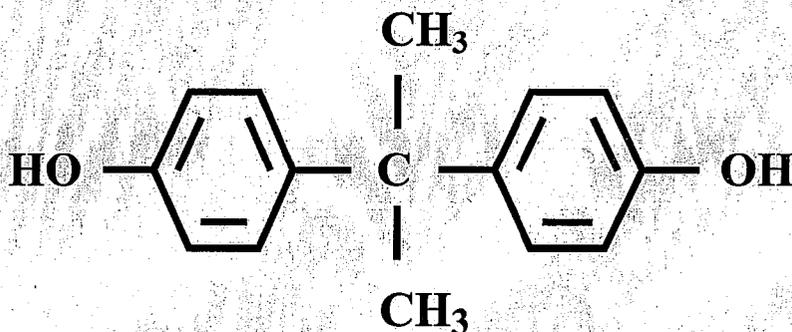


European Union Risk Assessment Report

CAS No: 80-05-7

EINECS No: 201-245-8

**4,4'-isopropylidenediphenol
(bisphenol-A)**



Cell free systems

In a briefly reported study (Olea et al., 1996), the relative binding affinity of bisphenol-A to oestrogen receptors was investigated. Cytosol from immature female rat uteri was incubated in medium containing various concentrations of bisphenol-A and 3 nM [³H]17 β -oestradiol, for 16 hours. In this assay the relative binding affinity of bisphenol-A to oestrogen receptors was approximately 4 orders of magnitude lower than that for 17 β -oestradiol.

In a briefly reported study (Maruyama et al., 1999), the binding affinity of bisphenol-A to oestrogen receptors in an oestrogen-responsive rat pituitary cell line, MtT/E-2, was investigated. Cytosol from MtT/E-2 cells was incubated with various concentrations of bisphenol-A and [³H]-oestrogen. The binding affinity of bisphenol-A to oestrogen receptors was approximately 4 orders of magnitude lower than that for oestrogen.

In a briefly reported study using rat uterine cytosol, the relative binding affinity of bisphenol-A to oestrogen receptors was approximately 3 orders of magnitude lower than that for 17 β -oestradiol (Feldman and Krishnan, 1995).

In a further briefly reported study, the relative binding affinity of bisphenol-A for oestrogen receptors was investigated by Dodge et al. (1996). Protein from MCF-7 cell lysates was incubated with various concentrations of bisphenol-A and 0.5 nM [³H]17 β -oestradiol, for 18 hours. In this assay, the relative binding affinity of bisphenol-A to oestrogen receptors was approximately 2 orders of magnitude lower than that for 17 β -oestradiol.

Recently, it has been found that the rat, mouse and human oestrogen receptor exists as two subtypes, ER α and ER β . The relative binding affinity of bisphenol-A to the receptors was investigated using human ER α and ER β protein in insect cell extracts attached to the wells of microtitration plates (Kuiper et al., 1998). Following adhesion, receptor proteins were incubated with various concentrations of bisphenol-A and [³H]17 β -oestradiol for 18 hours. The relative binding affinity of bisphenol-A for both the α and β oestrogen receptors was approximately 4 orders of magnitude lower than that for 17 β -oestradiol.

In a study reported as an abstract only (Zacharewski and Matthews, 2000), the ability of bisphenol-A and bisphenol-A glucuronide to compete with [³H]17 β -oestradiol for binding to α and β oestrogen receptors was investigated in 3 different preparations; mouse uterine cytosol, a bacterially expressed glutathione-S-transferase (GST)-ER fusion protein consisting of the human oestrogen α D, E and F domains and recombinant oestrogen β receptors. The binding affinity of bisphenol-A to oestrogen receptors was seen to vary between the preparations, and was approximately 2-4 orders of magnitude lower than that for 17 β -oestradiol. Bisphenol-A glucuronide did not competitively displace 17 β -oestradiol in any of the oestrogen receptor preparations.

In vitro systems

Cell proliferation assays

The oestrogenic activity of bisphenol-A was assessed in four MCF-7 cell strains (Villalobos et al., 1995). MCF-7 cells strains BUS, BB, ATCC and BB104 were cultured in the presence of human serum that had been treated with charcoal-dextran to remove endogenous oestrogens and so inhibit cell proliferation. Substances with oestrogenic activity can overcome this inhibition. Bisphenol-A elicited a proliferative response in each cell type in this assay. On a molar basis, it

was calculated that the oestrogenic potency of bisphenol-A, as measured in this assay, was approximately 4-5 orders of magnitude lower than that of 17 β -oestradiol.

In further studies using MCF-7 cells (strain not specified), and measuring the ability of putative oestrogen agonists to stimulate cell proliferation, it was calculated (on a molar basis) that the oestrogenic potency of bisphenol-A was approximately 3 (Brotons et al., 1995) and 4 (Olea et al., 1996) orders of magnitude lower than that of 17 β -oestradiol. In the Olea et al. (1996) study, hydroxytamoxifen, an oestrogen antagonist known to act via the oestrogen receptor, was shown to inhibit the activity of bisphenol-A, demonstrating that the assay response was due to interaction with the oestrogen receptor.

