Sensitivity of the Immature Rat Uterotrophic Assay to Mixtures of Estrogens

Helen Tinwell and John Ashby
Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire, United Kingdom

We have evaluated whether mixtures of estrogens, present in the mix at doses that are individually inactive in the immature rat uterotrophic assay, can give a uterotrophic response. Seven chemicals were evaluated: nonylphenol, bisphenol A (BPA), methoxychlor, genistein (GEN), estradiol, diethylstilbestrol, and ethinyl estradiol. Dose responses in the uterotrophic assay were constructed for each chemical. The first series of experiments involved evaluating binary mixtures of BPA and GEN at dose levels that gave moderate uterotrophic responses when tested individually. The mixtures generally showed an intermediate or reduced uterotrophic effect compared with when the components of the mixture were tested alone at the dose used in the mixture. The next series of experiments used a multicomponent (complex) mixture of all seven chemicals evaluated at doses that gave either weakly positive or inactive uterotrophic responses when tested individually in the assay. Doses that were nominally equi-uterotrophic ranged over approximately six orders of magnitude for the seven chemicals. Doses of agents that gave a weak uterotrophic response when tested individually gave a marginally enhanced positive response in the assay when tested combined as a mixture. Doses of agents that gave a negative uterotrophic response when tested individually gave a positive response when tested as a mixture. These data indicate that a variety of different estrogen receptor (ER) agonists, present individually at subeffective doses, can act simultaneously to evoke an ER-regulated response. However, translating these findings into the process of environmental hazard assessment will be difficult. The simple addition of the observed, or predicted, activities for the components of a mixture is confirmed here to be inappropriate and to overestimate the actual effect induced by the mixture. Equally, isobole analysis is only suitable for two- or three-component mixtures, and concentration addition requires access to dose–response data and EC50 values (concentration giving 50% of the maximum response) for the individual components of the mixture—requirements that will rarely be fulfilled for complex environmental samples. Given these uncertainties, we conclude that it may be most expedient to select and bioassay whole environmental mixtures of potential concern. Key words: anthropogenic estrogens, binary mixtures, complex mixtures, estrogenicity, immature rat uterotrophic assay, phytoestrogens, synthetic estrogens. Environ Health Perspect 112:575–582 (2004). doi:10.1289/ehp.6831 available via http://dx.doi.org/[Online 8 January 2004]

Recognition that exposure to environmental estrogens may cause adverse reproductive effects led to the development of assays capable of detecting such compounds. These include in vitro assays, such as binding to the estrogen or androgen receptor (ER and AR, respectively), and/or gene in vitro expression assays. For more refined hazard assessments, a variety of in vivo rodent assays have been described, such as the rodent uterotrophic and Hershberger assays [Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) 1998; Gray et al. 2002; Organisation for Economic Co-operation (OECD) 1998]. However, humans and wildlife are exposed to mixtures of chemicals, and the best way to determine the sum of the activities of the individual components of the mixture, leading to a holistic assessment of hazard, remains open to discussion.

There are several approaches to the assessment of mixtures, ranging from the bioassay of whole mixtures (e.g., Heindel et al. 1994; Jobling et al. 2002; Rodgers-Gray et al. 2001) to the more analytical component-based approaches (e.g., Payne et al. 2001; Silva et al. 2002). In whole-mixture approaches, the mixture is treated as if it were one single chemical entity, whereas in the component-based approach the mixture effects are derived from consideration of the activities of the individual constituents of the mixture. The present multicomponent experiments can be regarded as a surrogate mixture approach that lies between the whole-mixture and component-based approaches (“surrogate” because the mixture is re-created in the laboratory). The surrogate is illustrated by Heindel et al. (1994, 1995), who tested reconstituted mixtures of pesticides containing up to 100 times the concentrations measured in California and Iowa groundwater. They found that the mixtures were approximately as toxic as the most potent compound in the mixture for reproductive end points. Other methods include the simple addition of the individual effects [Waters et al. 1990; U.S. Environmental Protection Agency (EPA) 1989], the use of toxic equivalency factors (TEFs; Nisbet and LaGoy 1992; Safe 1998; Van den Berg et al. 1998), and isobole analysis in the case of two- or three-component mixtures for which knowledge exists regarding the dose–response relationships of the individual components of the mixture (Charles et al. 2002; Chen and Pounds 1998; Nellemann et al. 2003; Rajapake et al. 2002; Tully et al. 2000).

