



The Estrogenic Activity of Phthalate Esters *In Vitro*

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A large number of phthalate esters were screened for estrogenic activity using a recombinant yeast screen. A selection of these was also tested for mitogenic effect on estrogen-responsive human breast cancer cells. A small number of the commercially available phthalates tested showed extremely weak estrogenic activity. The relative potencies of these descended in the order butyl benzyl phthalate (BBP)>dibutyl phthalate (DBP)>diisobutyl phthalate (DIBP)>diethyl phthalate (DEP)>diisononyl phthalate (DINP). Potencies ranged from approximately 1×10^6 to 5×10^7 times less than 17β -estradiol. The phthalates that were estrogenic in the yeast screen were also mitogenic on the human breast cancer cells. Di(2-ethylhexyl) phthalate (DEHP) showed no estrogenic activity in these *in vitro* assays. A number of metabolites were tested, including mono-butyl phthalate, mono-benzyl phthalate, mono-ethylhexyl phthalate, mono-*n*-octyl phthalate; all were found to be inactive. One of the phthalates, ditridecyl phthalate (DTDP), produced inconsistent results; one sample was weakly estrogenic, whereas another, obtained from a different source, was inactive. Analysis by gel chromatography-mass spectrometry showed that the preparation exhibiting estrogenic activity contained 0.5% of the *ortho*-isomer of bisphenol A. It is likely that the presence of this antioxidant in the phthalate standard was responsible for the generation of a dose-response curve—which was not observed with an alternative sample that had not been supplemented with *o,p'*-bisphenol A—in the yeast screen; hence, DTDP is probably not weakly estrogenic. The activities of simple mixtures of BBP, DBP, and 17β -estradiol were assessed in the yeast screen. No synergism was observed, although the activities of the mixtures were approximately additive. In summary, a small number of phthalates are weakly estrogenic *in vitro*. No data has yet been published on whether these are also estrogenic *in vivo*; this will require tests using different classes of vertebrates and different routes of exposure. **Key words:** contaminated standards, estrogenicity, MCF-7, metabolites, phthalates, recombinant yeast screen, ZR-75. *Environ Health Perspect* 105:802–811 (1997).

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In recent years there have been a plethora of publications discussing man-made estrogen-mimicking chemicals, the so-called xenoestrogens. Reports of declining semen quality (1) have been followed by hypotheses that this phenomenon may be linked to an increase in the exposure of humans to xenoestrogens, specifically *in utero* (2). Suspect chemicals originate from a variety of backgrounds, many being anthropogenic in origin, such as pesticides, detergents, and plasticizers. One of the earliest endocrine disruptors to be identified was the pesticide DDT, the effects of which are discussed by Fry and Toone (3). Other man-made chemicals have since been recognized as possessing estrogenic properties. For example, 4-nonylphenol is the degradation product of one group of nonionic surfactants, the nonylphenol polyethoxylates, and exposure to it has been demonstrated to induce estrogenic effects both *in vitro* (4–6) and *in vivo* (7). However, naturally occurring xenoestrogens—including phytoestrogens, such as coumestrol and genistein, and mycoestrogens, such as zearalenone—also exist; these may be found in plant food stuffs, to which humans have always been exposed (8).

Phthalates are just one of the many classes of chemicals that have been implicated as having estrogenic properties. Evidence of the estrogenic behavior of certain phthalates *in vitro* has previously been reported (9–11). Furthermore, an *in vivo* study has shown the adverse effects of butyl benzyl phthalate (BBP) on rat testes size and sperm production (12). A report concerning an *in vivo* multigenerational study investigating the reproductive toxicity of dibutyl phthalate (DBP) in Sprague-Dawley rats has recently been published. In this study, Wine et al. (13) found that a number of reproductive parameters were adversely affected by exposure to DBP administered via feed and that, critically, the second generation appeared more adversely affected than the first generation in that most of the F₁ males were infertile. The mechanisms underpinning these adverse reproductive effects are unclear presently, but one possibility is that some phthalates are estrogenic *in vivo* and hence disrupt normal male development.

Phthalates are essentially used as plasticizers in the production of polymeric materials such as polyvinyl chloride (PVC), imparting flexibility and workability, both during the manufacturing process and to

the end product. When used in this way, they are not chemically bound to the product (14) and may therefore leach into the surrounding medium (15).

Phthalates are produced in extremely large volumes [the most widely used being di(2-ethylhexyl) phthalate (DEHP), at 400–500 thousand tons per annum in Europe alone; see Table 1] and have been identified in all environmental compartments. For example, they have been reported in water, sediment, air and biota sampled from the Gulf of Mexico (16), and river water and sewage effluent samples from the Greater Manchester area, United Kingdom (17). Food samples contaminated with phthalates have also been reported (18–21). The lipophilic nature of these chemicals indicates that fatty foods such as cream, cheese, and butter are most likely to be subject to contamination. Sharman et al. (21) discovered levels of up to 114 mg/kg total phthalate in cheese samples; however, the majority of samples contained 0.6–3.0 mg/kg DEHP and 4–20 mg/kg total phthalate. The authors suggested that these high levels might have arisen from environmental sources (for example, from the wrappers surrounding the cheese) rather than as a result of the diluted presence of the contaminant in the raw commodity, followed by its distillation in the fatty phase (21). Although these chemicals are no longer used in most direct contact food plastics and use in such materials has been regulated for many years based on toxicological data available and the fat content of the food concerned (22), it is possible that other sources of contamination during the manufacturing process, and from certain printing inks and adhesives used in packaging, may contribute to levels of phthalates found in more recently sampled foods (19).

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The possibility of such extensively used chemicals as the phthalates having a detrimental influence on reproductive systems, of either humans or wildlife, clearly causes public concern, as is evident from the considerable media coverage of this issue. However, when phthalates are discussed, they are often mistakenly referred to as a single group of chemicals, with the assumption that they all have similar properties, for example estrogenic activity. In this paper we investigate the ability of individual phthalate esters to produce an estrogenic response *in vitro* and attempt to relate this factor to their occurrence as environmental contaminants, as a partial contribution to an assessment of their risk as endocrine disruptors.

Materials and Methods

Chemicals tested. 17 β -estradiol was purchased from Sigma, Poole, United Kingdom.

Thirty-five phthalates, encompassing a variety of structural and behavioral differences and including the major phthalates of commercial importance, were purchased from Greyhound Chemservice, Merseyside, United Kingdom (Table 1). These were of 97–99% purity.

For comparison, a number of commercial preparations were also obtained as gifts from companies as follows: dibutyl phthalate (DBP, 99.7% pure), diisobutyl phthalate (DIBP, 99.6% pure), diethyl phthalate (DEP, >99.7% pure), and dioctyl phthalate (DOP, 99.9% pure), from BP Chemical Ltd., Hull, United Kingdom; diisodecyl phthalate (DIDP, 99.9%) and diisononyl phthalate (DINP, 99.9%) from EXXON Chemical Ltd., Fareham, United Kingdom; ditridecyl phthalate (DTDP) from EXXON Chemical Ltd., Courbevoie, France; and butyl benzyl phthalate (BBP, >98.5%) from Monsanto Europe S.A., Louvain-la-Neuve, Belgium. Purity of these preparations is given as provided by the company.