In two very briefly reported studies, bisphenol-A stimulated MCF-7 cell proliferation (Dodge et al., 1996; Coldham et al., 1997). No further details are available for either of these studies.

The oestrogenic activity of bisphenol-A was assessed in MtT/E-2 cells, an oestrogen-responsive rat pituitary cell line (Maruyama et al., 1999). A statistically significant increase in cell proliferation was observed at concentrations from 10⁻⁶ M bisphenol-A upwards. No further details are available.

Receptor assays

MCF-7 cells (strain not stated) were used in assessing the effect of adult human serum on the ability of bisphenol-A to bind to oestrogen receptors (Nagel et al., 1997). The reference competitor in these assays was non-radioactive 17 β -oestradiol. MCF-7 cells were cultured in several concentrations of bisphenol-A in the absence or presence of human serum in multiwell plates containing non-radioactive 17 β -oestradiol and [³H]oestradiol for 18 hours. Three independent tests were conducted, and the relative binding affinity of bisphenol-A to oestrogenic receptors in lysed MCF-7 cells was determined by scintillation counting or fluorometric measurement of DNA. The mean relative binding affinity of bisphenol-A was approximately 4 orders of magnitude lower than that of 17 β -oestradiol in both serum and serum-free media. The relative binding affinity of bisphenol-A in serum was 1.7 fold higher than that measured in serum-free medium. Thus, the presence of adult human serum produced a negligible increase in the oestrogenic activity of bisphenol-A in this assay.

In a briefly reported study, the interaction of bisphenol-A and 17 β -oestradiol with receptors for progesterone in MCF-7 cells was investigated (Olea et al., 1996). 17 β -Oestradiol (1 nM) was reported to increase progesterone receptor levels nearly 15 fold over the control value. Bisphenol-A was reported to increase progesterone receptor levels with no change in oestrogen receptor levels. No further information was provided. The results of this study indicate that *in vitro* bisphenol-A can also stimulate an increase in progesterone receptor levels, although the extent to which this occurs was not quantified.

Bisphenol-A was one of several substances tested in a yeast assay looking at interactions with oestrogenic receptors (Sohoni and Sumpter, 1998). The assay used a recombinant strain of yeast (*S. cerevisiae*), which contains an oestrogen-inducible expression system. In the presence of oestrogens, a reporter gene (Lac-Z) encoding for the enzyme β -galactosidase is expressed, which can be monitored by measuring a colour change reaction in the culture medium. A vehicle-only control was included in the test. The oestrogenic activity of the test substances was expressed as a potency relative to 17 β -oestradiol by determining the molar concentrations required to produce the same response. Bisphenol-A produced a positive response; the magnitude of the response was approximately 4 orders of magnitude lower than that of 17 β -oestradiol. Hydroxytamoxifen, an oestrogen antagonist known to act via the oestrogen receptor, was shown to inhibit the

In further studies, GH₃ pituitary cells were incubated with 10 nM oestradiol or 1 µM bisphenol-A for 7 days. Both 17β-oestradiol and bisphenol-A increased prolactin release by 2- to 3 fold in a time-dependent manner. Cell numbers were also observed to increase by 50-60% within 3-5 days.

Steinmetz et al. (1997) also investigated the induction of prolactin gene expression by bisphenol-A. GH₃ cells transfected with a rat prolactin reporter gene and a luciferase encoding sequence were incubated with 1 pM 17β-oestradiol, 1 nM bisphenol-A or 1 nM TRH (a known inducer of the prolactin gene) for 24 hours. Luciferase activity was determined in cell lysate by luminometry. Luciferase activity was increased 1.5- to 2.5 fold with both 17β-oestradiol and bisphenol-A. Higher doses of either compound were stated not to increase prolactin gene expression (data not available). TRH produced a 6- to 8-fold increase in prolactin gene expression. It was next examined whether bisphenol-A regulates transcription through the oestrogen responsive element (ERE). Anterior and posterior pituitary cells from untreated ovariectomised F344 rats were transfected with ERE/luciferase plasmid expressing the luciferase gene. Cells were incubated with 10 nM 17β-oestradiol or 1 µM bisphenol-A for 24 hours. Like 17β-oestradiol, bisphenol-A stimulated ERE-dependent gene expression, suggesting its binding to oestrogen receptors in both tissues.