A recent observation of particular interest is that a mixture of estrogens can cause estrogenic effects in vitro despite the individual components of the mixture being present at concentrations below their individual no observable effect levels (NOELs) for estrogenicity in vitro (Payne et al. 2001; Silva et al. 2002). Silva et al. (2002) used the phrase “something for nothing” in the title of their paper, thereby galvanizing interest in this topic. However, Edgren and Calhoun (1960) observed that the uterotrophic activity of strong estrogens is inhibited by the concomitant presence of weaker estrogens—an effect they referred to as biological buffering. Those data indicate that the observations made by Silva et al. (2002) in vitro may not automatically translate to the situation prevailing in estrogen-sensitive tissues in vivo.

The present studies were therefore designed to evaluate the activity of mixtures of estrogens using the immature rat uterotrophic assay. Initial studies were concerned with various binary mixtures of the synthetic estrogen bisphenol A (BPA) and the phytoestrogen genistein (GEN), using doses that were individually active in the assay. These studies were followed by investigation of a multicomponent mixture of seven estrogenic compounds. The seven chemicals were selected to include a range of anthropogenic, synthetic, and plant-derived estrogens and to cover approximately a million-fold range of potencies [from nonylphenol (NP; minimum detection level, 75 mg/kg) to ethinyl estradiol (EE; minimum detection level, 0.1 μg/kg)] in the immature rat uterotrophic assay. In those studies mixtures were tested such that their components were present in the mixture at doses that either gave a small but significant uterotrophic effect, or no effect, when tested individually.

Address correspondence to J. Ashby, Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, UK SK10 4TJ; Telephone: 01625-512833. Fax: 01625-590996. E-mail: John.Ashby@Syngenta.com

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Materials and Methods

**Chemicals.** Estradiol (E2; 98+% purity), diethylstilbestrol (DES; 99+% purity), EE (98+% purity), and arachis oil (AO; peanut oil) were purchased from the Sigma Chemical Company (Poole Dorset, UK). BPA (99+% purity) was purchased from Aldrich (Gillingham, Dorset, UK), GEN (98+% purity) from Ultrafine Chemicals (Manchester, UK), methoxychlor (MXC; ~98% purity) from ICN (Basingstoke, Hampshire, UK), and NP (95+% purity) from Schenectady International (Freeport, TX, USA). All compounds were either homogenized or, in the case of NP, dissolved in AO to give the appropriate stock solutions. MXC was ground to a powder using a pestle and mortar before homogenization in AO. A stock solution of each compound was prepared at the beginning of each study. Dosing solutions of the individual compounds were prepared once at the beginning of each study by diluting the appropriate stock solution, and dosing solutions of the mixtures were prepared fresh from the appropriate stock solutions on a daily basis. All solutions were stored at room temperature during the course of each study.

**Animals.** Immature female AP rats (19–20 days of age) were obtained from the barriered animal breeding unit (AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK). They were group housed at a maximum of six per cage in solid-bottomed polypropylene cages containing sawdust (Wood Treatments Ltd., Macclesfield, Cheshire, UK) and shredded paper as bedding for the duration of the experiment. Fun tubes and houses were provided as environmental enrichment. All animals were allowed RM1 diet (Special Diet Services Ltd., Witham, Essex, UK) and water ad libitum for the duration of the experiment.

**Uterotrophic assay.** All animals were weighed and then were terminated using an overdose of Halothane (Concord Pharmaceuticals Ltd, Dunmow, Essex, UK) followed by cervical dislocation. All terminations took place in the morning 24 hr after the last dose. Animals were removed from study in a blocked fashion, taking three animals/cage at a time. The uterus was removed from each animal, trimmed free of fat, gently blotted, and weighed as described previously (Odum et al. 1997). Each uterus was placed in a preweighed vial, dried overnight at 70°C, and then reweighed to obtain a dry weight measurement. Two people performed the necropsies while a third weighed all tissues and placed them into the appropriate vials. This allowed the termination of up to 180 animals (as in the final study) within 3 hr.

**Dosing.** Animals were exposed to all compounds (either individually or as a mixture) by single subcutaneous injection in the morning of 3 consecutive days using a dosing volume of 5 mL/kg body weight. With the exception of the first study (experiment 1), which had group sizes of 12, all other studies had group sizes of 8. The initial dose levels employed (detailed in Table 1) were based both on previously published data and data generated in-house. High doses were chosen to induce a clear positive response in the assay, whereas the lower doses were predicted to be inactive in the assay—the doses being adjusted during the course of the experiments to ensure such observations. For example, the highest dose of BPA used was 600 mg/kg (experiment 1); this was reduced to 75 mg/kg in later experiments (experiments 4–6). Similarly, the lowest dose of BPA used in the initial studies was 300 mg/kg (experiments 1 and 2), which was reduced to 30 mg/kg in the third study and was eventually lowered to 1.5 mg/kg in the final experiment (experiment 6).

