be expected to present a continuing challenge to all investigators in this field. This is primarily because the possible activities under study encompass virtually the whole of biology—few biological functions being totally independent of gender status and the degree of sexual development or senescence of the organism under study. Thus, a chemical may alter the body weight of a developing animal leading to a change in the timing of sexual maturation, and in such situations it will be difficult to associate the sexual change with either the body weight change or any intrinsic ED activities the chemical may possess. A related example is the demonstration by Steinmetz *et al.* (26) that both E_2 and bis phenol A (BPA) are able to increase plasma prolactin levels in F344 rats, but not in SD rats. It is easy to imagine results from these two strains of rat being separately derived and reported, thereby leading to the impression of a conflict between laboratories and investigators. It also remains possible that a subtle difference between sub-strains of Wistar rats may be at the root of the failure to replicate the ability of BBP to reduce testis size in rats (27,28). Adding to this potential complexity is the fact that little is currently known about many of the biological processes currently being implicitly investigated in ED research. For example, Shull *et al.* (29) have shown that ACI rats develop mammary gland cancer upon exposure to E_2, leading to the expectation that ovariectomized ACI rats, exposed to the same net plasma concentrations of E_2 experienced in the treated intact animals, would develop a similar incidence of mammary gland tumours. In fact, the ovariectomized animals were resistant to the carcinogenicity of oestradiol. This finding led Shull *et al.* (29) to consider the complementary role of progesterone in oestradiol-induced mammary gland carcinogenesis. The significance of these results lies in the fact that ovariectomy would hitherto have been considered to represent a potentially useful model for studying the effects of xenobiotic oestrogens on the rodent mammary gland—yet this is clearly not the case. Examples such as these illustrate that current attempts to study endocrine disruption are being made against a background of significant ignorance regarding many of the fundamental aspects of endocrine homeostasis. Such ignorance should not disable attempts to make progress in this field, but they should signal caution when suggesting testing strategies and regulatory testing requirements.

TESTING STRATEGIES

The development of practical and validated assays for ED effects is presently the subject of intensive international research, and within that context, the acceptance by EDSTAC that some of their preferred assays are only at the research stage of development, and the redundancy evident among the assays they listed, confirms the infant state of this field. This situation indicates that the most efficient way to proceed is to use the few currently available assays in a hierarchical way (30,31), and to keep under constant review the findings of current research. Thus, one suggested approach (32) is to gather data on the activity of major chemicals (of unknown reproductive toxicity) in *in vitro* assays for the expression of transfected ER and AR and to study further any active compounds in the available mammalian and wildlife ED assays. This will be an imperfect and temporary approach. For example, it may be that some ER agonists found inactive in the rodent uterotrophic assay may eventually be shown to exert a subtle but important effect on the rodent prostate gland, or that an agent may exert a specific ED effect in birds after having been defined as inactive in trout. Such findings could lead to a revision of the testing strategy. However, the alternative (precautionary) of seeking a perfect testing strategy using only the currently available assays may present greater eventual problems than those encountered with an evolutionary approach. The consensus represented by the International Program of Chemical Safety (IPCS) scheme for using short-term mutagenicity assays to predict carcinogens and germ cell mutagens (33) illustrates that an hierarchical approach to testing is a realistic prospect, albeit many compromises were required between those participants who were influenced primarily by precedents and those who were influenced primarily by possibilities. The final IPCS consensus was mediated by the discipline that proponents of the inclusion of a particular assay were required to name one mutagen/carcinogen that would remain undetected if it were to be omitted from the scheme. A similar requirement would help to justify the final battery of ED assays accepted by Regulatory authorities.

The large number of toxicological considerations relevant to the design of an efficient ED testing strategy are shown in Figure 2 (31). The final quadrant of Figure 2 is concerned with human/wildlife hazard assessment, the ultimate goal of all endeavours in this area. The present emphasis on assay selection and assay validation must therefore be seen as a means to the achievement of that goal, and not as an end in itself.

DOSE LEVEL SELECTION

It has been suggested by several investigators that the selection of dose levels for whole-animal ED studies will require a different approach to that adopted in other branches of toxicology. The examples cited include the plasticizer butyl benzyl phthalate (BBP) which has a no-effect level for rat liver enzyme induction (and an associated anti-carcinogenic effect in the rat mammary gland) of about 250 mg/kg bodyweight (34), yet it is reported to increase the weight of rat testis at a thousand times lower dose (27). Likewise, Nagel *et al*. (35) have discussed presumed ED activities of bisphenol A (BPA) occurring at orders of magnitude lower dose levels than those required to produce activity in the rat uterotrophic assay (36). The reality and implications of such effects for dose setting in ED studies require urgent and objective evaluation/validation before change are made to the usual criteria for dose selection.

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