Various phthalate metabolites were donated by R. Bos of the Department of Toxicology, University of Nijmegen, The Netherlands. These were mono-hexyl phthalate (MHP), mono-ethylhexyl phthalate (MEHP), mono-pentyl phthalate (MPP), mono-*n*-octyl phthalate (MnOP) and metabolites V, VI, and IX of DEHP (23). Also donated (by Monsanto Europe S.A.) were the primary metabolites of BBP, mono-butyl phthalate and mono-benzyl phthalate.

4-Nonylphenol, supplied by Schenectady International Inc. (Schenectady, NY), bisphenol A (Aldrich, Poole, U.K.), *o,p'*-DDT (Greyhound Chemservice, Merseyside, U.K.), and genistein (Sigma, Poole, U.K.) were tested in the recombinant yeast screen only, in order to demonstrate the activity and potency of some known xenoestrogens.

Table 1. All the parent phthalate esters tested using the recombinant yeast screen

Phthalate name	Abbreviation	European consumption (tons/annum)
Bis(2-ethylhexyl) phthalate	DEHP	400,000–500,000
Diisononyl phthalate ^a	DINP	100,000–200,000
Diisodecyl phthalate	DIDP	100,000–200,000
Butyl benzyl phthalate ^a	BBP	20,000–50,000
Dibutyl phthalate ^a	DBP	20,000–40,000
Diisobutyl phthalate ^a	DIBP	20,000–40,000
Ditridecyl phthalate ^a	DTDP	3,000–10,000
Diethyl phthalate ^a	DEP	10,000–20,000 (with DMP)
Dimethyl phthalate	DMP	10,000–20,000 (with DEP)
Diisohexyl phthalate	DIHP	<2,000
Diundecyl phthalate	DUP	<2,000
Butyl decyl phthalate	BDcP	<1,000
Butyl octyl phthalate	BOP	<1,000
Dicyclohexyl phthalate	DCHP	<1,000
Dihexyl phthalate	DHP	Not alone
Di- <i>n</i> -octyl phthalate	DnOP	Not alone
Bis(2- <i>n</i> -butoxyethyl) phthalate	DBoEP	Negligible
Bis[2-(2-ethoxyethoxy)ethyl] phthalate	DEoEoEP	Negligible
Bis(ethoxyethyl) phthalate	DEeEP	Negligible
Bis(2-ethylhexyl) hexahydrophthalate	DEHhP	Negligible
Bis(2-ethylhexyl) isophthalate	DEHIP	Negligible
Bis(methoxyethyl) phthalate	DMoEP	Negligible
Butyl cyclohexyl phthalate ^a	BCHP	Negligible
Butyl 2-ethylhexyl phthalate	BEHP	Negligible
Butyl isodecyl phthalate	BIDP	Negligible
Diamyl phthalate	DPeP	Negligible
Dimethyl isophthalate	DMIP	Negligible
Dioctyl isophthalate	DOIP	Negligible
Diphenyl phthalate ^a	DPhP	Negligible
2-Ethylhexyl isodecyl phthalate	EHIDP	Negligible
Hexyl decyl phthalate	HDP	Negligible
Hexyl 2-ethylhexyl phthalate	HEHP	Negligible
Isodecyl tridecyl phthalate	IDTDP	Negligible
Octyl isodecyl phthalate	OIDP	Negligible
Isohexylbenzyl phthalate ^a	IHBP	Negligible

A ballpark consumption figure for each phthalate is given; "not alone" indicates that these particular chemicals are not used individually but only in mixtures, in conjunction with other phthalates.

^aPhthalates found to possess estrogenic activity in the screen.

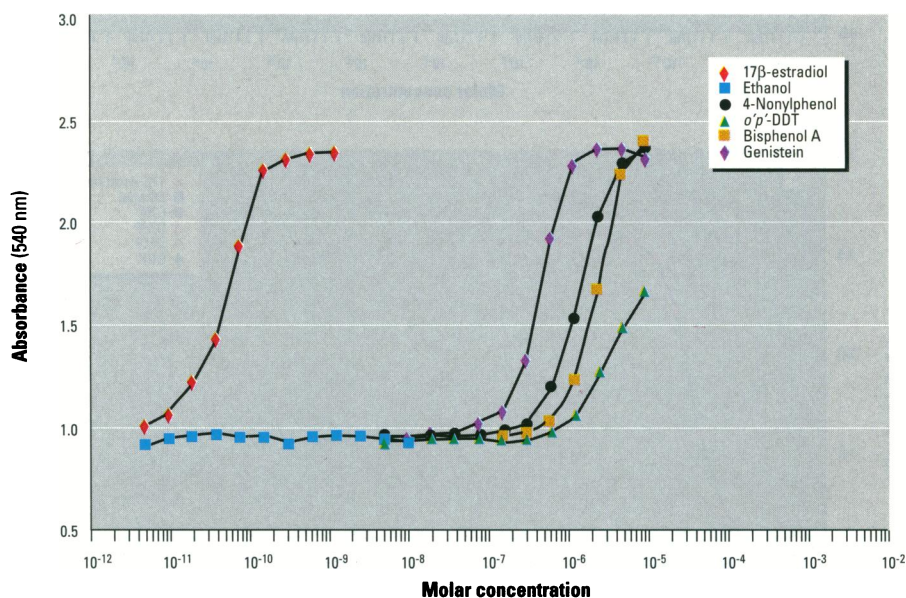
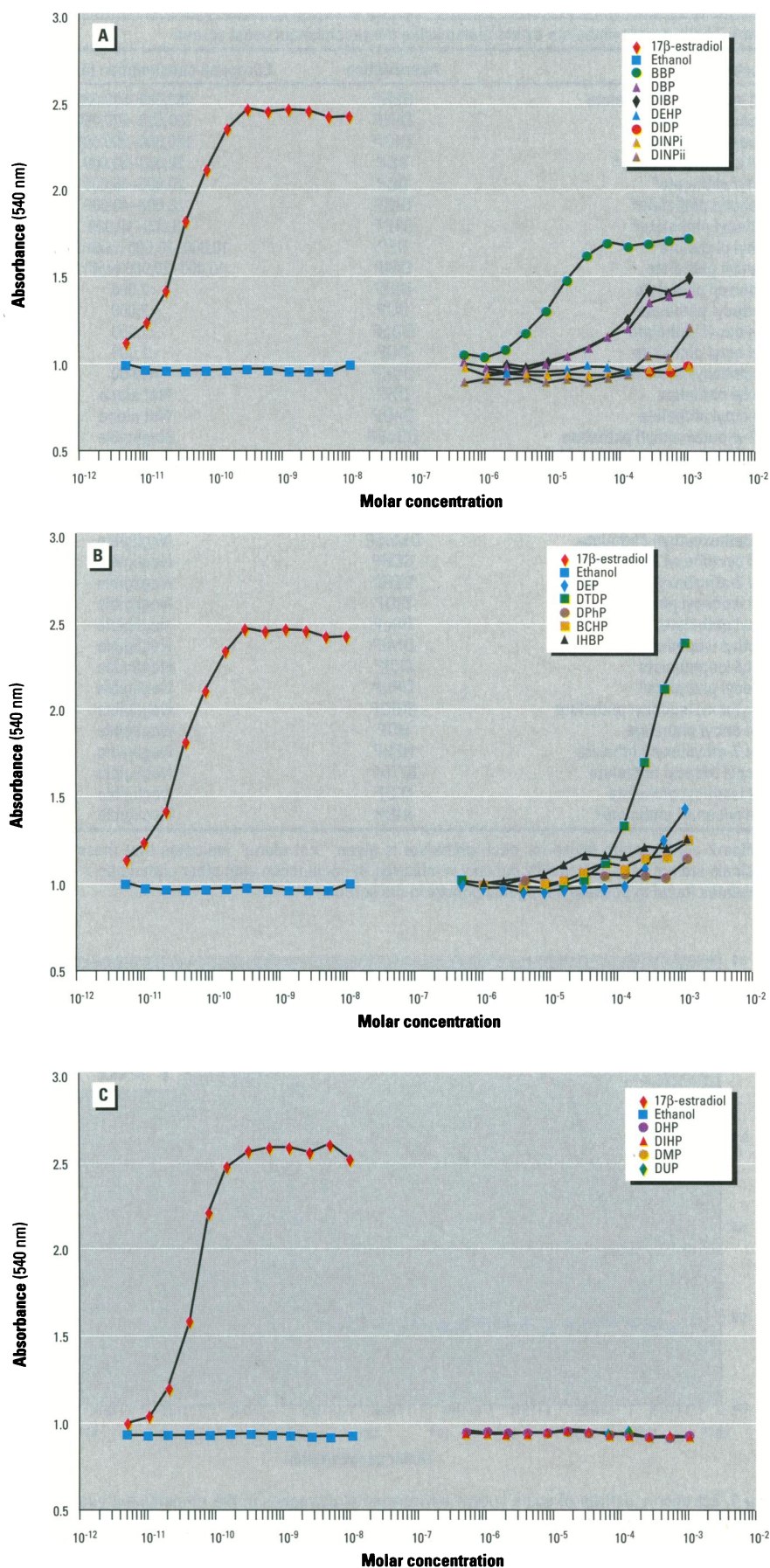