In a further study, Steinmetz et al. (1997) investigated the induction of prolactin regulating factor (PRF) by bisphenol-A. Posterior pituitary cells, which were removed from F344 and Sprague Dawley rats that had been subcutaneously exposed to about 0.25 mg/kg bisphenol-A or about 0.1 mg/kg 17β-oestradiol for 3 days, were co-cultured in the presence of GH₃ cells transfected with a rat prolactin reporter gene and a luciferase encoding sequence for 24 hours. Luciferase activity, designating induction of the prolactin promoter, was determined in cell lysate by luminometry. Posterior pituitary cells from controls of both strains of rat increased PRF activity 3 to 5 fold, indicating basal PRF activity. Cells harvested from 17β-oestradiol and bisphenol-A treated F344 rats increased PRF activity 15 to 17 fold. PRF activity in cells from Sprague Dawley rats treated with oestradiol or bisphenol-A was unchanged, indicating a marked strain difference (the author reports that results were observed with F344 rats only, as this strain is sensitive to exogenous oestrogens that induce hyperprolactinaemia). These results together with the results observed in F344 rats *in vivo* (see *in vivo* section) indicate that bisphenol-A can cause induction of PRF in the posterior pituitary leading to increased prolactin levels.

To summarise the *in vitro* oestrogenic data, bisphenol-A has oestrogenic activity in these systems and, overall, its activity is generally 3-5 orders of magnitude less than that of 17β-oestradiol. Bisphenol-A has also been shown to increase prolactin release, and there is limited evidence for anti-androgenic activity and stimulation of progesterone activity.

In vivo systems

The oestrogenic activity of bisphenol-A and its influence on prolactin release has been assessed *in vivo* in several studies generally using an assay based upon the uterotrophic response in the rat. These studies are presented below under sub-headings of the route of exposure used. Studies have also been grouped under sub-headings for the effect of bisphenol-A on the growth and development of the mammary gland and prolactin release.

Oral exposure

Rats

In an unpublished study for which the full test report is available (Central Toxicology Laboratory, 1999a), groups of 10 immature (21-22-day-old) female Alpk rats received daily doses of 0, 0.002, 0.02, 0.2, 1, 10, 100, 200 or 800 mg/kg bisphenol-A by gavage for three consecutive days. Animals were killed 24 hours after the final dose, the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels determined and the uterine wet and dry weight recorded together with a histopathological examination of the uteri. 17 β -Oestradiol (0.4 mg/kg) administered by the same route and dosing regime served as a positive control.

Clinical signs of toxicity were observed only in animals receiving 800 mg/kg bisphenol-A; comprising hunched posture, subdued behaviour, salivation and piloerection. A statistically significant increase (21%) in serum ALT was seen at 800 mg/kg bisphenol-A compared to controls. No increase in AST levels was seen in treated animals. Compared to controls, a statistically significant increase in uterine wet and dry weights was observed at 200 (30% and 26%, respectively) and 800 mg/kg bisphenol-A (114% and 76%). At necropsy, endometrial hypertrophy/hyperplasia, luminal epithelial apoptosis (800 mg/kg only), endometrial glandular epithelial apoptosis and increased stromal neutrophils were observed in the 200 and 800 mg/kg bisphenol-A dose groups. There were no treatment-related uterus changes at bisphenol-A doses of 100 mg/kg and below. For the positive control, statistically significant increases in the uterine wet (294%) and dry (203%) weight were observed, together with histopathological changes in the uteri consistent with those seen with bisphenol-A. Therefore, in this screening assay for oestrogenic activity using the oral route of exposure, changes to the uteri were observed in Alpk rats at 200 mg/kg bisphenol-A and above. No effects were observed at dose levels up to, and including, 100 mg/kg.

The activity of bisphenol-A was investigated in a rat uterotrophic study by Ashby and Tinwell (1998). Immature (21-22-day-old) female Alpk:AP rats (7-10 per dose level) received daily doses of 0, 400, 600 or 800 mg/kg bisphenol-A by gavage for 3 consecutive days. Animals were killed 24 hours after the final dose, the presence or absence of vaginal opening recorded, and the uterine wet and dry weight determined. DES (0.04 mg/kg), administered by the same route of administration and dosing regime, served as the positive control.

No clinical signs of toxicity or effects on body weight gain were observed with bisphenol-A. Compared to controls, uterine wet weights were increased by 31, 38 and 118%, and uterine dry weights by 40, 40 and 150%, following administration of 400, 600 and 800 mg/kg bisphenol-A, respectively. Premature vaginal opening was not observed in bisphenol-A treated groups. DES produced increases in uterine wet and dry weights of >250% and premature vaginal opening in 60% animals. Thus, an oestrogenic activity was observed with bisphenol-A in this immature rat uterotrophic assay following oral administration.

The effect of bisphenol-A on oestrus cyclicity was investigated in a uterotrophic assay by Rubin et al. (2001). Groups of 4-6 ovariectomised Sprague Dawley rats were administered 0, 0.2, 2.0 and 16.9 mg/kg bisphenol-A in the drinking water for 3 consecutive days. Vaginal cytology was conducted before and daily during treatment. Animals were sacrificed after treatment and uterine wet weights determined. Esterone (0.02 or 0.17 mg/kg) administered by the same route and dosing regime served as a positive control.

