Estrogens in Unexpected Places: Possible Implications for Researchers and Consumers

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Estrogenic activity originating in unexpected places was encountered on three occasions during an investigation of whether Saccharomyces cerevisiae synthesized estrogens. In each instance, estradiol found in the conditioned yeast culture medium originated from an exogenous source and was not synthesized by the yeast. In the first instance, yeast grown in the laboratory showed a time-dependent increase in estradiol in the conditioned medium. However, the culture medium supplement Bacto-peatone was found to contain large amounts of estrone. When added to yeast cultures in the form of YPD medium (yeast extract, Bacto-peatone, and dextrose), S. cerevisiae converted the estrone to estradiol leading to the accumulation of estradiol over time. In the second instance, commercially purchased S. cerevisiae grown in a molasses medium exhibited substantial amounts of estradiol. However, corn and beet molasses contained sufficient estrone and estradiol to account for the findings. As in the first instance, the yeast converted the estrone into estradiol. In the third instance, autoclaving culture medium in polycarbonate plastic flasks was found to cause an estrogenic substance to be added to the medium, whether yeast were present or not. It was determined that the autoclaving process leached bisphenol-A (BPA) out of the polycarbonate plastic. BPA was shown to bind to estrogen receptors and to induce estrogenic activity, including stimulation of MCF-7 breast cancer-cell proliferation and induction of the expression of progesterone receptors. The three instances highlight potential problems for investigators who might inadvertently add estrogens to experimental systems confounding their results. The BPA findings raise concerns about the possible addition of this estrogenic molecule to the food supply since polycarbonate plastic is used in myriad applications in the packaging of food and beverages. Although we are unaware of the substantial contamination of food products with BPA, we believe this possibility should be carefully investigated.

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Key words: estrogen receptor, estradiol, bisphenol-A (BPA), plastic, polycarbonate, yeast

Introduction

Many natural, environmental, or manufactured substances exhibit estrogenic activity (1). Since estrogens affect reproduction and cellular development and probably alter the risk of carcinogenesis, chronic environmental exposure of the population would be expected to have a major impact on health. In some cases, exposure to environmental estrogens has already been shown to cause disease in people or animals (2-6). In other cases, the unexpected presence of estrogens in research settings has led to laboratory anomalies and confounding results during experiments (7-10). In this paper, we describe the unexpected presence of estrogens and of an estrogenic molecule, bisphenol-A (BPA), in three laboratory settings and the ramifications these unexpected estrogens have had on our research (8,10).

BPA (4,4'-isopropylidenediphenol; CAS no 80-05-7; empirical formula, C15H14O2; mw, 228) is the monomer used in the manufacture of polycarbonate (Figure 1). Polycarbonate is used in a wide array of plastic products, with new applications continuously being developed. Current producers have the capacity to manufacture over a billion pounds of BPA in the United States. As detailed below, autoclaving polycarbonate flasks leaches BPA from the plastic and contributes estrogenic activity to the liquid contents of the flask.

We would like to provide some background information on why we were doing these experiments and how we came to identify estrogens in unexpected places. Our laboratory has been investigating the presence of estrogens in fungi that bind mammalian steroid hormones with high affinity and specificity. We have demonstrated the presence of a corticosteroid binding protein (CBP) in Candida albicans (11,12) and an estrogen binding protein (EBP) in both Candida albicans (13,14) and Saccharomyces cerevisiae (15,16). Although we had postulated that these steroid binding proteins might represent primitive steroid hormone receptor systems in yeast (15,17), we have subsequently cloned the genes and found these proteins likely to be flavin-containing oxidoreductases unrelated to the steroid receptors (12,14). We had also been interested in the corollary hypothesis that S. cerevisiae might produce an estrogenlike substance that could bind to estrogen binding protein (EBP) and act as a yeast hormone.

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Abbreviations used: BPA, bisphenol-A; CBP, corticosteroid binding protein; EBP, estrogen binding protein; RIA, radioimmunoassay; DCC, dextran-coated charcoal; YNB, yeast nitrogen base and dextrose; YTD, yeast extract, Bacto-tryptone, and dextrose; YPD, yeast extract, Bacto-peatone, and dextrose; HPLC, high-pressure liquid chromatography; ER, estrogen receptor; RRA, radioreceptor assay; NMR, nuclear magnetic resonance; PR, progesterone receptor.