Figure 1. Estrogenic activity of some known environmental estrogens in the recombinant yeast screen. 17 β -estradiol serially diluted from 10⁻⁸ M and ethanol were used as positive and negative controls, respectively. 4-Nonylphenol, *o,p'*-DDT, bisphenol A, and genistein are shown as standard curves serially diluted from 10⁻⁵ M.



o,p'-Bisphenol A was supplied by Dow Europe S.A., Horgen, Switzerland, and was tested in the recombinant yeast screen to assess the possible significance of its presence as a contaminant in one of the phthalate samples and its effect on the apparent estrogenicity of that sample.

The recombinant yeast screen. All chemicals were assessed for estrogenic activity using a recombinant yeast screen. This is a cost-effective, sensitive, and specific process for detecting estrogenic activity. The screen has been described and extensively validated elsewhere [see Routledge and Sumpter (24) for full details]. Essentially, a gene for the human estrogen receptor has been integrated into the main yeast genome and is expressed in a form capable of binding to estrogen response elements and controlling the expression of the reporter gene *lac-Z*. Thus, on activation of the receptor, the *lac-Z* gene is expressed, producing the enzyme β -galactosidase, which is secreted into the medium where it causes a color change of the chromogenic substance chlorophenol red- β -D-galactopyranoside (CPRG) from yellow to red. The intensity of the red color can be measured by absorbance.

The screen is highly specific for estrogens; androgens, progesterones and corticosteroids are either completely inactive in the screen or very weakly active at very high concentrations (24).

Details of the preparation of medium components and yeast stocks have been published previously (24).

Growth medium was prepared by adding 5 ml 20% w/v glucose solution, 1.25 ml 4 mg/ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml 24 mg/ml L-threonine solution, and 0.125 ml 20 mM copper (II) sulfate solution to 45 ml single strength minimal medium. The yeast culture was then prepared by seeding 50 ml growth medium

Figure 2. The estrogenic activity in the yeast screen of phthalate esters at concentrations ranging from 10^{-3} M to 5×10^{-7} M, compared to 17β -estradiol (serially diluted from 10^{-8} M). A) Illustrates the estrogenic activity of phthalates consumed in major volumes in Europe. B) Illustrates the estrogenic activity of DEP and DTDP, which are used commercially in Europe, and DPhP, BChP, and IHBP, which are of negligible commercial usage. C) Portrays the lack of estrogenic activity observed in the yeast screen when the cells were incubated with certain phthalates. Abbreviations: BBP, butyl benzyl phthalate; DBP, dibutyl phthalate; DIBP, diisobutyl phthalate; DEHP, bis(2-ethylhexyl) phthalate; DIDP, diisodecyl phthalate; DINP, diisononyl phthalate; DEP, diethyl phthalate; DTDP, ditiidecyl phthalate; DPhP, diphenyl phthalate; BChP, butyl cyclohexyl phthalate; IHBP, isohexyl/benzyl phthalate; DHP, dihexyl phthalate; DIHP, diisohexyl phthalate; DMP, dimethyl phthalate; DUP, diundecyl phthalate.

with 125 μ l yeast stock and incubating this overnight at 28°C on an orbital shaker. Assay medium contained 0.5 ml 10 mg/ml chlorophenol red- β -D-galactopyranoside added to 50 ml growth medium seeded with 1 ml of the above yeast culture.

All glassware was thoroughly washed with solvent. Test chemicals were made up in ethanol to 2×10^{-2} M (phthalates), 2×10^{-4} M (4-nonylphenol, bisphenol A, genistein, *o,p'*-DDT), or 2×10^{-7} M (17 β -estradiol) stock solutions and stored at 4°C.

Stock solutions were serially diluted in ethanol, and 10 μ l of each dilution was transferred to a 96-well microtiter plate (Linbro/Titertek, ICN FLOW, Bucks, U.K.). This gave a final concentration of 10^{-3} M to 4.8×10^{-7} M for the phthalates and their metabolites, 10^{-5} M to 4.8×10^{-9} M for other xenoestrogens, or 10^{-8} M to 4.8×10^{-12} M for 17 β -estradiol. Solvent controls were set up on each plate using 10- μ l aliquots of ethanol. The ethanol was allowed to evaporate and 200- μ l aliquots of assay medium (containing the yeast) was then added to each well. The plates were then sealed with autoclave tape, shaken for 2 min on a titer-plate shaker, and incubated at 32°C for 4–6 days in a naturally ventilated oven (WTB binder, BD-series; Jencons Scientific Ltd., Bedfordshire, U.K.). Plates were shaken on day 1 of incubation and again approximately 1 hr before taking absorbance readings (540 nm for color and 620 nm for turbidity), using a Titertek Multiskan MCC/340 plate reader (Life Sciences Int., Basingstoke, U.K.).

