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o,p'-DDT (1,1,1-trichloro-2 (*p*-chlorophenyl) 2-(*o*-chlorophenyl) ethane is a purely estrogenic agonist in the rat uterus *in vivo* and *in vitro*

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Like other chlorinated insecticides, *o,p'*-DDT and its analogs have been shown to exert estrogen-like action on avian oviduct and mammalian uterus [1–9].

That this results from an interaction of DDT itself, or of a metabolite, with the classical estrogen receptor system is indicated by the fact that the pesticides indeed compete with estradiol for the cytosolic receptor [10, 11] with an affinity that reflects their respective estrogenic potency [3, 8, 12]. This is also supported by the high degree of correlation between the level of nuclear receptor and the increase in uterine weight measured 3 and 24 hr, respectively, after administration of *o-p'*-DDT to immature rats [13].

Among the estrogenic substances, some, like estriol, are short-acting partial agonists; others, like the triphenylethylene derivatives (e.g. nafoxidine), are long-acting as compared to estradiol. This is associated with, respectively, shorter and longer nuclear retention of the estrogen receptor [14]. Both types of compounds interact with the estrogen receptor; both also can act as estrogen antagonists depending on the injection scheme [14].

In order to define the class of estrogenic compounds to which *o-p'*-DDT pertains, we compared its action on the rat uterus to that exerted by 17β-estradiol. This included time-course studies on *o-p'*-DDT induced changes in uterine wet weight and RNA, protein and glycogen contents together with measurement of eosinophils counts and cGMP content.

The effect of *o-p'*-DDT on two uterine responses that can be obtained *in vitro* with estradiol was also evaluated, namely the early increase in cGMP content [15] and the IP response, i.e. stimulation of the synthesis of the so-called "induced protein" [16, 17]. Induction of an IP-like response *in vitro* by the pesticide had been reported [18], and we wanted to characterize the IP thus stimulated, since we had previously shown [19] that the nafoxidine-stimulated IP in fact differs from the estradiol-stimulated one, which has a M.W. of 46,000 and corresponds to brain-type BB-creatine phosphokinase [20]. We also tested the effect of pretreatments with *o-p'*-DDT on further uterine responsiveness to 17β-estradiol. Treatments with the less estrogenic derivative, *p,p'*-DDT were included for comparison.

Materials and methods

Animals and treatments. Immature female rats (Wistar strain, from Proefdierencentrum K.U.L. Leuven, Belgium) 21–22 days old and weighing about 45 g were used. The animals were maintained on normal AO-4-chow, from Animalabo, Belgium.

The pesticides, *o-p'*-DDT and *p,p'*-DDT from Aldrich, Europe, were dissolved in dimethylsulfoxide-propylene glycol 1:1, at a final concentration of 12.5 μg per 100 μl which were injected intraperitoneally. Estradiol (from Sigma Chemical Co.) was dissolved in propylene glycol. Control animals received 100 μl of the solvent alone.

Biochemical determinations. DNA, RNA, protein and glycogen contents were measured respectively by the method of Burton [21], Dische [22] Lowry *et al.* [23] and Montgomery [24].

For cyclic GMP determination uterine horns, freed from adhering fat tissue, were immersed in 1.5 ml of distilled water, put in a boiling water-bath for extraction of the cyclic nucleotide and abolition of phosphodiesterase activity [25]. Cyclic GMP was quantitated as previously described [25], using the method of Steiner *et al.* [26].

Characterization of the IP synthesized under DDT influence, as compared to the classical "estrogen-induced protein" (Gorski's IP), was made as previously described [27].

Briefly, the uterine horns were incubated 3 hr at 37°, in Krebs–Ringer-bicarbonate medium, supplemented or not with either 17β-estradiol (2 nM), or *o-p'*- or *p-p'*-DDT (28 μM); ^{35}S-methionine (specific activity: 1101 Ci/mmole; from Amersham, U.K., at a dose of 50 μCi/ml) was added during the last 2 hr of incubation.