**Study design.** Six studies were performed in total and these are described in Table 1. The first three experiments were concerned with the

### Table 1. Dose levels used in the six experiments for the individual compounds when tested alone or in mixtures.

| Experiment number and procedure | Dose levels (per kilogram individually or as component of mixture) | Comments |
|--------------------------------|-------------------------------------------------|----------|
| 1 BPA and GEN tested individually | BPA (mg)   | GEN (mg) | NP (mg) | MXC (mg) | E2 (µg) | DES (µg) | EE (µg) | | Doses based on OECD validation studies (Kanno et al. 2003) |
| 2 BPA and GEN tested individually at all doses shown in binary mixtures of 300 mg BPA + GEN at each of the doses | 300 10 | | 30 15 | 30 20 | | 30 30 | | 30 40 | BPA dose level maintained at 300 mg/kg in mixtures while increasing the concentration of GEN; doses based on Kanno et al. (2003) |
| 3 BPA and GEN tested individually at doses shown and in the binary mixtures using a ratio of 30:1 BPA:GEN | 30 1 | 75 2.5 | 150 5 | 300 10 | | | | Maintenance of a constant ratio between BPA and GEN suggested by Kortenkamp (personal communication) |
| 4 α-Dose for individual compounds and contribution to mixture α/2 mixture | 75 5 | 37.5 2.5 | 25 25 | 0.5 0.025 | 0.075 | | | Doses for NP, BPA, MXC, and GEN based on Kanno et al. (2003); E2 dose based on Odum et al. (1997); DES dose based on in-house data; NP, GEN, and DES were inactive in the uterus at α-dose |
| α/5 for individual compounds and contribution to mixture | 15 1 | 10 2 | 10 0.2 | 0.01 0.03 | | | | |
| 5 α-Dose for individual compounds and contribution to mixture α/2 mixture | 75 10 | 37.5 7.5 | 25 25 | 0.5 0.125 | 0.05 | 0.05 | | Individual α-dose concentration marginally increased for GEN, NP, and DES to give α-doses (ensuring a positive uterotrophic response); EE α-dose marginally reduced to give α-dose; E2 active at α/10 |
| α/5 mixture | 15 2 | 15 10 | 0.2 0.05 | 0.05 | 0.02 | | |
| α/10-dose for individual compounds and contribution to mixture α/20 mixture | 7.5 1 | 7.5 5 | 0.5 0.025 | 0.01 | | | | |
| α/20 mixture | 3.75 0.5 | 3.75 2.5 | 0.05 0.0125 | 0.005 | | | | |
| α/50 mixture | 1.5 0.2 | 1.5 1 | 0.02 0.005 | 0.002 | | | | |
| 6 α-Dose for individual compounds and contribution to mixture α/2 mixture | 75 10 | 37.5 7.5 | 25 25 | 0.5 0.125 | 0.05 | 0.05 | | Identical to those in experiment 5; compounds tested individually at α/50 to ensure absence of uterotrophic response |
| α/5 mixture | 15 2 | 15 10 | 0.2 0.05 | 0.05 | 0.02 | | |
| α/10 mixture | 7.5 1 | 7.5 5 | 0.1 0.025 | 0.01 | | | | |
| α/20 mixture | 3.75 0.5 | 3.75 2.5 | 0.05 0.0125 | 0.005 | | | | |
| α/50-dose for individual compounds and contribution to mixture | 1.5 0.2 | 1.5 1 | 0.02 0.005 | 0.002 | | | | |
| α/100 mixture | 0.75 0.1 | 0.75 0.5 | 0.01 0.0025 | 0.001 | | | | |

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**Animals.** Immature female AP rats (19–20 days of age) were obtained from the barriered animal breeding unit (AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, UK). They were group housed at a maximum of six per cage in solid-bottomed polypropylene cages containing sawdust (Wood Treatments Ltd., Macclesfield, Cheshire, UK) and shredded paper as bedding for the duration of the experiment. Fun tubes and houses were provided as environmental enrichment. All animals were allowed RM1 diet (Special Diet Services Ltd., Witham, Essex, UK) and water ad libitum for the duration of the experiment.
interaction between BPA and GEN only (experiments 1–3, Tables 1 and 2). The initial study investigated the interaction between 300 or 600 mg/kg BPA and 15 or 50 mg/kg GEN, with the doses being based on those used for the OECD uterotrophic validation trials (Kanno et al. 2003). In the second study, BPA was maintained at 300 mg/kg and was mixed with increasing levels of GEN (10–50 mg/kg). The last of the BPA/GEN studies employed mixtures consisting of a fixed ratio of 30:1 BPA:GEN (experiment 3, Table 2) as described by Altenburger et al. (2000) and Backhaus et al. (2000) and as recommended by A. Kortenkamp (personal communication).