Mammalian cells. For comparison, the proliferative effects of all commercially available phthalates showing estrogenic activity in the recombinant yeast screen, as well as those that were negative but of major volume use, were tested using two estrogen-responsive human breast cancer cell lines, MCF-7 and ZR-75. As these cell lines are of human origin, they may be of particular relevance when considering the wide exposure of humans to the phthalates, which are ubiquitous in the environment (25) and can be found in such domestic products as vinyl flooring, children's toys, printing inks, and cosmetics (26).

The phthalate samples used in these assays were the analytical standards as supplied by Greyhound Chemservice. Cells were cultured in phenol red-free medium containing 5% v/v charcoal dextran stripped serum (DCC). They were then plated in 6-well microtiter plates (Falcon, Becton Dickinson, Lincoln Park, NJ) into the aforementioned medium 3–4 days prior to commencing the experiment. For the MCF-7 cells, medium was replaced with treated medium containing either 0.1% vehicle solvent (ethanol) as a negative control, 10^{-8} M 17 β -estradiol as a positive control, or 10^{-5}

M of each respective phthalate. Cells were trypsinized and counted using a Coulter Counter (Coulter Electronics, Harpenden, Herts, U.K.) on days 0, 2, 5, 8, and 12. Treatments were duplicated and the experiment was repeated twice. For the ZR75 cells, the treatments (control, 10^{-8} M, 10^{-10} M, and 10^{-12} M 17 β -estradiol and 10^{-5} M, 10^{-6} M, and 10^{-7} M of individual phthalates) were done in triplicate. Cells were counted at a single endpoint on day 11.

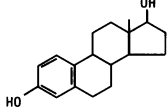
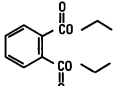
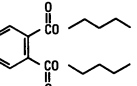
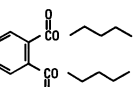
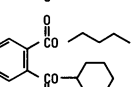
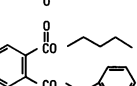
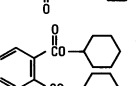
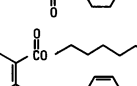
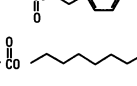
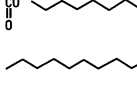
Results

Table 1 lists the phthalate esters tested, together with their consumption figures in

Europe, to give an idea of their importance relative to one another as industrial chemicals. Some phthalates generated a dose-dependent increase in β -galactosidase production in the yeast screen, indicating weak estrogenic activity.

In order to relate the significance of the activity of the estrogenic phthalates to that of other environmental estrogens, we assessed the response of the yeast screen to a range of environmental estrogens. The chemicals tested were bisphenol A (an antioxidant), genistein (a phytoestrogen), 4-nonylphenol (the degradation product of a surfactant), and *o,p'*-DDT (a pesticide);

Table 2. Phthalates found to give an estrogenic response in the yeast screen

Chemical	Structure	Approximate potency ^{a,b}	Maximum response (%) ^b
17 β -Estradiol		1	100
Diethyl phthalate		0.0000005	30
Dibutyl phthalate		0.0000001	35
Diisobutyl phthalate	 *	0.0000001	30
Butyl cyclohexyl phthalate		–	20
Butyl benzyl phthalate		0.000001	50
Diphenyl phthalate		–	10
Isohexylbenzyl phthalate	 *	–	20
Diisononyl phthalate	 *	–	15
Ditridecyl phthalate		0.0000001	95

Potency and response relative to 17 β -estradiol were calculated from data obtained on day 6 of the assay. Longer incubation times can increase the relative maximum response; thus, the values shown here apply only to a specific set of conditions.

^aIndicates the value was calculated at 25% of the maximum response.

^bAll data shown here were obtained using analytical standards.

*Alkyl chains are composed of various branched isomers.

the results are shown in Figure 1. These chemicals were tested over a concentration range of 10^{-5} M to 5×10^{-9} M, and were found to have potencies varying from approximately 10^4 – 10^5 times less than that of the main natural estrogen, 17β -estradiol.

The estrogenic activities of the major volume usage phthalates (those exceeding 20,000 ton/annum in Europe) in the yeast screen are shown in Figure 2A. Of these six major volume use phthalates, three possessed estrogenic activity (BBP, DBP, and

DIBP), two did not (DEHP and DIDP), and one (DINP) behaved unreproducibly in the screen. The former three phthalates were the most active of all those tested, and the latter three are the most extensively used in industry. Two dose–response curves were produced for DINP due to the slightly unreproducible behavior of this chemical in the yeast screen. DINP ii (Fig. 2A) shows the mean response of two standard curves in which a detectable increase in β -galactosidase production was observed. This pattern

was reproduced in three separate assays, but differed in a further three in which DINP appeared completely inactive (DINP i).

The phthalates of relatively low or negligible use in Europe (29 different ones) were assessed for estrogenic activity using the yeast screen only. Relatively few of these (five in total) possessed any estrogenic activity; all others were inactive, even at the highest concentration tested (10^{-3} M) (Fig. 2B, 2C). The results obtained from the five phthalates that showed estrogenic activity are illustrated in Figure 2B. Of these, only two (DEP and DTDP) are used commercially in Europe.

All of the phthalates that showed activity were very weak estrogens. The most potent, BBP, was approximately 1 million-fold less potent than estradiol (Table 2), making it considerably less potent than other environmental estrogens such as bisphenol A, nonylphenol, and *o,p'*-DDT. When chemicals are so weakly estrogenic, it is entirely feasible that it is not the chemical (in this case the phthalate) itself which is intrinsically estrogenic, but rather that an impurity in the chemical is estrogenic. Thus, before labeling a chemical as a weak estrogen, it is necessary to exclude the possibility that the chemical is contaminated with an estrogenic impurity. One way to address this issue is to test a number of samples, of different origin, of each phthalate possessing estrogenic activity. If all samples of a phthalate possess the same degree of estrogenic activity, it is likely that that particular phthalate is intrinsically active, whereas if the different samples of a phthalate possess considerably different potencies, it is then likely that the phthalate itself is not estrogenic, but that some samples contain varying proportions of one or more contaminants that are estrogenic.

To assess this possibility—that estrogenic contaminants might be present in some phthalates—commercial preparations of all the major volume usage phthalates, including DTDP and DEP, were assessed for estrogenic activity and their potencies compared to that of their respective analytical standards (data not shown). With one exception, no differences were observed; the estrogenic activities of the commercial preparations were equivalent to those of their respective analytical standards. However, contrary to the analytical standard, the commercial preparation of DTDP failed to produce a response, even when present at 10^{-3} M. Both samples of DTDP were subsequently analyzed by gel chromatography-mass spectrometry (GC-MS). The analytical standard (the active sample) was found to contain 0.5% of the *ortho*-isomer of bisphenol A. The inactive preparation of DTDP did not contain this chemical. A sample of *o,p'*-bisphenol A was then

