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 EC_{50} 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; Rajapakse 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 (E_2; 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) BPA (mg)	GEN (mg)	NP (mg)	MXC (mg)	E_2 (µg)	DES (µg)	EE (µg)	Comments
1 BPA and GEN tested individually	300	15						Doses based on OECD validation
	600	50						studies (Kanno et al. 2003)
BPA and GEN tested in binary mixtures	300	15						
	300	50						
	600	15						
	600	50						
2 BPA and GEN tested individually at all doses shown and in	300	10						BPA dose level maintained at
binary mixtures of 300 mg BPA + GEN at each of the doses		15						300 mg/kg in mixtures while
		20						increasing the concentration of
		40						GEN; doses based on Kanno et al.
		50						(2003)
3 BPA and GEN tested individually at doses shown	30	1						Maintenance of a constant ratio
and in the binary mixtures using a ratio of 30:1 BPA:GEN	75	2.5						between BPA and GEN suggested
	150	5						by Kortenkamp (personal
	300	10						communication)
4 α^*-Dose for individual compounds and contribution to mixture	75	5	50	50	1	0.05	0.15	Doses for NP, BPA, MXC, and GEN
$\alpha^*/2$ mixture	37.5	2.5	25	25	0.5	0.025	0.075	based on Kanno et al. (2003); E_2
$\alpha^*/5$ for individual compounds and contribution to mixture	15	1	10	10	0.2	0.01	0.03	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
5 α-Dose for individual compounds and contribution to mixture	75	10	75	50	1	0.25	0.1	Individual α^* concentration
$\alpha/2$ mixture	37.5	5	37.5	25	0.5	0.125	0.05	marginally increased for GEN, NP,
$\alpha/5$ mixture	15	2	15	10	0.2	0.05	0.02	and DES to give α-doses (ensuring
$\alpha/10$-dose for individual compounds and contribution to mixture	7.5	1	7.5	5	0.1	0.025	0.01	a positive uterotrophic response);
$\alpha/20$ mixture	3.75	0.5	3.75	2.5	0.05	0.0125	0.005	EE α^*-dose marginally reduced to
$\alpha/50$ mixture	1.5	0.2	1.5	1	0.02	0.005	0.002	give α-dose; E_2 active at $\alpha/10$
6 α-Dose for individual compounds and contribution to mixture	75	10	75	50	1	0.25	0.1	α-Dose for each compound
$\alpha/2$ mixture	37.5	5	37.5	25	0.5	0.125	0.05	identical to those in experiment 5;
$\alpha/5$ mixture	15	2	15	10	0.2	0.05	0.02	compounds tested individually at
$\alpha/10$ mixture	7.5	1	7.5	5	0.1	0.025	0.01	$\alpha/50$ to ensure absence of
$\alpha/20$ mixture	3.75	0.5	3.75	2.5	0.05	0.0125	0.005	uterotrophic response
$\alpha/50$-dose for individual compounds and contribution to mixture	1.5	0.2	1.5	1	0.02	0.005	0.002	
$\alpha/100$ mixture	0.75	0.1	0.75	0.5	0.01	0.0025	0.001	

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, E_2, 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 7× 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.

Statistical analyses. Organ weights were considered by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) on final body weight using SAS software (SAS Institute 1999). In addition, organ to body weight ratios were considered by ANOVA. Statistical outcomes shown in Tables 2 and 3 and in Figures 1–6 are based on ANCOVA.

