body (Dako). Seven PrPres$^-$ and six PrPres$^+$ brains were examined. Spongiform lesions and gliosis could not be seen in any brain region of PrPres$^-$ mice. The absence of localized PrPres deposits was confirmed by PrP immunohistochemistry.

30. Whole brain hemispheres were fixed overnight with a solution of 1% glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4). After 1 hour postfixation with 2% osmic acid, they were stained en bloc with uranyl acetate and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate before examination with a Philips CM10 electron microscope.
31. We thank R. Bradley for BSE-infected cattle brain homogenate, C. Weissmann and R. H. Kimberlin for helpful discussions, and R. Rioux and J. C. Mascaro for expert animal care. We also thank P. Fritch and M. Wasowicz, as well as L. Court, who encouraged our research on TSE. Supported by a grant from D.R.E.T. (Paris).

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TECHNICAL COMMENTS

# Potency of Combined Estrogenic Pesticides

Steven F. Arnold *et al.* found 150- to 1600-fold synergistic interactions between binary mixtures of the weakly estrogenic pesticides endosulfan, dieldrin, toxaphene, and chlordane in competitive estrogen receptor (ER) binding assays and in an estrogen-responsive assay in yeast (*1*). Less dramatic synergistic interactions between two weakly estrogenic hydroxy polychlorinated biphenyl congeners were also observed in the yeast assay and in human endometrial cancer cells. On the basis of these data, it was suggested "that the estrogenic potency of some environmental chemicals, when tested singly, may be underestimated" (*1*, p. 1491). The purported synergistic interactions of these compounds have important mechanistic and public health consequences (*2*). We reassessed the potential synergistic interactions of two weakly estrogenic pesticides, dieldrin and toxaphene, using the following estrogen-responsive assays: induction of uterine wet weight, progesterone receptor (PR) levels and uterine peroxidase activity in the immature female mouse; induction of cell growth and two estrogen-responsive reporter gene assays in MCF-7 human breast cancer cells; induction of reporter gene activities in two yeast-based assays that expressed either the human or mouse ER; and competitive binding to human and mouse ER. For these 10 different estrogen-responsive assays, the combined activities of dieldrin plus toxaphene were essentially additive. Moreover, interactions of all the binary mixtures of organochlorine pesticides reported by Arnold *et al.* (*1*) were reinvestigated in the two yeast-based assays.

The results we obtained in yeast transformed with an expression plasmid that contained the wild-type mouse ER and a reporter plasmid containing a single ERE linked to the *LacZ* gene (*3*) indicate that the estrogenic activities of all the binary mixtures of organochlorine pesticides were additive. These same binary mixtures were also investigated in a yeast-based human ER assay (*4*), which used the same yeast strain and reporter gene construct used by Arnold *et al.* (*1*). In contrast to that study, synergistic activity was not observed for any pesticide combination. The differences between our results and those reported by Arnold *et al.* (*1*) cannot be accounted for by differences in total ER expression, because varying this expression did not have any affect on synergy. These results demonstrate that synergism between weakly estrogenic chemicals is not universal, even within the same strain of yeast. The recent scientific, regulatory, and public concern regarding the potential adverse environmental and human health impacts from synergistic estrogen responses induced by organochlorine pesticide mixtures should be tempered by our results, which demonstrate that these compounds are weakly estrogenic and, in combination, their activities are additive (*5*).

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*Response*: It is difficult to compare the results of the study by Ramamoorthy *et al.* to ours because the assays they used, while appearing to be similar to ours, were in each case different. The differences, however, have been instructive in helping us frame some of the parameters that may be important in determining the synergistic action of weakly estrogenic chemicals.

In our mammalian and yeast cell assays (*1*), as well as in the ligand-binding experiments, the concentration of receptor molecules was low, while in the study by Ramamoorthy *et al.* the concentrations were high. For example, our mammalian cell culture experiments used Ishikawa uterine cancer cells that lack detectable ER and were transfected with only 20 ng of hER cDNA. In contrast, Ramamoorthy *et al.* used MCF-7 breast cancer cells that contained high levels of endogenous ERs [MCF-7 cells typically contain endogenous ER levels in the range of 30,000 ERs per cell (*2*) to 200,000 ERs per cell (*3*)] and that were transfected with an additional 4 to 5 μg of hER cDNA. Likewise, in the yeast-based assay used in our report, the number of expressed hERs was estimated to be 500 to 1000 receptors per cell, but the study by Ramamoorthy *et al.* appears to contain well in excess of 1000 ERs per cell. Finally, our in vitro competitive binding conditions used 0.4 nM concentrations of ERs (monomer concentrations), whereas the concentration of ER used by Ramamoorthy *et al.* was considerably higher and the assays were not performed according to our report (*1*). Therefore, because our results showed synergy and theirs did not, ER concentration may play an important role in the ability of mixtures of chemicals to synergize.