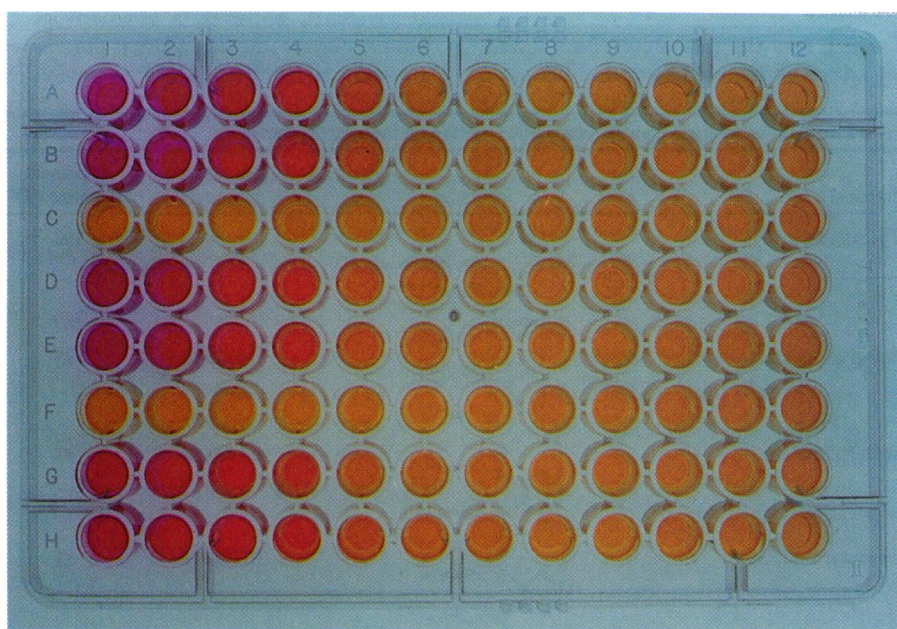


Figure 3. The activity of bisphenol A (rows A and B), *o,p'*-bisphenol A (rows D and E), and DTDP (rows G and H) in the recombinant yeast screen. Bisphenol A and the *ortho-para* isomer of this chemical were serially diluted (left to right) from 10^{-5} M. DTDP was serially diluted from 10^{-3} M. Rows C and F are controls (10 μ l ethanol added to each of these wells).

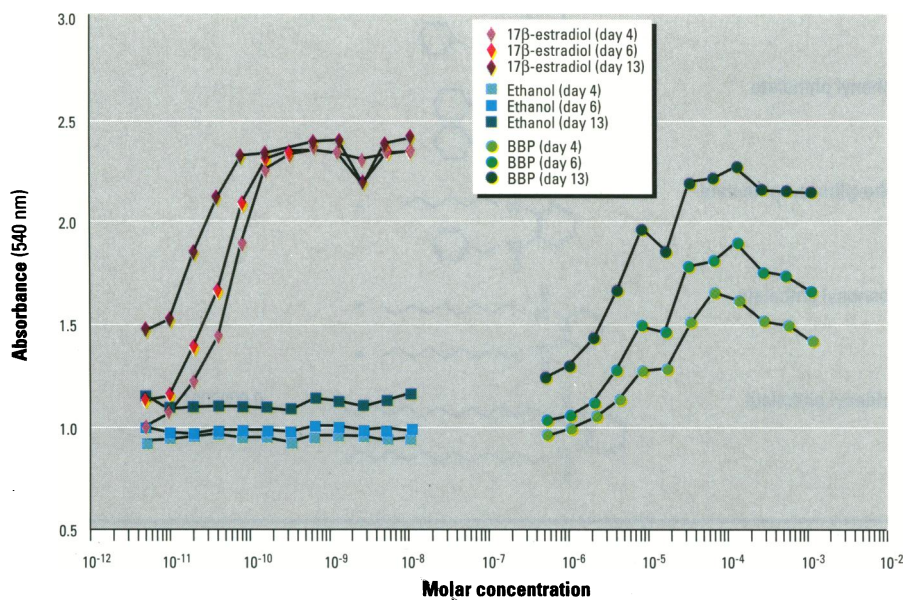


Figure 4. Development of the butyl benzyl phthalate (BBP) standard curve over time. The BBP standard curve can be seen to be developing to an almost maximal response in this yeast assay.

obtained and its response in the yeast screen was compared with that of the active DTDP sample. Figure 3 shows that *o,p'*-bisphenol A was about 100 times more potent than DTDP. Therefore, the presence of this chemical at just 0.5% in the DTDP sample would produce a response equivalent to that seen. Thus, it is likely that this chemical (*o,p'*-bisphenol A) was responsible for the weak activity observed in this phthalate sample (see Fig. 1); hence DTDP is not estrogenic.

The results shown in Figure 2A and 2B show that most of the active phthalates were unable to produce a maximal response in the yeast assay; only DTDP did so. For example, the response to BBP (the most estrogenic phthalate) reached a plateau at approximately 50% of the maximum response achieved with 17 β -estradiol. To determine whether this means that most of the phthalates are only partial estrogen agonists or whether other explanations account for the submaximal responses observed, a yeast screen containing BBP was incubated for longer than usual and the response was monitored daily. The results (Fig. 4) show that on day 4 (the usual incubation time for our yeast assays) BBP produced a shallow dose–response curve. However, by day 6, the response was greater. By day 13 the highest concentration of BBP had produced a maximal response. Note also that the dose–response curve to 17 β -estradiol moved approximately fourfold to the left between days 4 and 13 (i.e., the yeast screen became more sensitive), but the dose–response curve for BBP moved considerably further. Thus, the potency of BBP increased somewhat with time. For this reason, all the other phthalate data shown in this paper was obtained from yeast assays incubated for 6 days.

To assess whether the estrogenic responses observed in the yeast assay were reproducible in other estrogen assays, active phthalates (plus the major volume use phthalates, DEHP and DIDP) were also tested for their ability to stimulate proliferation of MCF-7 and ZR-75 cells. The results from these assays (Fig. 5 and Fig. 6), which are based on human breast cancer cell lines, are mostly comparable to those obtained from the yeast screen. However, DEP and DTDP failed to induce proliferation of ZR-75 cells at 10⁻⁵, 10⁻⁶, or 10⁻⁷ M (Fig. 5B) although they had been active in the yeast screen, albeit only at higher concentrations. Using the ZR-75 cells, DINP at 10⁻⁵, 10⁻⁶, and 10⁻⁷ M induced proliferation to a significantly greater extent than the control, which is in contrast to our findings for this chemical using the yeast screen. Growth curves for all estrogenic phthalates (i.e., those active in the yeast assay) and for DEHP and DIDP were obtained using MCF-7 cells. The results (Fig. 6) showed

that BBP was considerably more mitogenic than any of the other phthalates. DTDP, DIBP, and DBP were approximately equivalent in activity, and all the other phthalates tested showed relatively little activity. All these results are consistent with those obtained using the yeast assay.

Possible additive or synergistic effects between the most potent phthalates were investigated by incubating known concentrations of BBP, DBP, and 17 β -estradiol either individually or as simple mixtures in the yeast screen. The concentration of 17 β -estradiol used produced only a small response above background (Fig. 7), so that if additivity or synergism occurred, they could be observed

within the range of the assay. Two concentrations of each of the most active phthalates (BBP and DBP) were tested alone and in combination with 17 β -estradiol. In all cases, the response obtained was very close to that expected if additivity had occurred (Fig. 7); in no case was the response significantly greater than predicted if additivity had occurred, that is, no evidence of synergism was observed.