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

Experiment	Compound	Dose (/kg)	Uterine weight (mg) Blotted	Uterine weight (mg) Dry	Final body weight (g)
A	AO	4 mL	19.4 ± 2.1		51.9 ± 6.7
	BPA	10 mg	23.9 ± 3.3		52.1 ± 7.5
		100 mg	29.0 ± 3.8		50.8 ± 2.0
		300 mg	38.5 ± 6.3		52.6 ± 3.6
		600 mg	41.4 ± 7.8		51.5 ± 6.7
		800 mg	59.0 ± 8.6		49.6 ± 5.4
B	AO	4 mL	17.2 ± 1.5		52.3 ± 6.0
	GEN	1 mg	19.7 ± 1.3		51.1± 5.8
		15 mg	37.4 ± 7.6		51.9 ± 5.3
		35 mg	47.6 ± 9.3		51.1 ± 5.1
		50 mg	54.2 ± 9.0		51.6 ± 4.4
		80 mg	67.7 ± 8.2		52.6 ± 4.9
	EE	0.3 µg	49.3 ± 3.4		50.8 ± 4.5
		1.0 µg	91.9 ± 9.1		51.4 ± 3.6
1	AO	5 mL	21.7 ± 3.6	4.3 ± 0.7	51.1 ± 4.6
	BPA	300 mg	40.2 ± 6.1**	7.8 ± 1.0**	50.3 ± 4.7
		600 mg	45.8 ± 7.9**	9.1 ± 1.3**	50.9 ± 5.7
	GEN	15 mg	51.5 ± 7.1**	9.8 ± 1.0**	50.3 ± 4.5
		50 mg	82.8 ± 11.8**	15.0 ± 1.8**	49.3 ± 4.2
	EE	1 µg	101.7 ± 9.8**	17.8 ± 1.2**	50.2 ± 5.2
	BPA/GEN	300 mg/15 mg	47.6 ± 7.3**	9.9 ± 1.3**	49.7 ± 4.4
		300 mg/50 mg	67.7 ± 13.9**	13.0 ± 2.2**	49.4 ± 4.4
		600 mg/15 mg	57.9 ± 8.4**	11.6 ± 1.3**	48.7 ± 4.6
		600 mg/50 mg	68.3 ± 5.6**	13.0 ± 0.9**	48.9 ± 5.8
2	AO	5 mL	23.0 ± 2.3	4.2 ± 0.3	54.3 ± 3.3
	BPA	300 mg	39.6 ± 8.2**	7.5 ± 2.2**	52.7 ± 3.8
	GEN	10 mg	47.4 ± 3.4**	8.4 ± 0.6**	54.3 ± 4.2
		15 mg	51.2 ± 10.2**	9.1 ± 1.7**	54.0 ± 4.4
		20 mg	60.5 ± 8.7**	10.5 ± 1.6**	54.0 ± 3.4
		30 mg	70.1 ± 5.0**	12.2 ± 0.9**	55.0 ± 3.3
		40 mg	76.3 ± 9.7**	13.4 ± 1.6**	54.8 ± 4.3
		50 mg	86.4 ± 15.0**	14.6 ± 2.3**	54.2 ± 3.9
	EE	1 µg	96.7 ± 11.6**	15.6 ± 1.5**	54.0 ± 4.5
	BPA/GEN	300 mg/10 mg	50.8 ± 6.8**	9.1 ± 1.1**	54.5 ± 3.0
		300 mg/15 mg	46.1 ± 5.8**	8.5 ± 0.9**	53.3 ± 3.8
		300 mg/20 mg	56.0 ± 8.9**	9.9 ± 1.4**	52.9 ± 3.8
		300 mg/30 mg	64.1 ± 5.7**	11.3 ± 0.9**	54.5 ± 4.7
		300 mg/40 mg	65.3 ± 7.0**	11.1 ± 1.1**	53.2 ± 3.2
		300 mg/50 mg	66.3 ± 8.8**	11.4 ± 1.6**	53.1 ± 4.8
3	AO	5 mL	21.8 ± 3.6	4.1 ± 0.6	53.2 ± 3.5
	BPA	30 mg	31.7 ± 2.9**	6.2 ± 0.6**	53.2 ± 3.5
		75 mg	29.7 ± 3.9**	5.8 ± 0.9**	52.4 ± 4.1
		150 mg	32.3 ± 5.0**	6.3 ± 0.9**	51.0 ± 5.5
		300 mg	35.2 ± 3.4**	6.8 ± 0.6**	51.5 ± 3.9
	GEN	1 mg	23.4 ± 4.7	4.7 ± 1.0	51.7 ± 4.4
		2.5 mg	26.9 ± 4.1*	5.3 ± 0.8**	52.6 ± 4.3
		5 mg	26.8 ± 2.5*	5.4 ± 0.6**	51.7 ± 3.1
		10 mg	44.6 ± 7.4**	8.1 ± 1.5**	54.3 ± 2.5
	BPA/GEN	30 mg/1 mg	32.5 ± 2.9**	5.8 ± 0.5**	53.3 ± 4.1
		75 mg/2.5 mg	28.9 ± 2.4**	5.1 ± 0.6**	51.7 ± 6.2
		150 mg/5 mg	33.1 ± 3.4**	5.8 ± 0.6**	52.4 ± 4.3
		300 mg/10 mg	41.0 ± 5.8**	7.4 ± 1.1**	53.1 ± 3.8