Bisphenol-A had no effect on uterine wet weight or vaginal cytology. Compared to controls, a statistically significant increase in uterine wet weight (317%) was observed with 0.17 mg/kg estrone, along with cornified vaginal smears on the day of sacrifice.

Laws et al. (2000) conducted a series of experiments investigating the oestrogenic activity of a number of substances, including bisphenol-A, using different biological endpoints.

In a well reported experiment, a uterotrophic assay was conducted in immature (21-day-old) and ovariectomised Long Evans rats. Groups of 6 immature and 6 ovariectomised rats received 0, 100, 200 or 400 mg/kg bisphenol-A daily by gavage for 3 consecutive days. Animals were sacrificed at 6 hours, and 24 hours for immature animals, after the last dose and the uteri removed and weighed. Compared to controls, a statistically significant increase in uterine wet weight of approximately 260 and 310% was seen in immature rats 6 hours after administration of 200 and 400 mg/kg bisphenol-A, respectively. However, by 24 hours post-dosing in both treatment groups, uterine weight had returned to control levels. Bisphenol-A had no effect on uterine weight in ovariectomised animals.

The next study by Laws et al. (2000) was conducted to investigate the effects of bisphenol-A on vaginal opening. Groups of 7 or 8 immature Long Evans rats received 0, 50, 100, 200 or 400 mg/kg day bisphenol-A by gavage from 21 to 35 days of age and the day of vaginal opening was recorded. Bisphenol-A had no effect on body weight gain or on the time of vaginal opening.

In a further study by Laws et al. (2000) in Long Evans rats, groups of 6 ovariectomised and 9-15 "intact" females received 0 or 100 mg/kg day bisphenol-A by gavage for 25 days. Vaginal smears were taken daily to assess the effect of bisphenol-A on oestrus cyclicity. The authors report that oestrogenic activity in ovariectomised animals would result in persistent oestrous status, which would be reflected by the appearance of cornified epithelial cells in vaginal smears. No effect on vaginal cytology was observed in ovariectomised animals administered bisphenol-A. In "intact" animals, extended oestrous was observed in bisphenol-A treated animals so that the mean number of 4-5 day oestrous cycles was seen to decrease from 5.2 in controls to 3.7 in treated animals.

In the final study, Laws et al. (2000) compared the oestrogenic activity of bisphenol-A following oral and s.c. administration. Groups of 6 ovariectomised rats received 0 or 200 mg/kg bisphenol-A daily by gavage or s.c. injection for 3 consecutive days. Animals were sacrificed 6 hours after the last dose, the uteri removed and weighed. Compared to controls, a statistically significant increase in uterine wet weight of approximately 130 and 270% was seen following oral and s.c administration, respectively.

Overall, the Laws et al. (2000) studies demonstrate that the immature rat model was more sensitive than ovariectomised adult rats in detecting oestrogenic activity on the basis of the uterotrophic response. However, the activity seen in immature rats 6 hours post-dosing was observed to be short term, as uterine weight returned to control level values by 24 hours. Greater changes in uterine weight were seen when bisphenol-A was administered by the s.c. route compared with those following oral exposure. Assays based on the time to vaginal opening and vaginal cytology did not detect oestrogenic activity, although an effect of bisphenol-A was observed on oestrous cyclicity.

Bisphenol-A was one of a number of chemicals tested in the peripubertal male rat assay, which is being developed for the detection of anti-androgens, oestrogens and metabolic modulators (Ashby and Lefevre, 2000). Groups of 10 immature male Alpk:APfSD rats (21-22 or 32-33-day-old) were given 20 consecutive daily oral (gavage) doses of 0, 100 or 150 mg/kg bisphenol-A. The endpoints studied were changes in the weights of testes, epididymides, seminal vesicles,

prostate, liver, kidney and body weight. The day of prepuce separation and the influence of initial body weight on final organ weight were also evaluated. The study also evaluated DES, which is often used as a positive control in oestrogenic activity assays. No effect on any parameter measured was observed with bisphenol-A. DES (40 µg) reduced the weights of all reproductive organs and produced marked delays in the day of prepuce separation. Therefore, bisphenol-A had no effect on any endpoint associated with potential oestrogenic activity in this immature male rat study.

In a briefly reported study (Dodge et al., 1996), ovariectomised Sprague Dawley rats received 0, 0.1, 1.0, 10 or 30 mg/kg bisphenol-A daily by oral gavage for 4 days or 5 weeks. Oestrogenic activity was measured by changes in uterine wet weight after 4 days and 5 weeks of dosing. In addition, the effect of bisphenol-A on serum cholesterol levels was determined in rats for both dosing regimes, along with bone mineral density in rats dosed for 5 weeks. Compared to controls, the authors report that the maximum, and statistically significant, increases in uterine wet weights were observed after 4 days of dosing; 29% and 37% in the 10 and 30 mg/kg dose groups, respectively. The authors also report that bisphenol-A lowered serum cholesterol levels after 4 days of dosing. However, no results were presented for the effect of bisphenol-A on uterine wet weights and serum cholesterol levels after 5 weeks of dosing. No effects were observed on bone mineral density with bisphenol-A. Thus, the limited details of this study suggest that bisphenol-A increases uterine wet weight in ovariectomised rats.