The phthalate metabolites tested included 1) derivatives of the most abundant phthalate (DEHP), namely MEHP and metabolites V, VI, and IX (23); 2) MBzP and MBuP, which are primary metabolites of the most estrogenic phthalate (BBP); and 3) MHP, MnOP, and

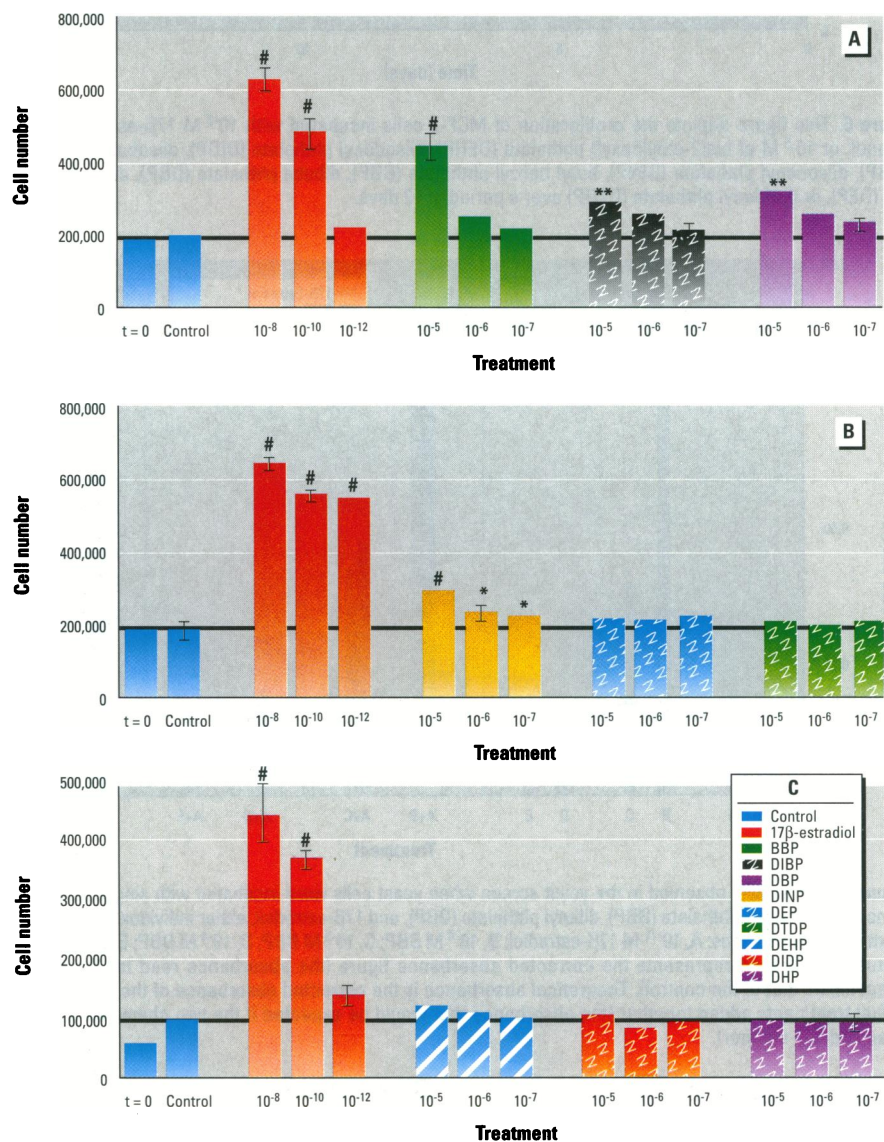


Figure 5. The proliferation of ZR-75 cells incubated with various phthalates and controls including time = 0 (t = 0), ethanol, and 17 β -estradiol (data obtained from three separate assays). A) Cells incubated with butyl benzyl phthalate (BBP), diisobutyl phthalate (DIBP), and dibutyl phthalate (DBP). B) Cells incubated with diisononyl phthalate; (DINP), diethyl phthalate (DEP), and dtridecyl phthalate (DTDP). C) Cells incubated with bis(2-ethylhexyl) phthalate (DEHP), diisodecyl phthalate (DIDP), and dihexyl phthalate (DHP). A simple ANOVA was performed on the data, followed by the Bonferroni/Dunn test for multiple comparisons. Cell numbers significantly greater than the control are denoted by * p <0.05; ** p <0.01; # p <0.001.

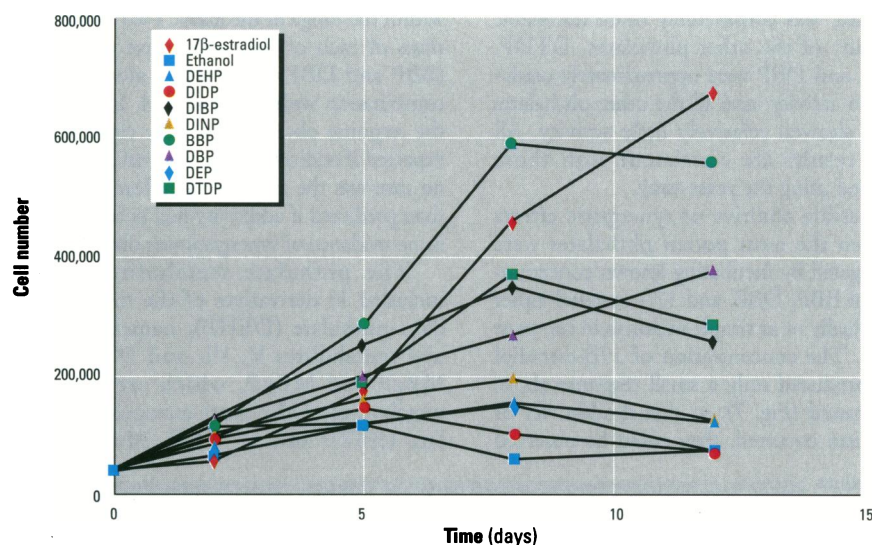


Figure 6. This figure depicts the proliferation of MCF-7 cells incubated with 10^{-8} M 17β -estradiol, 0.1% ethanol, or 10^{-5} M of bis(2-ethylhexyl) phthalate (DEHP), diisodecyl phthalate (DIDP), diisobutyl phthalate (DIBP), diisononyl phthalate (DINP), butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), diethyl phthalate (DEP), or ditridecyl phthalate (DTDP) over a period of 12 days.