Cytosols were then prepared and submitted to a two-step fractionation (DEAE-cellulose chromatography and SDS-polyacrylamide gel electrophoresis), according to Reiss and Kaye's modification of Laemmli's procedure [19]. Labeling intensity in the protein bands thus obtained from control and treated uteri were compared by fluorography, using Kodak X-O matt AR film.

Histology and autoradiography. The middle-third portion of one horn was fixed in neutral formalin and processed for paraffin embedding and staining of the eosinophils, or for autoradiography. In the latter case the animals were injected i.p. with 50 μCi of ^{3}H-thymidine (from Amersham, U.K.; s.p. 40–50 Ci/mmole), 60 min before sacrifice.

The tissue sections were covered with Ilford-K5 nuclear emulsion, by dipping in the melted emulsion, diluted by 1 vol. of distilled water. The autoradiographs were exposed for 15 days. The labeling index (per cent of labelled nuclei) was established on a total of 2000 cells for each cell population.

Results and discussion

Time-course studies. The dose of *o,p'*-DDT we used here (12.5 mg per animal) was chosen on the basis of previous work showing that it corresponds to maximal effects on uterine wet weight and DNA synthesis activity at 24 hr [8].

Our time-course study shows that with *o,p'*-DDT as with 17β-estradiol the changes in uterine wet weight and RNA, protein and glycogen content were transitory, with a high level at 24 hr and a decline to near-control value at 48 hr (Figs 1a–d). This contrasts with the 72 hr sustained effect of the antagonist nafoxidine ([14], review in [29]).

With *o,p'*-DDT these changes developed more slowly during the first 18 hr of the stimulation; this probably reflects the fact that translocation (or stabilisation) of nuclear receptors is also delayed with the pesticide as compared to estradiol [13].

The wet weight response during the first 6 hr following the injection, was the same with *o,p'*-DDT as with estradiol. This phase (phase I) of estrogenic stimulation is known to be dissociable from the late phase, or phase II, in which "true growth" (hypertrophy and hyperplasia) takes place, indicating that they correspond to distinct actions of estrogen (see rev. in [30]). Our results show that *o,p'*-DDT can mimick those two estrogenic actions on the rat uterus and that *p,p'*-DDT was much less efficient in this respect.

At 3 and 6 hr of *o,p'*-DDT treatment, the uterine cGMP content was increased to about twice the control level, i.e. to a comparable level as that observed with estradiol [27], whereas with *p,p'*-DDT there was no statistically significant change in this parameter (Table 1a). This is an agreement with the hypothesis of a relationship between these responses and the wet weight response [25, 31].

In vitro *experiments.* An increased cGMP content was also observed *in vitro*, 2 hr after incubation of uterine horns with 28 μM *o,p'*-DDT (Table 1b). This represents a second estrogenic effect obtained with the pesticide in a completely