In a well reported study (Gould et al., 1998b), groups of 4-5 immature (21-day-old) female Sprague Dawley rats received 0, 5, 10, 25, 50, 100 or 150 mg/kg bisphenol-A daily by gavage for 3 consecutive days. Animals were sacrificed 20 hours after the last dose, the uteri removed, weighed and then assays conducted to determine uterine progesterone receptor levels and peroxidase activity, two oestrogen-responsive proteins. Oestradiol (0.5 µg), administered by the i.p. route and using the same dosing regime, served as a positive control. Bisphenol-A had no effect on uterine wet weight. Compared to controls, a statistically significant increase in uterine peroxidase activity was seen at 100 (50%) and 150 mg/kg bisphenol-A (108%). A statistically significant but not dose-related increase in progesterone receptor levels of 34-76% was observed in all bisphenol-A treatment groups. Compared to controls, the positive control oestradiol produced a statistically significant increase in uterine wet weight (375%), peroxidase activity (717%) and progesterone receptor levels (514%). The toxicological significance of these observed increases in oestrogen responsive proteins is unclear.

The effect of bisphenol-A on the growth of prostate and seminal vesicles in the rat was investigated in an androgen and anti-androgen assay (Michna, 2000). In the androgen assay, groups of 7-10 orchietomised Wistar rats received 0, 3, 50, 200 or 500 mg/kg bisphenol-A by oral gavage daily for 12 days. A further group received 500 mg/kg bisphenol-A and the anti-androgen flutamid (3 mg/kg) by the same dosing regime. The assay also included a control group of "intact" males along with a positive control group receiving 1 mg/kg testosteronepropionat (TP) by s.c. injection. Animals were sacrificed 24 hours after the last dose and the kidney, liver, prostate and seminal vesicles removed, weighed and examined (no further details available). Blood was also taken from animals 5 minutes after the first and last dose, but the reasons for taking it are not reported.

A statistically significant decrease (22%) in body weight gain was observed with 500 mg/kg bisphenol-A and flutamid. No statistically significant increases or decreases were seen in absolute organ weights. However, after correction for body weight, a statistically significant increase in prostate weight was seen at 500 mg/kg in the absence (37%) and presence (16%) of flutamid compared to orchietomised controls. No statistically significant effects were seen in seminal vesicle, liver or kidney weights. Comparing the "intact" and orchietomised controls, a

statistically significant decrease in body weight (10%), prostate weight (89%) and seminal vesicle weight (89%) was seen in orchietomised males. Compared to orchietomised controls, TP produced a statistically significant increase in prostate (1313%), seminal vesicle (888%) and liver (12%) weight. The results of histopathological examination were not reported.

The anti-androgen assay used the same test protocol as the androgen assay but animals received 1 mg/kg TP (by s.c. injection) plus 0, 3, 50, 200 or 500 mg/kg bisphenol-A. A positive control group that received TP and flutamid was also included (but no "intact" control group).

A statistically significant decrease (10%) in body weight gain was seen at the top dose (500 mg/kg and 1 mg/kg TP). No statistically significant increases or decreases were seen in absolute organ weights. However, after correction for body weight, a statistically significant increase in prostate weight (15%) was seen at the top dose compared to controls. No statistically significant effects were seen on seminal vesicle, liver or kidney weight. A statistically significant decrease in prostate (55%) and seminal vesicle weight (63%) was seen with TP. Again, the results at histopathology were not reported.

The results of the Michna (2000) study show a stimulatory effect on prostate growth (37%) with 500 mg/kg bisphenol-A, which was antagonised by the anti-androgen flutamid. Bisphenol-A exhibited no activity in the anti-androgen assay. Thus, the data indicate androgen activity of bisphenol-A, albeit limited, at 500 mg/kg.

In a study cited in the BUA (1995) review (Bornmann and Loeser, 1959), oral administration of bisphenol-A in orchietomised rats resulted in "uncertain and very faint" oestrogenic activity, even after "high" doses. A single s.c. injection of 2400 mg/kg bisphenol-A apparently triggered oestrus in ovariectomised animals. No further information is provided. Consequently, the limited details available for this study mean that no reliable conclusions can be drawn from the data.

Mice

In a well reported study (Tinwell et al., 2000), a uterotrophic assay was conducted in immature (19-20-day-old) AP mice. Administration of 5-bromodeoxyuridine (0.8 mg/ml) in deionized water to these animals during the acclimatisation and dosing period also allowed uterine hyperplasia to be determined.