Figure 1. Structures of bisphenol A and polycarbonate.
In the pursuit of this line of investigation to find an endogenous ligand for EBP, we have found the presence of estrogens in complex laboratory media used for culturing yeast as well as in commercially prepared yeast grown in media containing corn and beet molasses. Furthermore, when we used culture media prepared using distilled water autoclaved in polycarbonate flasks, we detected estrogenic activity that was due to the presence of BPA that was leached from the plastic during the process of autoclaving. Careful analysis of the data in each of the three instances showed that the estrogens were not produced by yeast, and we were able to trace the origin of these estrogenic molecules to their unexpected sources.

**Materials and Methods**

We purchased 17β-[6,7-3H]estradiol (47 Ci/m mole) from Amersham Corporation (Arlington Heights, IL), yeast media from Difco (Detroit, MI), *S. cerevisiae* strains from the Yeast Genetics Stock Center (Berkeley, CA), and polycarbonate flasks from Nalgene (Rochester, NY). The molasses was a gift from the Western Sugar Company (Denver, CO). Authentic BPA was purchased from Aldrich Chemical Co. (Milwaukee, WI). All methods have been described in previous papers.

The various culture media used for the growth of *S. cerevisiae* and the constituents added to the media (peptone, gelatine, tryptone, and corn and beet molasses) were extracted, and the estrogen contents of these extracts were determined by reverse-phase high pressure liquid chromatography (HPLC) and radioimmunoassays (RIAs) using specific antibodies to estrone and estradiol. Autoclaving was performed in polycarbonate flasks (1000 ml capacity) with 450 ml distilled water (pH 5.5) for 30 min at 120 to 125°C with a slow exhaust cycle for a total of 75 min. [3H]Estradiol radioimmunoassays used a dextran-coated charcoal (DCC) method. Progesterone receptors were assayed by DCC with [3H]R5020 (New England Nuclear, Boston, MA) as ligand.

**Results**

**Presence of Estrogens in Culture Media Supplements**

In attempts to determine whether *S. cerevisiae* produces estrogens or other substances that could serve as an endogenous ligand for EBP, we grew cultures of yeast in our laboratory and examined extracts of both the medium and cells for an activity that could compete with [3H]estradiol for occupancy of EBP. In addition, we assayed the extracts for the presence of estradiol by a sensitive RIA. Initial experiments showed great variability of RIA results; control medium possessed detectable estradiol, but conditioned medium possessed substantially higher levels. As shown in Table 1, the abundance of estradiol in the conditioned medium was determined by the nature of the yeast medium used. Conditioned media from yeasts grown on YNB (yeast nitrogen base and dextrose) and YPD (yeast extract, Bacto-tryptone, and dextrose) had trace levels of estradiol (pg/liter) while YPD (yeast extract, Bacto-peptone, and dextrose) had higher levels (ng/liter). This led us to suspect that the estrogen content was determined by the medium supplement used. As shown in Table 2, Bacto-peptone was found to be the source of the higher levels of estrogens in YPD medium. In fact, estrone was substantially more prominent than estradiol, but both steroids were present in peptone. This led us to consider whether the yeast could convert estrone to estradiol to explain the high levels of estradiol we had found in the conditioned medium. As shown in Figure 2, yeast grown in YPD medium caused a time-dependent conversion of estrone to estradiol. When yeast cultures were grown in YNB, a simple defined medium, only negligible levels of estrogens could be detected. In summary, Bacto-peptone contains both estrone and estradiol, and yeast exhibit the capacity to convert estrone to estradiol, thus explaining the presence of estradiol in yeast conditioned medium. In experiments employing Bacto-peptone, investigators should be cognizant of this finding and recognize that the medium supplement contains estrogens.