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 5). Data from experiments 1–3 were analyzed for statistical significance by both 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 1–7. We used 1 µg/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

and GEN (Table 2, Figures 1–3). Significant increases in uterine weight (blotted and dry) were induced by both compounds administered individually, with the lowest active dose for GEN being 2.5 mg/kg (experiment 3; Table 2) and that for BPA being 30 mg/kg (experiment 3; Table 2). In experiment 1 (Figure 1), the mixture of BPA and GEN gave intermediate uterine weights—greater than the effect of BPA alone but less than the effect of GEN alone. In experiment 2 (Figure 2), addition of a fixed dose of 300 mg/kg BPA to different doses of GEN (10–50 mg/kg) attenuated the GEN dose response, with effects at the top two doses being significantly reduced. In experiment 3 (Table 2, Figure 3), a fixed ratio of 30:1 BPA:GEN was evaluated over a dilution range. The activity of the mixture was not significantly different from the response given by BPA alone.

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 (/kg)	Contribution to mixture	Uterine weight (mg) Blotted	Uterine weight (mg) Dry	Final body weight (g)
4	AO	5 mL	–	24.2 ± 3.8	4.9 ± 1.0	53.6 ± 5.7
	NP	10 mg	$\alpha^*/5$	24.2 ± 4.6	4.7 ± 0.7	55.9 ± 10.4
		50 mg	α^*	24.8 ± 4.3	4.9 ± 0.6	54.5 ± 6.8
	MXC	10 mg	$\alpha^*/5$	23.1 ± 4.2	4.6 ± 0.6	51.0 ± 6.9
		50 mg	α^*	34.5 ± 9.4**	6.4 ± 1.5*	53.7 ± 4.1
	BPA	15 mg	$\alpha^*/5$	29.0 ± 1.6	5.9 ± 0.4	54.0 ± 4.3
		75 mg	α^*	32.0 ± 4.2*	6.4 ± 0.7**	52.2 ± 6.0
	GEN	1 mg	$\alpha^*/5$	24.9 ± 5.0	4.9 ± 0.8	52.0 ± 7.2
		5 mg	α^*	26.0 ± 4.9	5.0 ± 1.0	53.3 ± 6.5
	E_2	0.2 µg	$\alpha^*/5$	26.6 ± 6.6	5.2 ± 1.2	52.2 ± 6.0
		1.0 µg	α^*	39.1 ± 9.4**	7.4 ± 1.6**	53.2 ± 6.7
	DES	0.01 µg	$\alpha^*/5$	23.6 ± 4.5	4.9 ± 0.9	55.3 ± 9.2
		0.05 µg	α^*	28.1 ± 5.7	5.7 ± 1.1	54.3 ± 5.8
	EE	0.03 µg	$\alpha^*/5$	24.6 ± 4.5	4.9 ± 0.6	54.4 ± 4.4
		0.15 µg	α^*	46.9 ± 7.0**	8.6 ± 1.2**	54.6 ± 4.7
		1.0 µg	–	116.4 ± 32.1**	20.5 ± 5.1**	52.4 ± 9.2
	Mixture		$\alpha^*/5$	41.3 ± 5.0**	7.7 ± 0.8**	54.8 ± 6.2
			$\alpha^*/2$	49.0 ± 7.2**	9.3 ± 1.4**	53.2 ± 6.7