Groups of 12 immature (19-20-day-old) female AP mice received 0, 0.5, 1, 5, 10, 50, 100, 200 or 300 mg/kg bisphenol-A daily by gavage for 3 consecutive days. Animals were sacrificed 24 hours after the last dose, the uteri removed and weighed. Uterine samples were then taken, stained for bromodeoxyuridine and labelling index determined by light microscopy. Uterine hypertrophy was determined by light microscopy. DES (10 µg/kg), administered by the same route of administration and dosing regime, served as a positive control. Bisphenol-A had no effect on body weight gain or uterine wet weight, nor did administration of bisphenol-A lead to uterine hypertrophy; no significant increase was seen in the height of the uterine epithelium or endometrium. However, compared to controls, a statistically significant increase in the number of cells in the uterine epithelium (181%) and endometrial stroma (166%) was seen at 200 mg/kg, and in the uterine epithelium (384%) and endometrial glands (54%) and stroma (172%) at 300 mg/kg. Compared to controls, DES produced a statistically significant increase in uterine wet weight (317%), height of the uterine epithelium (161%) and endometrium (79%); and in the number of cells in the uterine epithelium (618%), and endometrial glands (49%) and stroma (325%). Thus, the only effect observed on the uterus in this immature mouse uterotrophic assay was hyperplasia at 200 mg/kg and above. No effect on the uterus was seen at concentrations up to, and including, 100 mg/kg bisphenol-A.

Parenteral routes of exposure

Rats

A number of older studies are available which were designed to investigate the oestrogenic potential of bisphenol-A. These reports are generally brief and lack significant information on the test protocol and effects observed.

Bisphenol-A showed an oestrogenic effect (cornification of the vagina) after injection (by an unstated route) of 100 mg bisphenol-A in oily solution to ovariectomised rats (Dodds and Lawson, 1936; 1938). An oestrus response was also observed in ovariectomised rats injected with an unstated amount of bisphenol-A six times over three days (Campbell, 1940). Reid and Wilson (1944) also reported that bisphenol-A had shown oestrogenic activity in the rat. No further details are available for any of these studies.

In a recent unpublished study for which the full test report is available (Central Toxicology Laboratory, 1999b), groups of 10 immature (21-22-day-old) female Alpk rats received daily doses of 0, 0.002, 0.02, 0.2, 1, 10, 100, or 800 mg/kg bisphenol-A by s.c injection for three consecutive days. Animals were killed 24 hours after the final dose, the serum ALT and AST levels determined and the uterine wet and dry weight recorded together with a histopathological examination of the uteri. 17 β -Oestradiol (0.4 mg/kg) administered by the same route and dosing regime served as a positive control.

Clinical signs of toxicity were observed only in the top dose (800 mg/kg) group; fur staining in several animals and a statistically significant decrease (10%) in body weight gain compared to controls. A statistically significant decrease in serum ALT levels was seen in animals at 800 mg/kg (16%). Serum AST levels in treated and control animals were similar. Compared to controls, a statistically significant increase in wet uteri weight (117%) was seen at 800 mg/kg bisphenol-A only. In bisphenol-A-treated animals, no increase in uterine dry weight achieved statistical significance when compared to controls. At necropsy, endometrial hypertrophy/hyperplasia, luminal epithelial apoptosis, endometrial glandular epithelial apoptosis and increased stromal neutrophils were seen at 100 mg/kg bisphenol-A and above. Uterine luminal epithelial apoptosis and endometrial glandular epithelial apoptosis were also seen in animals at 10 mg/kg. For the positive control, statistically significant increases in the uterine wet (218%) and dry (166%) weight were observed, together with histopathological changes in the uteri consistent with those seen with bisphenol-A. Therefore, in this screening assay for oestrogenic activity using the s.c route of exposure, bisphenol-A produced changes in the uteri in Alpk rats.

The activity of bisphenol-A was investigated in a recent rat uterotrophic study using the s.c route of administration (Ashby and Tinwell, 1998). Immature (21-22-day-old) female Alpk:AP rats (7-10 per dose level) received daily injections of 0, 400, 600 or 800 mg/kg bisphenol-A for 3 consecutive days. Animals were killed 24 hours after the final dose, the presence or absence of vaginal opening recorded, and the uterine wet and dry weight determined. DES (0.04 mg/kg), administered by the same route of administration and dosing regime, served as the positive control.

No clinical signs of toxicity or effects on body weight gain were observed with bisphenol-A. Compared to controls, uterine wet weights were increased by 50, 71 and 103%, and uterine dry weights by 50, 67 and 100%, following administration of 400, 600 and 800 mg/kg bisphenol-A, respectively. Premature vaginal opening was observed in 0, 0, 57 and 47% rats at 0, 400, 600 and 800 mg/kg bisphenol-A, respectively. DES produced increases in uterine wet and dry weights of

>200% and premature vaginal opening in 30% animals. Thus, an oestrogenic activity was observed with bisphenol-A in this immature rat uterotrophic assay following s.c. administration.