A complex mixture, consisting of seven compounds (NP, MXC, BPA, GEN, E2, DES, and EE) was investigated in the final set of experiments. The top dose of each mixture component is referred to as either the α*-dose in experiment 4 (Tables 1 and 3) or the α-dose in the last two studies (Tables 1 and 3), with α* and α being distinct from each other as follows. In the first complex mixture study (experiment 4, Tables 1 and 3), the top doses (α*) were chosen to induce a moderate increase in blotted uterine weight, based on previously published data. However, the absence of a positive response for some of the compounds in this study led to marginal adjustments of the top dose levels for the mixture components (experiment 5, Tables 1 and 3). These highest concentrations, referred to as α*-dose levels, were also used in the final study (experiment 6, Tables 1 and 3). Several dilutions of both α* and α were also studied to determine the NOEL for each mixture component.

Stock solutions of the individual compounds, which were 7-fold more concentrated than the highest (α*/α) dose to be used, were diluted to give α, α*, α*/5, α/10, and α/50 dosing solutions for each compound as appropriate to the study (Table 1). The highest concentration mixture dosing solution (α* for experiment 4 and α for experiments 5 and 6) was prepared by homogenizing equal volumes of the individual 7α concentrated α*/α stock solutions together. Serial dilutions of this top mixture gave dosing solutions of α*/2 and α*/5 for experiment 4 and a series of solutions ranging from α/2 to α/100 for experiments 5 and 6. EE at 1 µg/kg was used as a maximal positive response control in all binary and complex mixture studies.

Table 2. Uterine and body weights (mean ± SD) from five independent immature rat uterotrophic assays.

| Experiment | Compound | Dose (mg) | Uterine weight (mg) | Final body weight (mg) |
|-----------|----------|-----------|---------------------|-----------------------|
| A         |          |           | Blotted             | Dry                   |                        |
| 1         | A        | 5 ml      | 21.3 ± 2.3          | 4.3 ± 0.7             | 51.1 ± 9.3             |
| 2         | BPA      | 10 mg     | 23.9 ± 3.3          | 5.1 ± 1.7             | 52.1 ± 6.7             |
| 3         | 15 mg    | 29.0 ± 3.8 | 5.8 ± 0.9          | 50.8 ± 2.0             |
| 4         | 300 mg   | 38.5 ± 6.3 | 5.2 ± 3.6         | 52.6 ± 3.6             |
| 5         | 600 mg   | 41.4 ± 7.8 | 5.1 ± 6.7          | 51.5 ± 7.6             |
| 6         | 800 mg   | 59.0 ± 8.6 | 46.5 ± 5.4         | 49.6 ± 4.6             |
| B         | GEN      | 1 mg      | 19.7 ± 1.3          | 51.1 ± 5.8             |
| 7         | 15 mg    | 37.4 ± 6.5 | 5.1 ± 9.3          | 52.1 ± 6.7             |
| 8         | 30 mg    | 47.6 ± 9.3 | 5.1 ± 5.1          | 52.1 ± 6.7             |
| 9         | 50 mg    | 54.2 ± 9.0 | 5.1 ± 4.4          | 51.5 ± 6.7             |
| 10        | 80 mg    | 67.7 ± 8.2 | 52.6 ± 4.9         | 49.6 ± 4.6             |
| 11        | EE       | 0.3 µg    | 48.9 ± 3.4          | 52.1 ± 6.7             |
| 12        | 1 µg     | 91.9 ± 9.1 | 51.4 ± 9.3         | 49.6 ± 4.6             |

Table 2: Uterine and body weights (mean ± SD) from five independent immature rat uterotrophic assays.

Experiments A and B: data generated in this laboratory for the OECD evaluation of the uterotrophic assay (Kanno et al. 2003) and used as part of the dose–response curves (Figure 8). Data from experiments 1–3 were analyzed for statistical significance by ANOVA and ANCOVA.

*p < 0.05 and **p < 0.01 by ANCOVA.