			α^*	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	$\alpha/10$	24.7 ± 4.9	4.9 ± 0.9	54.2 ± 5.5
		75 mg	α	36.3 ± 8.0**	6.7 ± 1.2**	54.1 ± 6.8
	MXC	5 mg	$\alpha/10$	25.3 ± 6.8	4.9 ± 1.2	53.2 ± 5.2
		50 mg	α	33.3 ± 6.4**	6.3 ± 1.2**	54.1 ± 5.7
	BPA	7.5 mg	$\alpha/10$	26.0 ± 3.1	5.3 ± 0.4**	52.3 ± 7.4
		75 mg	α	33.4 ± 3.8**	6.7 ± 0.8**	53.0 ± 7.6
	GEN	1 mg	$\alpha/10$	24.9 ± 5.0	5.0 ± 0.8	54.1 ± 6.6
		10 mg	α	51.9 ± 6.6**	9.6 ± 1.2**	53.8 ± 7.4
	E_2	0.1 µg	$\alpha/10$	28.3 ± 8.2*	5.5 ± 1.4	53.9 ± 6.9
		1.0 µg	α	50.0 ± 9.9**	8.9 ± 1.6**	53.8 ± 5.9
	DES	0.025 µg	$\alpha/10$	25.2 ± 6.3	5.4 ± 1.1	53.9 ± 7.1
		0.25 µg	α	47.9 ± 1.4**	8.5 ± 0.3**	52.3 ± 5.8
	EE	0.01 µg	$\alpha/10$	22.3 ± 5.2	4.5 ± 1.3	53.6 ± 8.7
		0.1 µg	α	31.4 ± 4.8**	5.9 ± 1.0*	55.7 ± 5.9
		1.0 µg	–	102.7 ± 10.1**	17.1 ± 1.4**	54.9 ± 5.4
	Mixture		$\alpha/50$	26.5 ± 5.5	5.5 ± 1.0	54.5 ± 6.9
			$\alpha/20$	28.1 ± 4.8*	5.5 ± 0.9*	51.6 ± 6.5
			$\alpha/10$	37.4 ± 4.1**	7.4 ± 0.7**	53.0 ± 7.0
			$\alpha/5$	44.0 ± 5.2**	8.4 ± 1.0**	52.7 ± 7.2
			$\alpha/2$	49.5 ± 5.3**	9.3 ± 1.2**	53.4 ± 6.6
			α	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	$\alpha/50$	23.7 ± 1.5	4.7 ± 0.2	55.0 ± 6.1
		75 mg	α	35.7 ± 10.0**	6.7 ± 1.4**	54.3 ± 4.3
	MXC	1 mg	$\alpha/50$	23.2 ± 5.9	4.6 ± 1.1	55.0 ± 6.6
		50 mg	α	32.9 ± 8.6**	6.2 ± 1.4**	55.1 ± 6.8
	BPA	1.5 mg	$\alpha/50$	24.4 ± 5.5	4.8 ± 0.9	54.8 ± 5.2
		75 mg	α	35.3 ± 3.2**	6.9 ± 0.7**	56.2 ± 5.5
	GEN	0.2 mg	$\alpha/50$	24.3 ± 7.4	4.5 ± 1.3	54.0 ± 6.9
		10 mg	α	42.6 ± 4.2**	7.9 ± 0.9**	54.1 ± 6.6
	E_2	0.02 µg	$\alpha/50$	22.8 ± 3.0	4.3 ± 0.6	54.3 ± 7.5
		1.0 µg	α	50.6 ± 7.9**	8.6 ± 1.0**	54.8 ± 3.9
	DES	0.005 µg	$\alpha/50$	23.4 ± 2.8	4.3 ± 0.5	55.2 ± 6.4
		0.25 µg	α	45.3 ± 5.9**	7.8 ± 0.9**	54.8 ± 7.4
	EE	0.002 µg	$\alpha/50$	25.7 ± 4.7	5.0 ± 0.9	55.9 ± 7.7
		0.1 µg	α	34.6 ± 5.5**	6.2 ± 0.9**	56.0 ± 6.5
		1.0 µg	–	98.3 ± 23.8**	16.2 ± 4.3**	55.2 ± 7.9
	Mixture		$\alpha/100$	23.0 ± 5.7	4.4 ± 1.0	55.2 ± 7.1
			$\alpha/50$	29.7 ± 6.7*	5.5 ± 1.1*	54.7 ± 6.5
			$\alpha/20$	28.3 ± 3.3	5.4 ± 0.7*	54.7 ± 6.5
			$\alpha/10$	37.1 ± 5.6**	6.8 ± 1.2**	53.5 ± 10.1
			$\alpha/5$	45.3 ± 3.8**	8.5 ± 0.8**	54.8 ± 5.3
			$\alpha/2$	53.4 ± 10.9**	10.0 ± 2.3**	55.4 ± 7.2
			α	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.