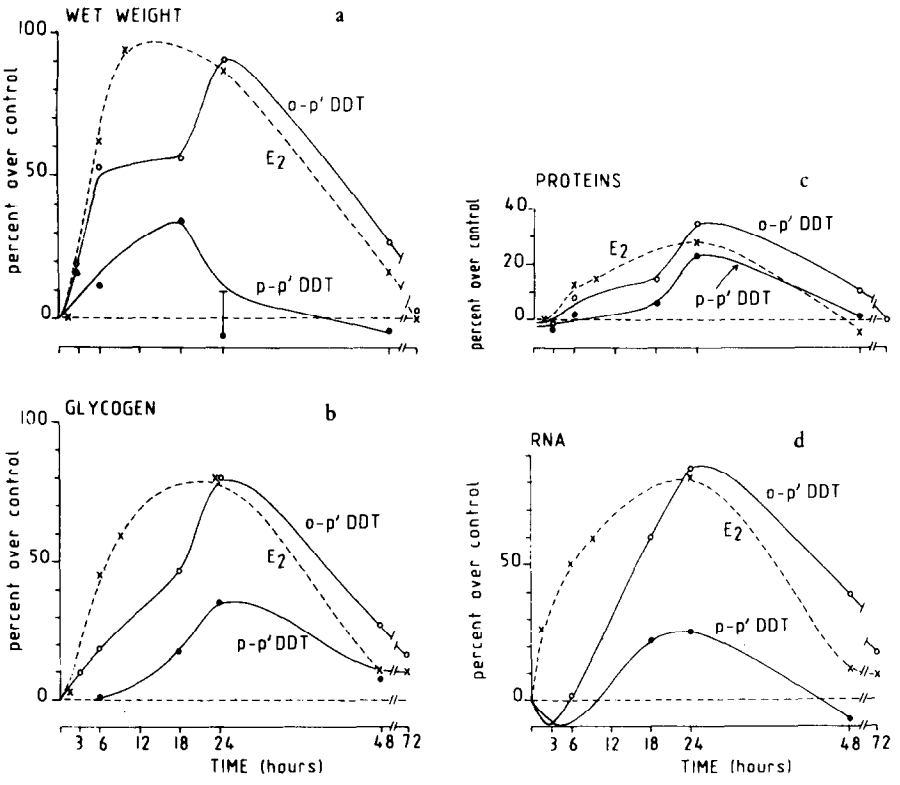

Fig. 1. (a, b). Time course effects of *o-p'*-DDT (●) or *p-p'*-DDT (○) (12.5 mg/animal for each), on uterine wet weight (a) and glycogen (b) protein (c) and RNA (d) content. The effect of 0.05 μg 17β-estradiol (taken from previous work—see Ref. 30) are illustrated for comparison (×; dashed line). Five animals were used for each point; S.E.M. value did not exceed 5–7% of the indicated value, except when illustrated. To facilitate comparison of the intensity of the various responses, the results are given as per cent over control levels.

Table 1. Effect of *o-p'*-DDT and *p-p'*-DDT on cGMP content in the immature rat uterus (pmoles cGMP/mg DNA) *in vivo* and *in vitro**

(a) *In vivo* Treatment duration	Control	*p-p'*-DDT (12.5 mg)	*o-p'*-DDT (12.5 mg)
3 hr	2.60 ± 0.65 (N = 5)	1.52 ± 0.26 (N = 5)	5.49 ± 1.05 (N = 5) (p = 0.04)
6 hr	3.33 ± 0.46 (N = 5)	4.25 ± 0.57 (N = 5)	6.46 ± 1.49 (N = 5) (P = 0.07)
(b) *In vitro* (2 hr incubation) Control	17-β-estradiol (10^{-9} M)	*p-p'*-DDT (28 μM)	*o-p'*-DDT (28 μM)
5.4 ± 1.6 (N = 25)	17.7 ± 2.7 (N = 27) (P = 0.0002)	12.0 ± 1.4 (N = 26) (P = 0.003)	12.5 ± 2.1 (N = 27) (P = 0.0002)

* P value for the difference with control are given; they were calculated by Student's *t*-test.

in vitro system (the first being the IP response [18] and this work).

In vitro, *p,p'*-DDT was as efficient as *o,p'*-DDT whereas *in vivo* its estrogenic potency for this and other responses was much in accord with its lowest affinity for the estrogen receptor. We already reported on a similar paradoxical observation with 17α-estradiol [15]. As we then suggested, this might reflect a change in the interaction of the compound with the receptor system *in vitro*, similar to that reported for testosterone [32]. Considering now the nature of the "IP" protein stimulated by *o,p'*-DDT, our two-step characterization indicated (Fig. 2) that it has the same charge and molecular weight as the estradiol-induced IP, BBCK; it therefore differs from any of the nafoxidine-