**Figure 2.** Conversion of estrone to estradiol by *S. cerevisiae* grown in YPD medium. An overnight culture of *S. cerevisiae* α-cells were resuspended in fresh YPD medium at a concentration of 3×10^7 cells/ml. Cultures were grown at 29°C with vigorous shaking. At 2-hr intervals, an aliquot (500 ml) of the culture was withdrawn and the medium and cells were separated and processed for the determination of estrogen levels. Data are given as picograms of steroid per liter of medium where 1 liter contains 10 g peptone.

**Table 1.** Estradiol in three yeast culture media.

| Medium | Control | Conditioned |
|--------|---------|-------------|
|        | n | Mean | Range | n | Mean | Range |
| YNB    | 5 | 47   | 11–86 | 25 | 132  | 16–246 |
| YPD    | 3 | 215  | 200–236 | 3 | 2040 | 1560–2310 |
| YTD    | 3 | 63   | 0–128 | 3 | 94   | 90–101 |

**Table 2.** Presence of estrogens in culture media supplements.

| Medium supplement | Estradiol, pg/10 g | Estrone, pg/10 g |
|-------------------|-------------------|-----------------|
|                    | n | Mean | Range | n | Mean | Range |
| Peptone            | 10 | 451  | 200–1030 | 10 | 4360 | 1690–6980 |
| Gelatine           | 2  | 32   | 22–41  | 2  | 252  | 248–256  |
| Tryptone           | 6  | 140  | 0–359  | 6  | 0    | 0       |

n, number of experiments. Steroid levels have been corrected for [3H]estradiol recovery and are expressed as pg/10 g supplement (the equivalent of 1 liter of medium at a concentration of 1% supplement). Modified from Miller et al. (8).
conditioned medium of this yeast preparation for estrogens. Substantial quantities of estradiol were found in these extracts. We therefore sought to determine whether, like YPD, the medium in which the yeast were grown supplied either the estrogen itself or a substrate that the yeast could convert into an estrogen. The commercial supplier used a molasses-based medium of uncertain content. We formulated a yeast growth medium based on a commonly used formula (22) incorporating cane (3%) and beet (12%) molasses. As detailed in Table 3, the medium contained substantial amounts of estradiol and estrone. After growth of yeast in the molasses medium, the conversion of estrone to estradiol could be demonstrated in the conditioned medium. However, all of the estrogen found in the molasses-conditioned medium could be accounted for by the estrone plus estradiol in the starting medium. Again our findings indicate that the yeast are accumulating and converting estrogens provided in the medium and not carrying out de novo synthesis of estradiol.

Presence of Estrogenic Activity in Medium Autoclaved in Polycarbonate Flasks

In a third series of experiments to determine whether yeast produces an endogenous ligand for EBP, we examined extracts of conditioned medium from yeast cultures grown in our laboratory in a chemically defined medium. The extracts were fractionated over an HPLC column and assayed for ligand activity by competition with [3H]estradiol for binding sites on either EBP or mammalian estrogen receptor (ER) derived from MCF-7 human breast cancer cells (10). Estrogen-binding activity detected by displacement of [3H]estradiol in an ER-ligand binding assay will be referred to as radioreceptor assay (RRA) activity.

**Table 3. Presence of estrogens in molasses, molasses medium, and yeast grown in molasses medium.**

| Strain | Estradiol, pg/liter | Estrone, pg/liter | Estradiol, pg/liter | Estrone, pg/liter |
|--------|---------------------|-------------------|--------------------|-------------------|
| Cane molasses, 3% | 470 | 450 | 920 |
| Beet molasses, 12% | 1760 | 1170 | 2930 |
| Molasses medium, 15% | 2230 | 1630 | 3860 |
| Yeast cells, grown in molasses medium | 2540 | 0 | 2540 |

Steroid concentrations are corrected for recovery and are expressed as picograms per liter medium. Modified from Miller et al. (6). *The net weight of cells after overnight growth in 1 liter molasses medium was about 27 g.

Very early in these studies, it became clear to us that the yeast culture medium contained RRA activity but that the active principle was not being produced by the yeast. Control experiments showed that autoclaving medium or distilled water in plastic flasks yielded estrogenic RRA activity in the medium or water and that the presence of yeast was not required. However, autoclaving in plastic flasks was required. Using RRA activity as a bioassay, we purified the putative endogenous ligand by a series of sequential column chromatography and HPLC steps (10). The purified material was identified by mass spectrometry and NMR as BPA, a major constituent of polycarbonate (Figure 1). It should be emphasized that the polycarbonate flasks employed in these experiments are marketed as autoclavable.