Mixtures of seven compounds. EE (1 µg/kg) gave a maximum positive uterotrophic response (both blotted and dry uterine weight) in all of the complex mixture experiments (Table 3). Three studies were performed comparing the effects on the uterus of a mixture containing seven compounds (NP, BPA, MXC, GEN, E_2, DES, and EE), with the effects induced individually by each of the mixture components (Table 3, Figures 4–6). Doses were based on previously published data or generated in-house as described in Table 1. Minor adjustments to these initial dose levels, as well as the inclusion of additional doses, allowed dose–response curves for the individual compounds to be established, and these showed a high degree of concordance with previously published data (Figure 5).

In the initial complex mixture study (experiment 4; Table 3, Figure 4), the highest dose for each of the compounds was chosen to induce a small, but statistically significant, increase in uterine blotted weight (the α^*-doses, approximating individual lowest effective dose levels). The lower individual doses (the $\alpha^*/5$-doses) were predicted to be inactive in the assay based on the published dose–response data (Figure 5). BPA, MXC, E_2, and EE individually produced significant increases in uterine weight (blotted and dry) at the α^*-doses ($p < 0.01$). However, NP, GEN, and DES failed to increase uterine weight significantly. No increases in uterine weight were observed at individual $\alpha^*/5$-doses (Table 3, Figure 4). Mixtures of the seven compounds at the α^*, $\alpha^*/2$, or $\alpha^*/5$ doses each induced a significant increase in uterine weight relative to the control weights ($p < 0.01$).

In the subsequent complex mixture studies, the α^*-doses of DES, GEN, and NP were

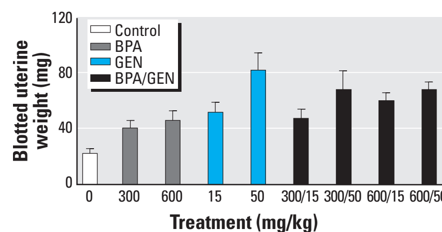

Figure 1. Uterotrophic activity of BPA (300 mg/kg or 600 mg/kg), GEN (15 mg/kg or 50 mg/kg), and mixtures of the two compounds. Error bars indicate SD. Individual compounds and the binary mixtures induced significant increases ($p < 0.01$) in blotted uterine weight (by ANOVA and ANCOVA).

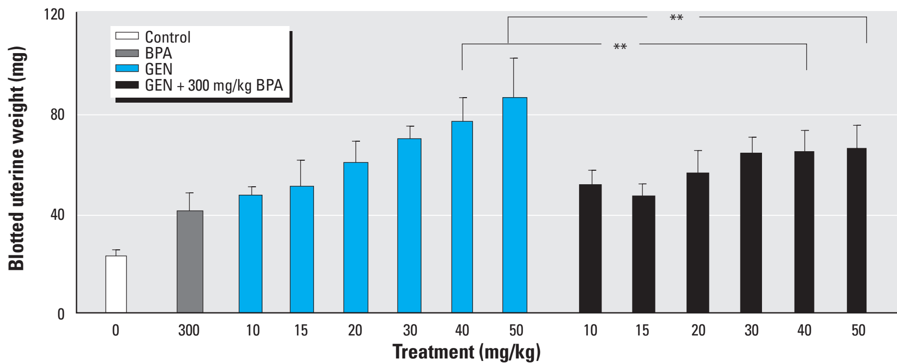

Figure 2. Uterotrophic activity of a dose range of GEN in the presence or absence of 300 mg/kg BPA. Error bars indicate SD. Data were analyzed for statistical significance by both ANOVA and ANCOVA.
** $p < 0.01$ by ANCOVA. A significant increase ($p < 0.01$) in blotted uterine weight was observed for BPA alone and for all individual doses of GEN. In addition, all BPA/GEN mixtures produced a significant increase ($p < 0.01$) in uterine weight relative to BPA alone. The mixtures produced reductions in uterine weight relative to the appropriate dose of GEN; results for the 40 and 50 mg/kg doses were statistically significant, as shown above.