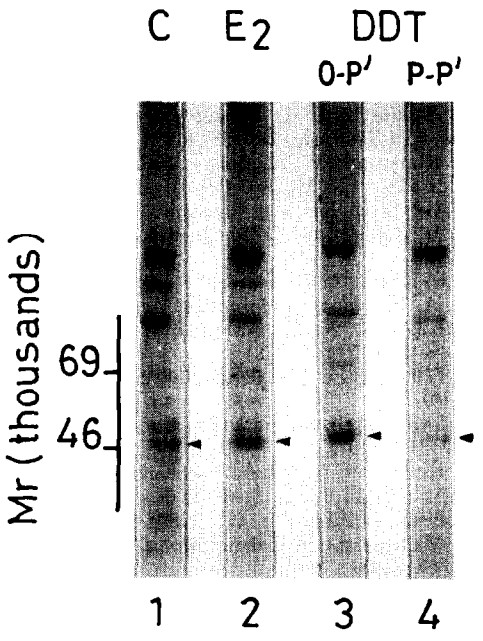

Fig. 2. Characterization of *o'-p*-DDT and estradiol-stimulated cytosolic proteins. The figure shows fluorograms from SDS polyacrylamide gel electrophoregrams, obtained by loading the gels with the fractions from DEAE-cellulose chromatography corresponding to the peak of BB-creatine phosphokinase activity. Each column was loaded with the same total radioactivity of 3.10^6 c.p.m. (see Methods for details). Treatments were as follows: (1) control; (2) 17β-estradiol (2.10^{-9} M); (3) *o-p'*-DDT, 28 μM; (4) *p-p'*-DDT, 28 μM. The arrow indicates the position of the IP: this corresponded to a M.W. of 46,000.

induced proteins, which have M.W. of 27,000 and 30,000 D [29]. Thus our data from time-course and *in vitro* studies indicate that the reported interaction of *o,p'*-DDT with the estrogen receptor and the resulting agonistic effects [3, 8, 10–12] are kinetically and qualitatively similar to those of long acting estrogens (like estradiol) and distinct from those of antagonists which, like triphenylethylene derivatives, exhibit a partial agonistic potency, associated with prolonged nuclear retention [14].

Pretreatment experiments. This conclusion is also supported by the pretreatment experiments in which we tested the effect of a 24 hr or 48 hr pretreatment with 12.5 μg *o-p'*-DDT on further uterine responsiveness to estradiol. The results (Figs 3a–d) indeed show that following *o-p'*-DDT pretreatment, a 24 hr estradiol treatment increased the uterine wet weight and RNA, protein or glycogen contents, to levels identical to those observed in saline pretreated-estradiol treated animals. In the 24 hr *o-p'*-DDT pretreated group, this was obtained by a slight incremental effect of estradiol over the remanent effect of the pretreatment itself. This might indicate the attainment of an inbuilt limit to the uterine ability to accumulate these different constituents. Perhaps more likely, it might also indicate saturation of the acceptor sites involved in those responses; if so, this would imply that the complexes formed by DDT and estradiol with the receptor, act on the same acceptor sites.

As after 48 hr there remained no effect of *o-p'*-DDT, the response to estradiol in this group was equal, in absolute terms, to that observed in saline pretreated animals, unambiguously indicating full restoration of uterine responsiveness. A pretreatment with 12.5 μg *o-p'*-DDT by itself induced a marked uterine eosinophilia at 24 hr (7.4 ± 1.3