We next pursued the question of whether authentic BPA exhibited estrogenic activity. We selected three biochemical criteria for this determination: a) could BPA bind to ER and displace [3H]estradiol; b) could BPA stimulate proliferation of MCF-7 human breast cancer cells; and c) could BPA induce progesterone receptors (PR) in MCF-7 cells and would the effect be blocked by tamoxifen, an estrogen antagonist.

In experiments to determine whether BPA could bind to the ER, we compared the abilities of commercially obtained BPA and estradiol to compete with [3H]estradiol for binding sites to the ER. An extract made from autoclaved water was also tested in this assay. Rat uterine cytosol was incubated with [3H]estradiol (1 nM) ± 250-fold excess unlabeled estradiol to measure non-specific binding. The ability of increasing concentrations of unlabeled estradiol, extract of autoclaved water, and authentic BPA (Aldrich) to compete for specific [3H]estradiol binding sites is shown. For these experiments, 4 liters of distilled water samples were autoclaved in polycarbonate flasks for 30 min at 125°C, and the extract was purified as described by Krishnan et al. (10). As shown in Figure 3, BPA competes for ER binding sites in a rat uterine cytosol preparation at a ratio of approximately 1:2000 the potency of estradiol. Extracts of water autoclaved in polycarbonate flasks showed a parallel competition profile. In similar experiments using MCF-7 cells as a source of ER, a potency ratio of BPA to estradiol of 1:1000 was found (data not shown).

We next assessed the ability of authentic BPA to stimulate MCF-7 cell proliferation.

MCF-7 cells were grown in the presence or absence of various doses of estradiol or BPA for 6 days; at the end of the 6 days, [3H]thymidine incorporation was measured (10). As shown in Figure 4, estradiol stimulates proliferation at concentrations as low as 1 pM. BPA stimulates proliferation at 5 to 10 nM and the stimulation achieves clear significance at 25 nM.

Induction of PR is considered a highly specific estrogenic action. MCF-7 cells were grown in the presence of various concentrations of estradiol or BPA for 6 days. Control cells received ethanol vehicle throughout the experiment. At the end of treatment, cells were processed and PR levels were determined by [3H]R5020 binding (10). In MCF-7 cells, estradiol at

![Figure 3. Comparison of extract from autoclaved water, authentic BPA, and estradiol by receptor assay (RRA). From the RRA activity it can be estimated that the extract contained approximately 3 μg of BPA/liter of autoclaved water. Reproduced with permission from Krishnan et al. (10).](image-url)

![Figure 4. Effects of estradiol and BPA on [3H]thymidine incorporation in MCF-7 cells. Values are given as mean ± SE from three to five experiments and represented as a percent of control (cells grown on ethanol vehicle) that was 1274 ± 305 dpm/μg DNA. Statistical analysis was done using analysis of variance and applying the Bonferroni-Dunn correction. Asterisks represent values significantly different from control: *p < 0.01; **p < 0.001. Reproduced with permission from Krishnan et al. (10).](image-url)
concentrations of 10 pM induced PR, and BPA induced PR at 25 nM (Figure 5). In experiments to test whether the stimulation of PR could be blocked by tamoxifen, a significant rise in PR was detected at 10 nM BPA, which was completely blocked by the simultaneous addition of 1 μM tamoxifen (data not shown).

The BPA detection limit in safety assays used by manufacturers to detect BPA contamination is 10 parts per billion (ppb) (50 nM). Our data show that BPA exhibits estrogenic activity in vitro at concentrations of 10 to 25 nM (2–5 ppb), which would be below the level of detectability in the safety assays.