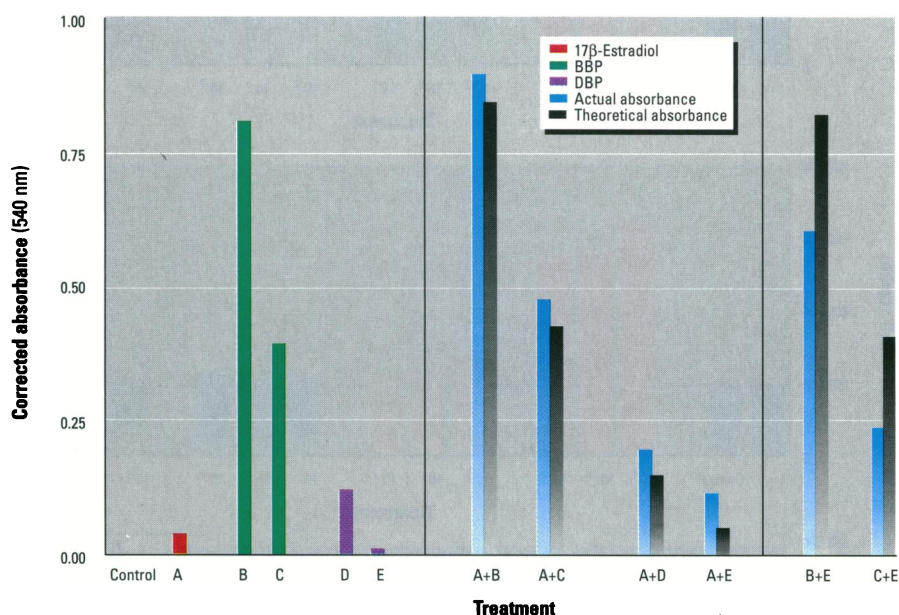


Figure 7. The activity observed in the yeast screen when yeast cells were incubated with single concentrations of butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), and 17β -estradiol, either individually or in simple mixtures. Abbreviations: A, 10^{-11} M 17β -estradiol; B, 10^{-4} M BBP; C, 10^{-5} M BBP; D, 10^{-4} M DBP; E, 10^{-5} M DBP. Actual absorbance represents the corrected absorbance figure (the absorbance read for the treated yeast minus that of the control). Theoretical absorbance is the corrected absorbance of the relevant individual treatments added together (the absorbance that would be expected if the two chemicals behaved in an additive manner).

MPeP. All were serially diluted from 10^{-3} M to 4.8×10^{-7} M, and none showed any signs of estrogenic activity in the yeast screen (data not shown).

Table 2 summarizes the relative potency and the magnitude of the responses (compared to 17β -estradiol) of all phthalates that were active in the yeast screen, together with their structures.

Discussion

In this paper, we investigate the possible estrogenic behavior of a large number of phthalate esters *in vitro*. As far as we are aware, this is the first paper to address individual estrogenic potencies for such a comprehensive spectrum of this class of chemicals.

The phthalates studied are used by industry in variable amounts, the greatest

of which is for DEHP, at up to 500,000 tons/annum in Western Europe. The worldwide production of another class of chemicals, the alkylphenol polyethoxylates, was 360,000 tons/annum in the late 1980s (27), which puts into perspective the large scale use of phthalate esters as industrial chemicals, as well as their potential environmental importance.

In terms of their estrogenic behavior, it seems that those phthalates requiring further scrutiny include 1) the shorter chain phthalates, namely BBP, DBP, and DIBP, which are used by industry in smaller quantities (Table 1), but are more estrogenically active; and 2) the longer chain phthalate DINP, which although extremely weakly estrogenic *in vitro*, is used in large quantities (up to 200,000 tons/annum in Europe). The estrogenic behavior of the phthalates in these assays compares favorably to that previously reported (9-11), where the potency of BBP (approximately 1 millionfold less potent than estradiol in the yeast screen) was similar to that reported by Soto et al. (10,11) in the E-SCREEN assay (3 millionfold less potent than estradiol) and the relative strengths of the phthalates reported to be estrogenic by Jobling et al. (9) correspond to that observed in the yeast screen (BBP > DBP). It must also be noted that, generally speaking, the activities of the phthalates in the recombinant yeast screen were reproduced in the mammalian assays, thus implying that these are real estrogenic effects, and not artifactual. There were occasional discrepancies between assays: DTDP and DEP were not found to be mitogenic in the ZR-75 cell line, but they had shown slight mitogenic activity in the MCF-7 assay and a positive response in the recombinant yeast screen. The yeast cells are more robust than mammalian cells and so could be exposed to higher concentrations of phthalates with no adverse effects, hence, the observation of activity at the higher concentrations applied in the yeast screen. The reasons for discrepancy between the two mammalian assays are unclear, but may be a result of the enhanced proliferation of the MCF-7 cell line in the presence of growth factors (the identity of which is not known), as compared to the ZR-75 cell line, which is more estrogen specific.

All active chemicals, however potent, are said to be active because they cause a response above the baseline. However, for all active phthalates, only a partial dose response was observed after the usual incubation time. For example, for DINP, the most used of all the active phthalates, the maximum response was just 15% of the maximum response obtained with 17β -estradiol. A possible explanation for results such as these, which suggest partial agonistic

behavior of the phthalates, is that these chemicals were not fully solubilized in the water-based medium employed in these assays. This is a situation frequently encountered when applying highly organic compounds to *in vitro* assays and is entirely feasible since, generally speaking, the solubility values for phthalates are lower than the concentrations used in these trials. Thus, it is plausible that some of the phthalates tested are actually more potent than they appear to be. However, it must be noted that the chemical treatments were added to the medium of the mammalian cell assays in ethanol, thus leading to greater homogeneity throughout, and still only a partial response was observed. Conversely, contamination of a chemical with an estrogenic compound can imply a weak estrogenicity of the substance in question when it is, in fact, the contaminant that is generating the observed response and the chemical under investigation is not estrogenically active. This phenomenon was detected in the case of DTDP, where the weakly estrogenic preparation was found to be contaminated with the *ortho*-isomer of bisphenol A. Hence, caution must be applied when labeling a chemical a weak estrogen, particularly if the chemical is not pure (which is usually the case, especially with industrial chemicals).

It has been reported that there is a relationship between the structure of a chemical and its estrogenic behavior (28). Of the total number of phthalates tested in our study, five possessed a secondary ring structure (BBP, BChP, DPhP, IHBP, DChP); of these, the first four were all weakly estrogenic, albeit with varying potencies. However, by no means was this the key to estrogenicity. Of those considered to be estrogenically active, there were several that possessed alkyl side-chains, and of these, a greater maximum response was obtained with DBP, DIBP, and DEP than by those with a secondary ring structure. It appeared that the majority of the active phthalates were among the lower molecular weight species, but again there were inconsistencies with this observation, with many of the lighter phthalates being inactive in the recombinant yeast screen. It is therefore difficult to deduce, from their two-dimensional structures alone, which phthalate esters will elicit estrogenic responses.

If a chemical exhibits only weak estrogenic activity *in vitro*, it does not necessarily follow that the effect of the same chemical will be insignificant when applied to a whole organism. Unfortunately, results of the nature obtained here cannot be directly extrapolated to an *in vivo* situation. It is not known at present whether any phthalates are estrogenic *in vivo*, and it will be neces-

sary to test these chemicals *in vivo* via different routes of exposure before reaching conclusions. Although *in vitro* assays give us an idea of the potential strength of a chemical as a xenoestrogen, they cannot simulate changes to the chemical within an organism and differences in the systems of individual organisms, which may influence the potency and/or bioavailability of the chemical. Metabolic processes will vary greatly, depending on the route of uptake and on the characteristics of both the chemical and the organism concerned.