Results

All of the raw data generated have been recorded in tabular form to allow others to reanalyze the database. However, in order to render this complex set of experiments intelligible, primary reliance has been placed here on Figures 1–6. We used 1 mg/kg EE as a positive control in most experiments (Tables 2 and 3). Full dose–response relationships were established for all of the chemicals studied (Figure 5), and these are consistent with the available literature for each chemical (Table 1, Figure 5). Figure 7 compares the observed increases in blotted uterine weight after exposure to a mixture with the predicted outcome assuming an additive response. To calculate the additive effect, the group mean control uterine weight was subtracted from the group mean uterine weights recorded for each of the concurrent individual components. The resultant values, as well as the group mean control weight, were then added together to give a final weight, which represented the predicted outcome.

BPA/GEN studies. Initial studies investigated the estrogenicity of a mixture of BPA...
Table 3. Uterine and final body weight (mean ± SD) of the seven compounds when tested alone or as part of a mixture.

| Experiment | Compound | Dose (µg) | Contribution to mixture | Blotted uterine weight (g) | Dry uterine weight (g) | Final body weight (g) |
|------------|----------|-----------|-------------------------|---------------------------|-----------------------|----------------------|
| 4          | AO       | 5 mL      | α/5                     | 24.7 ± 5.8               | 4.9 ± 1.0             | 53.6 ± 5.7           |
|            | NP       | 10 µg     | α/5                     | 24.2 ± 4.6               | 4.7 ± 0.7             | 55.9 ± 10.4          |
|            | MXC      | 50 µg     | α/5                     | 48.9 ± 3.8               | 4.9 ± 0.6             | 54.5 ± 6.8           |
|            | BPA      | 15 µg     | α/5                     | 23.1 ± 4.2               | 4.6 ± 0.6             | 51.0 ± 6.9           |
|            | GEN      | 75 µg     | α/5                     | 24.9 ± 5.0               | 4.9 ± 0.8             | 52.0 ± 7.2           |
|            | 5 µg     | α/5       |                         | 26.0 ± 4.0               | 5.0 ± 1.0             | 53.3 ± 6.5           |
|            | E2       | 0.1 µg    | α/5                     | 23.1 ± 5.4               | 4.9 ± 0.9             | 55.3 ± 9.2           |
|            | DES      | 0.05 µg   | α/5                     | 28.1 ± 5.7               | 5.7 ± 1.1             | 54.3 ± 5.8           |
|            | EE       | 0.03 µg   | α/5                     | 24.6 ± 4.5               | 4.9 ± 0.6             | 54.4 ± 4.4           |
|            | 1.0 µg   | α/5       |                         | 116.4 ± 52.1             | 20.5 ± 5.1            | 52.4 ± 9.2           |
|            | Mixture  | 0.1 µg    | α/5                     | 41.3 ± 5.0*              | 7.7 ± 1.4*            | 54.8 ± 6.2           |
|            |          | 0.025 µg  | α/5                     | 49.0 ± 7.2*              | 9.3 ± 1.4*            | 53.2 ± 6.7           |
|            |          | 0.05 µg   | α/5                     | 59.9 ± 4.9*              | 11.4 ± 0.9*           | 54.6 ± 5.1           |
| 5          | AO       | 5 mL      | –                       | 21.7 ± 5.0               | 4.5 ± 0.8             | 52.3 ± 6.5           |
|            | NP       | 7.5 mg    | α/10                    | 24.7 ± 4.9               | 4.9 ± 0.3             | 54.2 ± 5.5           |
|            | MXC      | 5 mg      | α/10                    | 36.3 ± 8.0**             | 6.7 ± 1.2**           | 54.1 ± 6.8           |
|            | BPA      | 5 mg      | α/10                    | 25.6 ± 6.8               | 4.9 ± 1.2             | 53.2 ± 5.2           |
|            | GEN      | 7.5 mg    | α/10                    | 33.6 ± 6.4**             | 6.3 ± 1.2**           | 54.1 ± 5.7           |
|            | 1 mg     | α/10      |                         | 24.9 ± 5.0               | 5.0 ± 0.8             | 54.1 ± 6.6           |
|            | E2       | 0.1 µg    | α/10                    | 28.3 ± 8.2               | 5.5 ± 1.4             | 53.9 ± 6.9           |