With regard to the animal studies, our earlier work showed synergistic responses to weakly estrogenic chemicals in turtles that were treated early in development (*4*). The study by Ramamoorthy *et al.* was performed in the uterus of female mice that had already undergone sexual differentiation. Our contention has been that developmentally exposed animals are more likely to demonstrate synergistic responses to estrogenic chemicals. Nonetheless, inspection of the data provided by Ramamoorthy *et al.* sug-

gests that dieldrin and toxaphene, at the lowest doses used, appeared to have induced the progesterone receptor, an estrogen-specific marker in mice, in a synergistic manner; no indication of this effect was seen when measuring uterine weight or uterine peroxidase activity. This suggests that some estrogen-dependent phenomena are better markers than others for revealing synergistic responses. Consistent with this idea is the observation that a combination of estradiol and 3,4,3′,4′,-tetrachlorobiphenyl synergistically induces pS2, an estrogen-regulated protein, but not another estrogen responsive marker, in the human breast cancer cell line, ZR-75-1 (5). Indeed, synergy observed in one cell line (ZR-75-1) was not seen in another (MCF-7) in the same study (5); this underscores the importance of cell type in determining estrogenic responses.

A mechanism underlying these synergistic effects remains to be determined. One of our working hypotheses is that under conditions in which the ER tends to exist as a monomer, the binding characteristics of two interacting molecules are different from that observed at high receptor concentrations. We contend that this low ER experimental condition better approximates ER concentrations found during early development [the ER content of uterine epithelial cells is low in fetal or newborn mouse (6) or rat (7), a period critical for estrogen-associated disorders (8)]. During these sensitive periods, chemical interactions resulting in synergy may occur at conditions in which critical ligand-receptor or receptor-receptor combinations occur.

Synergism between weakly estrogenic chemicals may not be universal, as Ramamoorthy *et al.* suggest. However, synergy in biological systems has a long history. Synergy has been observed between steroid hormones, different nuclear receptors (9), membrane and nuclear receptors (10), drugs and hormones (11), and temperature and hormone response (12). Synergistic interactions have also been observed between drugs and temperature (13) and weakly estrogenic compounds (4). Our discovery of synergy of natural and synthetic estrogens was made by observing the effects of these compounds on the sexual development of turtle embryos. We demonstrated synergy between a combination of two polychlorinated biphenyls (4), and, more recently, two steroidal estrogens (14). We also have recently reported that the binding of chemical mixtures to the estrogen receptor from the American alligator occurs in a synergistic manner (15). Our laboratory has shown that a combination of phytoestrogens produced a synergistic response in yeast (16). In addition, in cell culture studies of fish hepatocytes (17) as well as mammalian cells (18), mixtures of weakly estrogenic chemicals were shown to act synergistically in stimulating estrogenic responses appropriate to the species. These findings together suggest that the synergistic action of weak estrogens may be phylogenetically conserved and therefore fundamental.

We currently are evaluating the occurrence of synergistic interactions of chemicals with the ER in different yeast strains, mammalian cells, and biological systems. We have noted synergy in some yeast strains, but not others, as well as an apparent relationship to ER concentrations (19). We have likewise found a synergistic interaction between ovarian steroidal estrogens in both a yeast-based assay and the developing turtle (14). These latter studies both confirm and extend our previous report (1) and suggest a mechanism for synergy. We look forward to the continued clarification of this important issue.

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# Redox Stabilization of the Atmosphere and Oceans and Marine Productivity

Philippe Van Cappellen and Ellery D. Ingall provide a coupled biogeochemical box model to investigate whether negative feedbacks between the global cycles of phosphorus and oxygen might have stabilized the amount of atmospheric $O_2$ during the Phanerozoic (1). We have duplicated these results (1), but have found that slight modifications to the treatment of tectonic uplift and resultant weathering rates dramatically affect the outputs of the model.

Van Cappellen and Ingall set the rate of $O_2$ consumption during weathering to be proportional to the global rate of uplift. The rate of $O_2$ production is highly sensitive to marine reactive P availability through interactions with the carbon cycle. Van Cappellen and Ingall assume that the rate of P input to the oceans depends only on the size of the terrestrial lithosphere reservoir of this element and not on weathering rates. This assumption virtually decouples the rate of oxidative weathering from that of P transfer to the oceans on time scales of tens to hundreds of millions of years and accounts for the rapid depletion in atmospheric $O_2$ in the model after an increase in uplift rate (Fig. 1). It seems more likely that the flux of P to the oceans also depends on the rate of uplift. Today, refractory, detrital P phases account for less than 25% of the total solid-phase P in most marine sediments (2), and changes in total continental P weathering rates have apparently led to comparable changes in the chemical weathering of P phases over at least the last 100 million years (My) (3). When the model (1) is run with P and Fe oceanic inputs coupled to uplift rates, atmospheric $O_2$ is found to rise slightly rather than decrease dramatically in response to an increase in the uplift rate (Fig. 1.)

The output of the model (1) is also adversely influenced by the assumption that the C:P ratio in oxic sediments is much smaller (200) than in anoxic sediments (400 to 4000). Lower C:P ratios in oxic sediments are attributed to relative enrichment in P during organic matter remineralization by aerobic benthic bacteria (4), a