Our final experiments were directed at the question of whether the amount of BPA leached out of a flask by autoclaving was high enough to alter the estrogenic environment of the cells cultured in the autoclaved medium. Phenol red-free RPMI medium was prepared with water autoclaved in glass or polycarbonate flasks after preadjusting the pH to 7.4. The medium was supplemented with 5% charcoal-stripped calf serum and used to culture MCF-7 cells. The cells reached confluence in 6 to 8 days, at which time basal PR levels were determined by specific $[^3H]R5020$ binding (10). As shown in Table 4, the PR content of cells grown in medium autoclaved in plastic flasks is higher than when grown in medium autoclaved in glass.

**Discussion**

Our data describe three instances in which unexpected estrogenic activity was detected during laboratory experiments. In each case, the estrogenic activity had the potential to mislead our investigation and confuse our results. We report these experiences to alert other researchers about these events and perhaps, by informing the research community, to help avoid these unexpected estrogenic activities from confounding the research of other investigators.

The presence of estrogens in Bacto-peptone should not be surprising considering that the material is prepared from bovine and porcine sources. It is not clear how often the presence of estrogens in culture media prepared with Bacto-peptone might have given misleading experimental results. However, YPD is a very commonly used culture medium.

The source of estrogens in corn and beet molasses remains problematic. There are published reports indicating that plants can synthesize estrogens (22,23). However, given the experiences we have encountered in finding unexpected estrogens in various laboratory settings, one must be circumspect in assessing such reports.

Finally, we turn to the BPA results. Since polycarbonate is so universally used in multiple consumer applications, our findings raise the possibility that polycarbonate plastic may be a source of environmental estrogens. Several factors should be mentioned that suggest this may not be a major public health problem. First, BPA is a weak estrogen, about 1/2000th the potency of estradiol (10). However, when the concentrations are high enough, BPA appears to be a full agonist. Second, our data suggest that heat is required to leach the BPA out of the plastic. It is known that the carbonate linkages are subject to electrolytic attack at elevated temperatures. It is also clear that hydrolysis is accelerated at high pH and retarded at pH 5 or below. Since acid pH protects and alkaline pH increases the leaching process, the nature of the contents and the washing process may greatly affect the rate of BPA leaching into the contents of the plastic container. Third, we have not examined the estrogenic potency of BPA when ingested orally. It is not yet known how much estrogenic potency is exerted by BPA when it is consumed and exposed to possible inactivation in the digestive tract or whether it is metabolized to inactive forms.

On the other hand, multiple opportunities exist in the consumer world for plastic containers to contaminate their food and beverage contents and thereby perhaps to cause exposure of the population to BPA. For example, large water jugs containing purified water are made of polycarbonate. Reusable bottles for soda, beer, and other beverages may be manufactured from polycarbonate. The packaging of various items for babies including food and juice containers, baby bottles, and baby food warmers, all of which might be heated in their routine use, is commonly made of polycarbonate. Finally, many metal cans used to market soups, fruits, vegetables, condensed milk, and other foods are lined with polycarbonate. Since heating may be used to sterilize or cook foods in the can, the risk of BPA contamination of the contents may be high. There are many other examples of plastics coming into intimate contact with items that people eat or drink.

We raise this issue not to cause undue alarm but merely to alert the proper agencies to evaluate the extent of this potential problem. Historically, it was well known that BPA exhibited estrogenic activity ([24–27]; Bond et al., unpublished data). Our data raise the question: To what extent is BPA leached from polycarbonate containers into the food supply, possibly causing exposure of the population to this estrogenic activity? We have no evidence at this time that plastic is contributing to the pool of environmental estrogens. However, as reported by so many laboratories at this meeting, many possible sources of environmental estrogens exist, and the estrogenic burden is likely to be cumulative. The potential for BPA to be leached from plastic and to cause estrogenic actions in the population is real, and we believe further studies are warranted to evaluate this possibility.

| Table 4. Effect of autoclaving culture medium in plastic flasks on PR levels in MCF-7 cells. |
|------------------------------------------|
| Experiment number | Glass fmoles/mg | Polycarbonate fmoles/mg |
|------------------|----------------|------------------------|
| 1                | 28             | 42                     |
| 2                | 20             | 34                     |
| 3                | 18             | 26                     |

Paired t-tests were performed for three experiments (n=1 each experiment) and p<0.05. Modified from Krishnan et al. (10).

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