|            | DES      | 0.25 µg   | α/10                    | 22.7 ± 4.6               | 5.4 ± 1.1             | 53.9 ± 7.1           |
|            | EE       | 0.01 µg   | α/10                    | 22.3 ± 5.2               | 4.5 ± 1.3             | 53.6 ± 8.7           |
|            | 0.1 µg   | α/10      |                         | 31.4 ± 4.8*              | 5.9 ± 1.0*            | 55.7 ± 5.9           |
|            | Mixture  | 0.1 µg    | α/10                    | 102.7 ± 10.1**           | 17.1 ± 1.4**          | 54.9 ± 5.4           |
|            | 1.0 µg   | α/10      |                         | 26.5 ± 5.5               | 5.6 ± 1.0             | 54.5 ± 6.9           |
|            |          | 0.025 µg  | α/10                    | 21.8 ± 4.8**             | 5.5 ± 0.9**           | 51.6 ± 6.5           |
|            |          | 0.05 µg   | α/10                    | 37.4 ± 4.1**             | 7.4 ± 0.7**           | 53.0 ± 7.0           |
|            |          | 0.1 µg    | α/10                    | 44.0 ± 5.2**             | 8.4 ± 1.0**           | 52.7 ± 7.2           |
|            |          | 0.2 µg    | α/10                    | 49.5 ± 5.3**             | 9.3 ± 1.2             | 53.4 ± 6.6           |
|            |          | 1.0 µg    | α/10                    | 63.0 ± 10.4**            | 12.0 ± 1.8**          | 54.0 ± 6.4           |
| 6          | AO       | 5 mL      | –                       | 23.6 ± 5.3               | 4.4 ± 0.9             | 54.5 ± 6.9           |
|            | NP       | 1.5 mg    | α/20                    | 23.7 ± 15                | 4.7 ± 0.2             | 55.0 ± 6.1           |
|            | MXC      | 1 mg      | α/20                    | 23.2 ± 5.9               | 4.6 ± 1.1             | 55.0 ± 6.6           |
|            | BPA      | 1.5 mg    | α/20                    | 24.4 ± 5.5               | 4.8 ± 0.9             | 54.8 ± 5.2           |
|            | GEN      | 0.2 mg    | α/20                    | 24.3 ± 7.4               | 4.5 ± 1.3             | 54.0 ± 6.8           |
|            | E2       | 0.02 µg   | α/20                    | 42.6 ± 4.2**             | 7.9 ± 0.9**           | 54.1 ± 6.6           |
|            | DES      | 0.005 µg  | α/20                    | 23.4 ± 2.8               | 4.3 ± 0.5             | 55.2 ± 6.4           |
|            | EE       | 0.25 µg   | α/20                    | 46.2 ± 5.9**             | 7.8 ± 0.9**           | 54.8 ± 7.4           |
|            | 0.05 µg  | α/20      |                         | 50.6 ± 7.9**             | 8.6 ± 1.0**           | 54.8 ± 3.9           |
|            | Mixture  | 0.1 µg    | α/100                   | 23.0 ± 5.7               | 4.4 ± 1.0             | 55.2 ± 7.1           |
|            |          | 0.01 µg   | α/50                    | 29.7 ± 6.7               | 5.5 ± 1.1**           | 54.7 ± 6.5           |
|            |          | 0.1 µg    | α/50                    | 38.3 ± 3.3               | 5.4 ± 0.7**           | 54.7 ± 6.5           |
|            |          | 0.02 µg   | α/50                    | 37.1 ± 5.6**             | 6.2 ± 0.9**           | 53.5 ± 10.1          |
|            |          | 0.05 µg   | α/50                    | 43.5 ± 8.2**             | 8.5 ± 0.8**           | 54.8 ± 5.3           |
|            |          | 0.1 µg    | α/2                     | 53.4 ± 10.9**            | 10.0 ± 2.3**          | 55.4 ± 7.2           |
|            |          | 0.2 µg    | α/2                     | 62.2 ± 9.0*              | 11.5 ± 1.5**          | 54.7 ± 5.5           |

Data were analyzed for statistical significance by both ANOVA and ANCOVA.

*p < 0.05 and **p < 0.01 by ANCOVA.
increased in order to obtain a positive response for each when tested individually at that dose; the α*-dose of EE was also reduced from 0.15 µg/kg to 0.1 µg/kg. The remaining three chemicals were maintained at their original α*-dose. Given these changes, the top doses in the next two experiments (experiments 5 and 6; Table 3, Figures 5 and 6) were referred to as the α-doses, as opposed to α*-doses (as described in Tables 1 and 3).