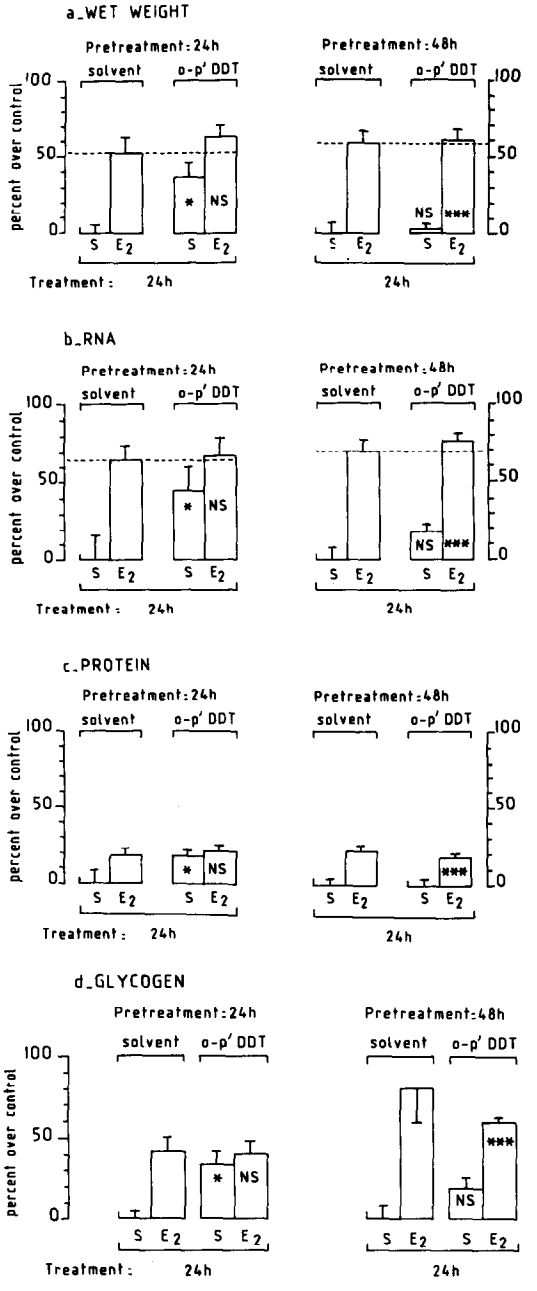

Fig. 3. (a–d). Effect of pretreatment with *o-p'*-DDT (12.5 μg/rat), or with the solvent, as indicated on top of each group of blocks, on the 24 hr response to further treatment with 5 μg 17β-estradiol (E2) or with saline (S). The results are expressed as per cent over basal control (solvent-saline) values; they represent the mean for five animals in each group. Bars indicate ± S.E.M. values. All differences between E2 values (whatever the pretreatment) and basal values are highly significant. The statistical significances of the differences between E2 values in *o-p'*-DDT pretreated and saline pretreated animals are indicated in the E2 block. The statistical significance of the difference between *o-p'*-DDT-saline values and saline-saline (basal) values are indicated on the S block. The code is as follows: N.S., not significant; *P level <0.05, **P level <0.01, ***P level <0.001.

eosinophils/section vs 1.07 ± 0.15 in controls); it did not suppress that provoked by a further 24 hr treatment with estradiol (3.8 ± 0.6 eosinophils/section, with 5 μg estradiol alone; 13.8 ± 3.4 in *o-p'*-DDT pretreated estradiol treated rats).

In summary, for all parameters investigated, *o-p'*-DDT quantitatively and qualitatively behaves like estradiol, i.e. as a long acting, purely agonistic estrogen, thus distinct from estrogenic compounds that elicit short (like estriol) or overprolonged (like triphenylethylene derivatives) nuclear retention of the estrogen receptor.

Therefore any adverse effect that pharmacological doses of *o-p'*-DDT might have on rat uterine physiology would be attributable to undue (but otherwise normal) estrogenic stimulation rather than to an estrogen-antagonist action.

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The inhibitory effect of *p*-trifluoromethyl substitution on the hepatic microsomal metabolism of benzyl phenyl sulphide

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Many drugs contain sulphur and readily undergo metabolic *S*-oxidation and *S*-dealkylation which may result in inactivation and render the molecule more polar, facilitating excretion. This unwanted metabolism may perhaps be modified by the introduction of appropriately placed fluorine substituents since the inclusion of fluorine into a molecule can inhibit metabolism up to several carbon atoms removed from the fluorine atom [1–4]. In this study we have used a rat hepatic microsomal system to investigate the inhibitory effect of substituting *p*-trifluoromethyl groups into the model compound benzyl phenyl sulphide (**Ia**, see Fig. 1). The extents of overall metabolism and amounts of sulphoxides and sulphones formed have been determined. The results obtained demonstrate that while *C*-oxidation is markedly inhibited by fluorine substitution there is only a minor effect on *S*-oxidation.