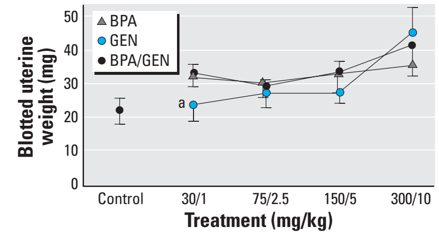

Figure 3. Uterotrophic assay dose–response curves for BPA (30–300 mg/kg), GEN (1–10 mg/kg), and dilutions of a 30:1 mixture of BPA and GEN. Error bars indicate SD. Data were analyzed for statistical significance by both ANOVA and ANCOVA. With the exception of 1 mg/kg GEN (labeled "a"), both compounds induced a significant increase in blotted uterine weight, either individually or as a mixture; ($p < 0.05$ for 2.5 and 5 mg/kg GEN; $p < 0.01$ for top dose of GEN, all mixtures and all doses of BPA).

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 E_2 ($p < 0.05$; 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 penultimate study.

Because both BPA and E_2 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,

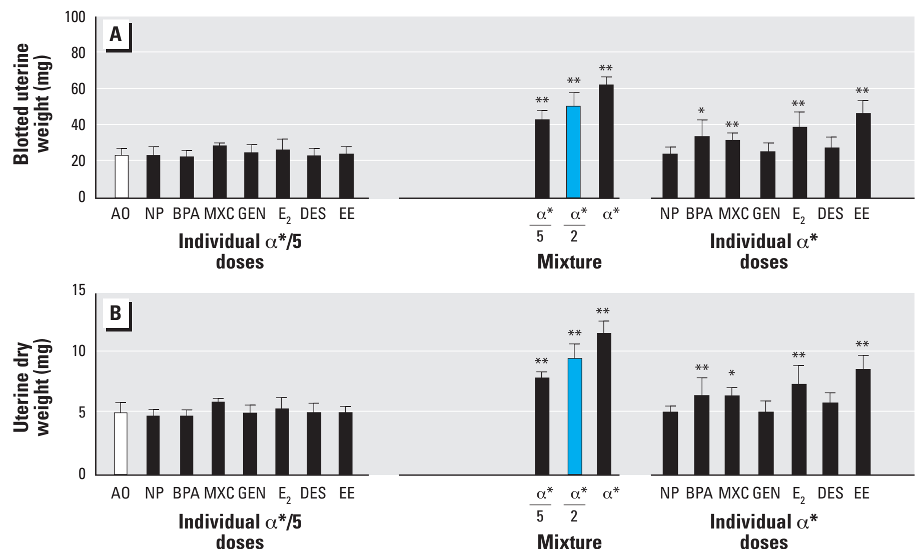

Figure 4. Uterotrophic activity of individual chemicals and their mixtures (experiment 4; Table 3) shown as (*A*) blotted uterine weight and (*B*) uterine dry weight. Error bars indicate SD. The top dose (α*) of each compound was based on Table 1 and was chosen to induce a weak increase in uterine weight. Data were analyzed for statistical significance by both ANOVA and ANCOVA.
* $p < 0.05$ and ** $p < 0.01$ by ANCOVA.

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 significant or nonsignificant increases seen for 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; Kortenkamp 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,