In experiment 5 the components were tested individually at their α- and α/10 doses. The mixture was tested at the α-dose, and the α/2, α/5, α/10, α/20 and α/50 doses. All compounds individually produced significant increases (p < 0.01) in uterine weight (blotted and dry) at the α-dose (experiment 5; Table 3, Figures 5 and 6). At the α/10-dose five chemicals were negative, but a small increase in blotted uterine weight was seen for E2 (p < 0.05; Figures 5 and 6) and a small increase in dry uterine weight for BPA (p < 0.01; Figure 6). Significant increases in both blotted and dry uterine weight were seen for all mixtures down to the α/20 dose, with no effects being observed at the α/50-dose in this present study.

Because both BPA and E2 produced uterine effects at their α/10 dose levels in experiment 5, all seven compounds were tested individually at their α- and α/50 doses in the final experiment (experiment 6; Table 3, Figures 5 and 6). The mixtures were the same as in experiment 5 (α-α/50) with the addition of an α/100 mixture dose. All compounds individually induced a significant (p < 0.01) increase in uterine weight at their α-dose and were inactive at their individual α/50-dose (blotted and dry; Figures 5 and 6). Significant increases (p < 0.01) in uterine weight (blotted and dry) were recorded for the mixtures at α/10 dose levels and above. Increases were also seen for the dry weight measurements of both the α/20 and the α/50 mixture doses (experiment 6; Table 3, Figure 6). In addition, a significant increase (p < 0.05) in uterine blotted weight was recorded for the α/50 mixture dose. No effects were observed at the α/100 mixture dose.

The data in Figures 5 and 6 reveal that, in general, effects considered to be statistically insignificant are marginally greater than the concurrent control levels. To evaluate if the effects of mixture doses merely reflected the sum of the individual components of the mixture, these individual increases were summed, added to the control level, and shown as a “predicted” effect in Figure 7. Such additions represent an invalid method of predicting the activity of mixtures (Berenbaum 1981, 1989; Korstenkamp and Altenburger 1998), but because they are likely to be employed by others, the method was evaluated again here. This additive approach led to an overestimation of the final outcome in most of the cases (binary mixtures experiments 1–3 and complex mixtures at α*, α/10, and α-dose levels in experiments 4–6). In a few cases, the additivity approach led to a slight underestimation of the observed outcome (α*/5 mixture of experiment 4; α/50 mixture of experiment 6). There was only one situation where the prediction outcome matched the observed data (30:1 BPA:GEN mixture of experiment 3).

Discussion

The present studies were conducted using large group sizes to increase the chance of observing small changes in mean uterine weight. This,
coupled to the large size of the total database (836 individual data points), and the repeat studies conducted, enables the properties of mixtures of estrogens in vivo to be considered visually by reference to Figures 1–7. Nonetheless, all of the data are presented in tabular form to enable others to conduct alternative statistical analyses.

The uterotrophic potency of the seven chemicals used in these studies varied by more than 1,000,000-fold (Figure 5). The derivation of nominally equi-uterotrophic doses for the individual agents was therefore a critical requirement for these experiments. For example, the positive α-dose complex mixture contained equi-uterotrophic doses of NP (75 mg/kg) and EE (0.1 µg/kg)—to mix each chemical at 75 mg/kg would have generated a maximal positive uterotrophic response because of the dominance of the EE dose. Likewise, to mix them at 0.1 µg/kg would not have significantly affected the original EE response because of the absence of uterotrophic activity for NP at that dose.

The average control uterine blotted weights for these experiments was approximately 20 mg, and the maximum uterine weight possible for the assay was approximately 100 mg (as induced by 1 µg/kg EE). Thus, the reach of the assay involves a maximum of a 5-fold increase in uterine weight. In the first two binary mixture experiments (Figures 1 and 2), the individual components gave medium uterotrophic responses, yielding 2- to 4-fold increases in uterine weight (uteri weighing between 40 and 80 mg). Under these conditions the mixtures generally gave an intermediate or reduced uterotrophic response compared with those of the individual components.

The third binary mixture experiment used individual dose levels giving only an approximately 2-fold increase in uterine weight, and the mixture of BPA and GEN was kept at a constant ratio of 30:1 (Figure 3). The response given by dilutions of the mixture was the same as that given by BPA alone, except for at the highest dose, where the response was midway between those given individually by BPA and GEN. Given the absence of additive effects in these experiments, the remaining experiments were designed to evaluate the properties of mixtures whose constituents were present at doses that were either weakly active, or inactive, in the assay when tested alone (the situation most likely to prevail in environmentally relevant mixtures).