A further study investigating the activity of bisphenol-A in a rat uterotrophic assay following s.c. administration is available (Goloubkova et al., 2000). Groups of ovariectomised Wistar rats (6 per dose group) received daily injections of 0, 11, 78, 128 and 250 mg/kg for 7 days. The study also included a control group of sham surgery animals. Animals were killed after treatment (it is not reported how long after the final injection), serum prolactin levels and uterine wet weight determined, and prolactin-expressed cells were identified in pituitary glands by immunohistochemical staining. It is not reported whether a positive control was used in this study.

A statistically significant decrease in body weight gain (6%) was observed in animals receiving 250 mg/kg compared to controls. A statistically significant and dose-related increase in mean uterine wet weight was observed at 11 (approx 35%), 78 (50%), 128 (80%) and 250 mg/kg (155%) compared to controls. Even at 250 mg/kg bisphenol-A, the uterine wet weights were not restored to levels seen in the sham control animals. Compared to controls, statistically significant increases in mean anterior pituitary gland weights (approx >40%) and mean prolactin levels (approx >700%) were seen at 128 and 250 mg/kg bisphenol-A. A statistically significant increase in prolactin-immunopositive cells was also seen at 250 mg/kg (data not presented). The number of prolactin-immunopositive cells was seen to decrease in ovariectomised animals; 24% decrease in ovariectomised controls compared to sham control animals.

An immature rat uterotrophic assay using both the s.c. and oral route of administration is available (Yamasaki et al., 2000). Female CD rats (8 animals per dose group) were given daily s.c. injections of 0, 8, 40 or 160 mg/kg bisphenol-A in sesame oil, or daily oral (gavage) doses of 0, 40, 160 or 800 mg/kg bisphenol-A in sesame oil, on postnatal days 18-20. Animals were sacrificed 24 hours after the final dose and uteri removed and weighed. A repeat study using the same experimental protocol was conducted. The initial study also determined plasma concentrations of bisphenol-A in 4 females per dose group 1 hour after the last injection. These results have not been reported in this summary.

In both the initial and repeat study, no clinical signs of toxicity, effects on body weight gain or immature vaginal opening were observed for either the oral or s.c. route of administration. In the initial study, a statistically significant and dose-related increase in absolute wet and dry uterine weight (44%) was seen following oral administration of 800 mg/kg bisphenol-A, compared to controls. Statistically significant increases in the relative wet and dry uterine weights ($\geq 13\%$) were seen at 160 mg/kg and above. In the repeat study, statistically significant increases in absolute and relative wet and dry uterine weights ($\geq 14\%$) were seen at 160 mg/kg and above.

In the initial study using s.c. administration, a statistically significant and dose-related increase in absolute and relative wet and dry uterine weight ($\geq 14\%$) was seen at 8 mg/kg and above, compared to controls. In the repeat study, statistically significant increases in absolute wet and dry uterine weights ($\geq 47\%$) were seen at 40 mg/kg and above, compared to controls. Increases in relative wet and dry uterine weights ($\geq 14\%$) were statistically significant at 8 mg/kg and above.

Yamasaki et al. (2000) investigated time course changes in uterine weight after s.c. administration of bisphenol-A. This study employed the same dose levels and experimental procedure described above, with the addition that animals were sacrificed 6, 12, 18 and 24 hours after the last injection. Statistically significant increases were seen in uterine wet and dry weight for all sample times at 40 and 160 mg/kg, compared to controls. The increases at 6 hours were greater than those observed at 24 hours, but the coefficient of variation was lower at 24 hours

than at 6 hours. Thus, the authors suggest that autopsy at 24 hours after final administration of the test substance is more suitable, based on the coefficients of variation at low-dose levels.

A series of exploratory studies examining the growth, differentiation and gene expression in the female rat reproductive tract was conducted by Steinmetz et al. (1998).

In a briefly reported experiment, groups of ovariectomised F344 rats (number not reported) received a single i.p. injection of 0, 19, 37.5, 75, 150 or 200 mg/kg bisphenol-A. 17β -Oestradiol (10 μ g/kg) administered by the same route, served as a positive control. Animals were injected i.p. with 5-bromodeoxyuridine 19 hours after administration of bisphenol-A or oestradiol, and killed 1 hour later. Uteri and vaginas were removed and cell proliferation determined in these tissues by bromodeoxyuridine immunostaining. A statistically significant increase in the number of labelled epithelium cells in both uteri and vagina was observed at 37.5 mg/kg bisphenol-A and above. Maximum labelling in the uterine epithelium was observed at 75 and 150 mg/kg. A secondary increase in vaginal epithelium labelling was observed at levels causing toxicity in some animals, 200 mg/kg, and at an additional dose level of 300 mg/kg bisphenol-A (no further data provided). The limited details prevent any conclusions to be drawn on the possible cause of this secondary increase. The authors report that labelled epithelial cells in both uteri and vagina were observed with the positive control. This study demonstrates that bisphenol-A increases cell proliferation in the uteri and vaginas of ovariectomised rats.