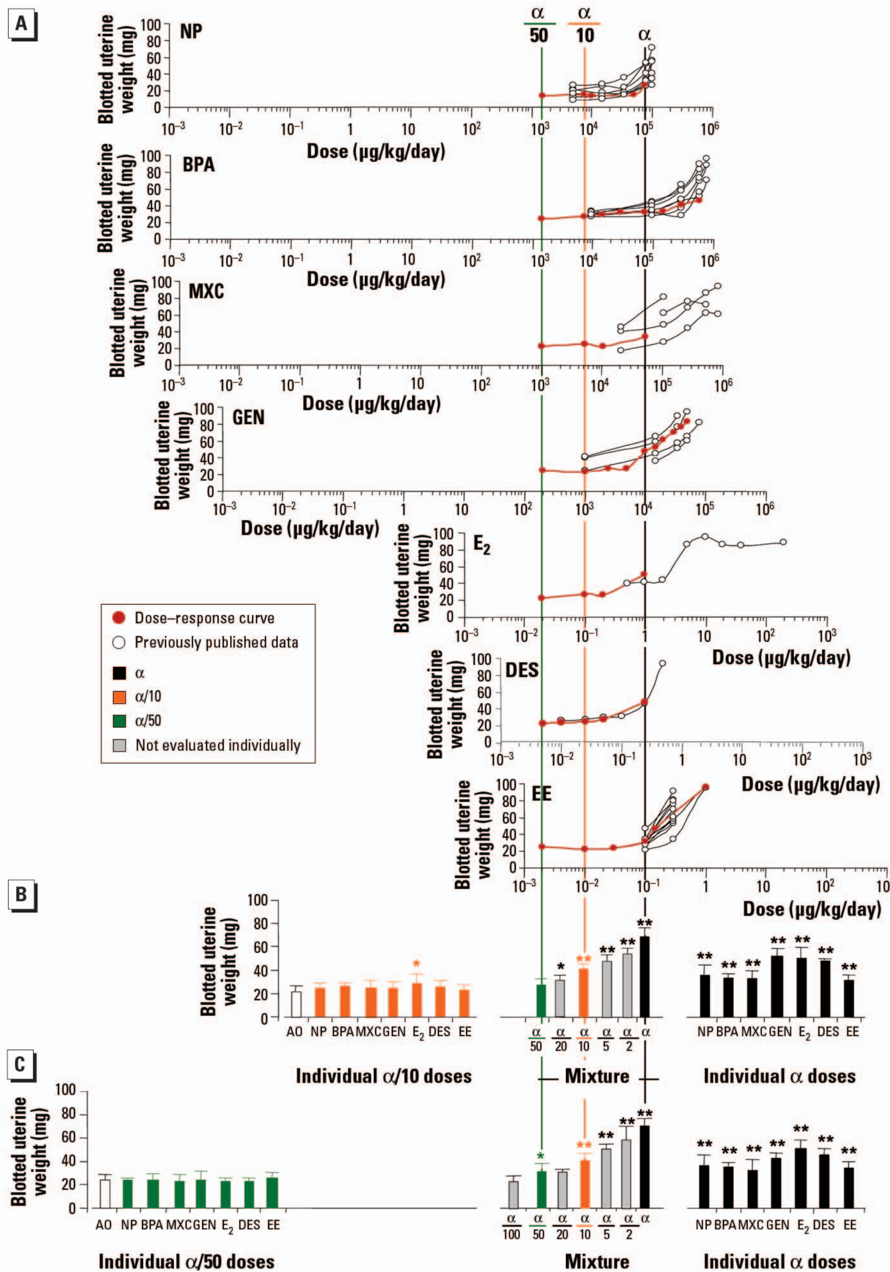

Figure 5. (*A*) Dose–response curves derived for the seven compounds used in experiments 1–6. These dose responses are compared either with previously published data: NP, BPA, MXC, and GEN (Kanno et al. 2003); EE (Kanno et al. 2001); E_2 (Odum et al. 1997); and DES (in-house data). (*B*) Uterotrophic data from experiment 5. (*C*) Uterotrophic data from experiment 6. Error bars indicate SD. The concentrations defined as α, α/10, and α/50 are highlighted because these were investigated both in mixtures and as individual agents (experiments 5 and 6; Table 3). Data were analyzed for statistical significance by both ANOVA and ANCOVA. *$p < 0.05$ and **$p < 0.01$ by ANCOVA.

and each gave a positive uterotrophic response ($p < 0.01$). With two exceptions, the individual α/10 doses (experiment 5) were nonuterotrophic. These exceptions were the blotted uterine weight for the α/10 dose of E_2 and the dry uterine weight for the α/10 dose of BPA (both $p < 0.05$). The α/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 α/20 dose in experiment 5 and to the α/50 dose in experiment 6. The fact that the α/50 mixture dose was active in experiment 6, yet inactive in experiment 5, and that the α/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 nonuterotrophic.

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; Lijinski 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.