Based on the individual chemical dose–response data shown in Figure 5, an attempt was made to select individual doses that would be either weakly uterotrophic or non-uterotrophic (the α* and α*/5 doses, respectively; Figure 4). The α* doses of NP, GEN, and DES selected were too low to trigger uterotrophic responses, and all of the α*/5 doses were inactive. Mixtures of the α*, α*/2, and α*/5 were clearly uterotrophic, in terms of both blotted and dry uterine weight (Figure 4). The α* dose mixture gave only a marginally higher response than did the individual components, consistent with the earlier binary mixture data (Figures 1–3). Nonetheless, the effect of the α* dose mixture was significantly higher (p < 0.01) than the highest effect of the individual α* responses (EE). The positive response given by the α*/5 dose mixture, with each of the individual components at α*/5 doses being inactive, clearly established the potential of the effects reported by Silva et al. (2002) in vitro to be seen also in vivo. The final two experiments were designed to elaborate this finding using greater dilutions (lower doses) of the mixture and with adjustments to the α*-doses of NP, GEN, and DES for them to be individually positive.

The revised α*-doses shown in Figures 5 and 6 are hereafter referred to as the α-doses,
and each gave a positive uterotrophic response \((p < 0.01)\). With two exceptions, the individual \(\alpha/10\) doses (experiment 5) were non-uterotrophic. These exceptions were the blotted uterine weight for the \(\alpha/10\) dose of E2 and the dry uterine weight for the \(\alpha/10\) dose of BPA (both \(p < 0.05\)). The \(\alpha/50\) individual doses were all non-uterotrophic (experiment 6). The dose-related uterotrophic response (both blotted and dry uterine weights) given by the mixtures extended to the \(\alpha/20\) dose in experiment 5 and to the \(\alpha/50\) dose in experiment 6. The fact that the \(\alpha/50\) mixture dose was active in experiment 6, yet inactive in experiment 5, and that the \(\alpha/20\) mixture dose was active in experiment 5 but inactive in experiment 6, probably reflects the fact that the uterotrophic responses in that region are very weak and are slipping in and out of statistical significance. Nonetheless, these mixture data confirm that uterotrophic effects can be seen for mixtures of chemicals under conditions where the doses of the components of the mixture are non-uterotrophic.

The data shown in Figure 7 confirm that the addition of individual uterotrophic responses for chemicals does not provide a useful estimate of the likely uterotrophic activity of the mixture—the greater the magnitude of the uterotrophic responses being summed, the greater becomes the overestimate of the predicted response for the mixture—an effect referred to by Edgren and Calhoun (1960) as “biological buffering.” These data therefore confirm earlier demonstrations of the inappropriateness of this approach (Berenbaum 1981, 1989; Kortenkamp and Altenburger 1998).

The present data have confirmed that it is legitimate to consider the potential hazard posed by exposure to mixtures, even though the components of the mixture may be present at individually inactive doses. At the molecular level these data indicate that a variety of ER agonists can act simultaneously to evoke an ER-regulated response once a critical concentration of the combined agonists is reached. This would be consistent with the observation that at least 10–20% of uterine ERs must be occupied for at least 4–6 hr in order to stimulate sustained uterine hyperplasia (Anderson et al. 1972, 1975; Clark and Peck 1979; Lan and Katzenellenbogen 1976). However, translating this finding into the process of environmental hazard assessment will be difficult. The greatest problem will be assessing the individual potency of the components of a mixture. For example, the potency of the seven chemicals used in these studies varied by approximately 1,000,000-fold. Further, it is confirmed here that the simple addition of the individual activities of the components of a mixture will overestimate the actual effect induced by the mixture. Equally, the most detailed of methods for combining effects, isobole analysis, is only suitable for two- or three-component mixtures. Given these uncertainties, we conclude that it may be most expedient to select and bioassay whole mixtures of potential concern in the environment, as illustrated by the studies by Rodgers-Gray et al. (2001) and Jobling et al. (2002).

Finally, consideration of the potential activity of mixtures is not unique to estrogenicity. The potential hazard posed by occupational/environmental exposure to carcinogenic and/or mutagenic mixtures has been studied (Ashby and Kettle 1987; Ashby et al. 1988; Feron et al. 2001; Krewski and Thomas 1992; Lagorio et al. 2000; Salamone et al. 1979; Taylor et al. 1995), as has the carcinogenicity to rodents of complex mixtures of carcinogens (Ito et al. 1969; Lijinsky et al. 1983; Takayama et al. 1989). Experience gained in these other areas may prove useful when considering the potential activities of mixtures of estrogens.
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