Another difficulty in estimating the environmental hazard posed by phthalate esters is the lack of data documenting the exposure of humans or wildlife to these chemicals. The fact that phthalates are used in a wide variety of extensively used goods is indisputable. It is also known that they can exude from these products. For example, DBP has been found to leach from dentures (29), as has DINP from milk tubing (30). Furtmann (31) has suggested that the main source of phthalates are the consumer products themselves and that there is some justification in the inference that, following dumping or incineration of these products, there are considerable phthalate emissions into the environment. The estimated total loss of phthalate esters in Western Europe has been put at 7,740 tons/annum, or approximately 1% of total consumption (32). However, the use of such data in the analysis of environmental hazard assessment for individual chemicals is problematic because the data is generalized and estimates refer to total phthalates.

By far the most frequently reported phthalate, and that found at highest concentrations in the environment, is DEHP. This is to be expected, considering its high usage and greater likelihood of persistence relative to the shorter chain phthalates. For this reason, one would also expect DIDP and DINP to be apparent in environmental samples, but reports concerning these phthalates are sparse. Other phthalates that have been regularly documented in food (19,20), air (33,34), sediments (31,35), and river water (36,37) include the lower molecular weight phthalates such as DMP, DEP, DBP, and BBP. These are less stable as plasticizers and are therefore liable to migrate from a polymer matrix, particularly when this material is subjected to elevated temperature or surrounded by a lipophilic medium. For this reason, despite lower consumption of these phthalates compared to the higher molecular weight species, it is perhaps not surprising for them to be commonly detected, albeit at very low concentrations, in environmental samples. The solubility and environmental persistence of individual

phthalates is somewhat dependent upon the chain length of the phthalate concerned. [For a more detailed discussion of the behavior of phthalates in the aquatic environment, see Furtmann (31)]. It must also be considered that these chemicals are not present in isolation in environmental systems. In any one system, various mixtures of toxic organic chemicals can be found. For example, a cocktail of trace organics was documented in alligator eggs in Lake Apopka, Florida (38). Phthalates themselves have been found in environmental samples alongside polychlorinated biphenyls, *p,p'*-DDT, and *p,p'*-DDE (34). Certain of the PCB congeners, for example 3,4,3',4'-tetrachlorobiphenyl, have been identified as estrogen mimics (39), whereas *p,p'*-DDT and *p,p'*-DDE have both been reported to possess antiandrogenic properties (40). In addition, various combinations of phthalates have been found to be present in environmental samples (37,41). With the possibility that any contaminated environmental sample will contain more than one endocrine disrupting chemical, it seems necessary to investigate whether the effect of a combination of these chemicals will be additive, more than additive, or antagonistic. This issue was addressed by incubating simple combinations of 17 β -estradiol and two estrogenic phthalates (BBP and DBP) in the recombinant yeast screen. Jobling et al. (9) found DBP and BBP, in the presence of 17 β -estradiol, to have an agonistic, as opposed to antagonistic, effect on the stimulation of transcriptional activity in transfected MCF-7 cells. We demonstrate in this paper that the activity of combinations of two phthalates, DBP and BBP, at the concentrations shown (Fig. 7) are, in fact, slightly less than additive. When these chemicals were incubated in the presence of 17 β -estradiol (with BBP at a concentration that would induce a less than maximal response), the behavior of the combination was again additive rather than synergistic.

Another factor influencing the occurrence of phthalates in the environment is their potential for persisting and accumulating in organic matrices. This would be expected to be high because phthalates are hydrophobic chemicals; thus, it might be possible to predict their environmental fate pattern based on that of other man-made organic chemicals. For example, the polychlorinated biphenyls (42) and 4-nonylphenol (43) bioaccumulate in organisms that are exposed to these chemicals over a period of time, and they also biomagnify through the food chain. However, phthalates appear to be more readily metabolized than these persistent chemicals, particularly by enzymes in the gut (44) and in sewage treatment works,

although their rate of degradation does appear to be influenced by the length of their side chains (45,46). It is not known whether the yeast strain employed in the assays shown in this paper is capable of metabolizing complex organic chemicals, although methoxychlor has shown a positive response in the recombinant yeast screen (47); and it has been reported that this chemical must be metabolized before it becomes estrogenically active (48), thus suggesting that the yeast strain is capable of degrading certain organic chemicals. A small number of phthalate metabolites were tested in the recombinant yeast screen, including monobutyl phthalate (the primary metabolite of DBP and DIBP) and monobenzyl phthalate (which, with monobutyl phthalate, are the primary metabolites of BBP). All metabolites tested were inactive in this assay, suggesting that it is the parent compounds which are estrogenic. This is significant in that, as previously discussed, the phthalates appear to be metabolized following oral exposure, and hence the monoesters are more likely to be the bioavailable form of phthalates.

It is conceivable that the route of exposure of an organism to phthalates is an important parameter when considering metabolism of these chemicals *in vivo*. It seems probable that phthalates are readily metabolized in the gut, such that oral exposure would not lead to accumulation of high concentrations of these chemicals. However, there is little data available on the metabolism of this group of chemicals following inhalation or dermal exposure. It is perhaps necessary to investigate the fate of phthalates within an organism following administration via these routes, judging by the presence of these chemicals in a wide array of contact media. In addition, uptake via the gills, hence directly into the blood system, as occurs in fish, may elicit responses that other routes of exposure would not.

In summary, a small number of commercially available phthalate esters (BBP, DIBP, DBP, DEP, DINP) are capable of acting as extremely weak estrogens *in vitro*. How this is relevant to the environment cannot yet be directly estimated, partly because comprehensive data concerning the environmental fate and behavior of these individual phthalates is not available and partly due to the impracticalities involved with extrapolating *in vitro* data to a whole animal situation. The phthalate most widely used by the plastic industry, and that reported on with greatest frequency, is DEHP. This phthalate did not show estrogenic activity in the assays employed in this paper. Laboratory biodegradation studies, particularly of the shorter chain phthalates (that is, those which are the more potent

xenoestrogens), might imply that concentrations in the environment as a whole, and within an organism, would not reach values high enough to be of significant danger. Although the potential exists for the above-mentioned chemicals to generate adverse effects when present within the system of an organism, the concentrations and the conditions of exposure required to do so are unknown. Also note that this paper has investigated one mechanism of action only, that is, the ability of phthalates to act as estrogen agonists. This may be just one of many pathways that might lead to adverse reproductive effects in animals exposed to these chemicals. The results of *in vivo* experiments, such as those reported by Sharpe et al. (12) and Wine et al. (13), may not be due solely to the weak estrogenic activities of the particular phthalates administered, but may involve other, and possibly more important, mechanisms of action. For example, DEHP has been recognized for many years to be a reproductive toxicant (49–52), yet this particular phthalate demonstrated no estrogenic behavior in the assays employed in this study. It may also transpire that it is not simply a matter of the response of a parent organism to the chemical concerned, whether exposure is acute or chronic, but that any effect may not be detected until subsequent generations. This possibility has been very clearly demonstrated by Wine et al. (13), who found that adverse reproductive effects induced by DBP in Sprague-Dawley rats were most pronounced in the second generation although the mechanisms generating these responses are unknown.

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