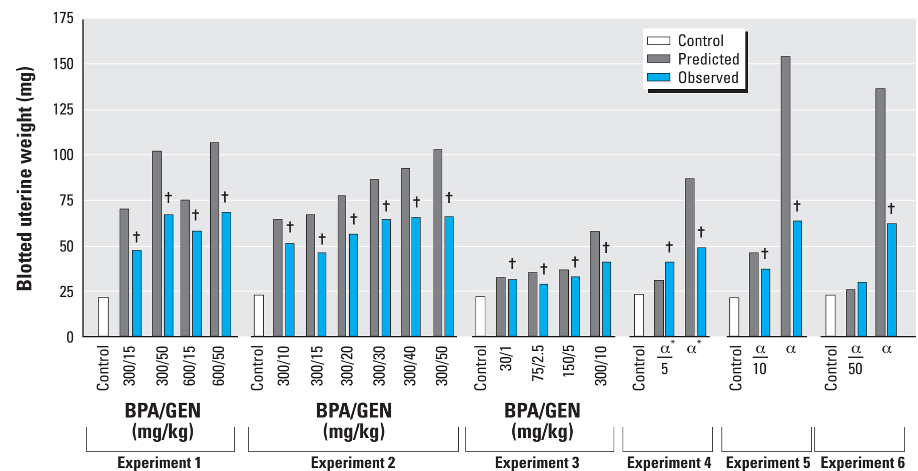

Figure 7. Comparison of observed responses for the mixtures shown with predicted outcomes. The predicted outcomes were calculated assuming additivity and represent the sum of the increases in uterine weight over concurrent controls for the components of a mixture plus one control weight.
†Observed mixture responses are significantly different from the concurrent control ($p < 0.05$ or $p < 0.01$ as described in Tables 2 and 3).

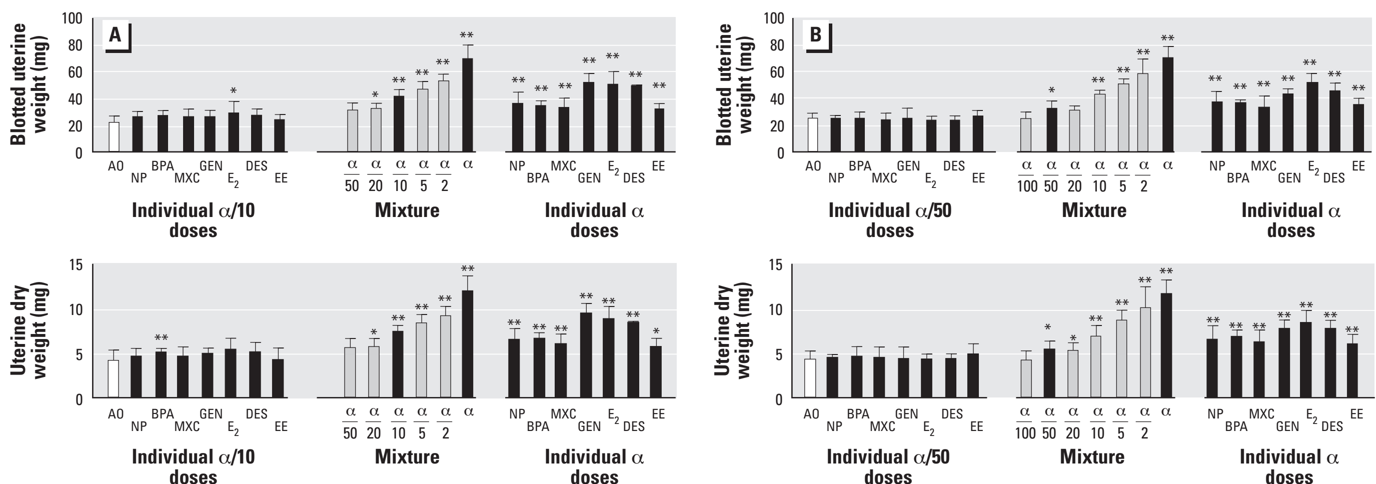

Figure 6. Comparison of blotted uterine weights with dry uterine weights for (*A*) experiment 5 and (*B*) experiment 6 (Table 3 and Figure 5). Error bars indicate SD. Data were analyzed for statistical significance by both ANOVA and ANCOVA. Mixtures, whose individual components were not evaluated, are shown as gray bars.
*$p < 0.05$ and **$p < 0.01$ by ANCOVA.

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