The next study was conducted to compare the ability of bisphenol-A and 17β -oestradiol to induce *c-fos* gene expression in the F344 rat uterus and vagina. Groups of ovariectomised rats (number not reported) received a single i.p. injection of 0 or 50 mg/kg bisphenol-A. 17β -Oestradiol (10 μ g/kg), administered by the same route, served as a positive control. Animals were killed 2, 6 and 24 hours later. Uteri and vaginas were removed and *c-fos* gene expression determined in each. Both bisphenol-A and 17β -oestradiol increased *c-fos* messenger RNA levels in the uterus 14- to 17-fold and 7- to 9-fold in the vagina above control values within 2 hours. In the uterus, *c-fos* expression returned to basal levels after 6 hours following both bisphenol-A and 17β -oestradiol treatment. In the vagina, bisphenol-A-induced *c-fos* expression remained elevated for up to 6 hours compared to transient increases observed with 17β -oestradiol. The results of this study show the potential of bisphenol-A to induce *c-fos* gene expression in the uterus and vagina of F344 rats.

In the final study, groups of ovariectomised F344 and Sprague Dawley rats (number not reported) were implanted subcutaneously with capsules containing crystalline bisphenol-A or 17β -oestradiol. Controls received empty capsules. Animals were killed after 3 days and uterine sections examined, the heights of the luminal epithelial cells being measured. The rate of release of bisphenol-A and 17β -oestradiol from the capsules was estimated to be approximately 0.3 and 0.006 mg/kg/day, respectively. In F344 rats a statistically significant increase of 2.5-fold and 3.5-fold were observed in epithelial cell height compared to controls following bisphenol-A and 17β -oestradiol treatment, respectively. Compared to controls, a statistically significant increase in uterine wet weight of nearly 2-fold and 3-3.5 fold was observed following treatment with bisphenol-A and 17β -oestradiol, respectively. Similar to 17β -oestradiol, bisphenol-A resulted in hypertrophy of the luminal epithelium and stimulated mucus secretion in the uterus, and hyperplasia and cornification of the vaginal epithelium. However, in Sprague Dawley rats uterine cell height and uterine wet weight were significantly altered only with 17β -oestradiol. The results of this study show a marked strain difference in the oestrogenic activity of bisphenol-A, but not of 17β -oestradiol. Based on the estimated release of bisphenol-A and oestradiol, the potency of bisphenol-A in this study is approximately 50-fold less than that of oestradiol.

Overall, the Steinmetz et al. (1998) studies demonstrate that the molecular and morphological alterations induced by bisphenol-A in the uterus and vagina are qualitatively similar to those induced by 17 β -oestradiol. The studies also indicate that the reproductive tract of F344 rats appears to be more sensitive than that of Sprague Dawley rats to the effects of bisphenol-A, although there is no information to indicate which strain is more relevant to humans.

Long et al. (2000), investigated if the rat vagina (an oestrogen target tissue) responds to bisphenol-A in a strain-specific manner in F344 and Sprague Dawley rats. However, the data reported for F344 rats are from the Steinmetz et al. (1998) study. Only the results in Sprague Dawley rats are reported here.

Groups of 4 ovariectomised Sprague Dawley rats were administered a single i.p. injection of 0 or 0.2-150 mg/kg bisphenol-A and cell proliferation in vaginal epithelium determined 19 hours later. Animals were injected with bromodeoxyuridine 1 hour prior to sacrifice, vaginal tissue taken, stained for bromodeoxyuridine and labelling index determined by light microscopy. 17 β -Oestradiol (0.02-2 μ g/kg) administered by the same route, served as a positive control. No significant increase in the number of labelled epithelial cells was seen in Sprague Dawley rats treated with bisphenol-A (in comparison, in F344 rats, a statistically significant increase in labelled vaginal epithelial cells was seen at 37.5 mg/kg and above in the Steinmetz et al. (1998) study). The positive control produced a significant increase in labelled cells.

The Long et al. (2000) study also conducted further experiments investigating potential strain differences between F344 and Sprague Dawley rats; metabolic clearance of bisphenol-A, stimulation of *c-fos* gene expression by bisphenol-A and vaginal epithelium concentration of oestrogen receptors. However, since the study did not concurrently investigate the activity of bisphenol-A on vaginal epithelium in F344 rats, these experiments are considered to be of limited value. Consequently, they are not discussed in detail here, except to report that clearance of [³H]-bisphenol-A from blood (administered by i.v. injection) followed the same time course in both rat strains, stimulation of *c-fos* gene expression by bisphenol-A showed no strain difference and analysis of [³H]-bisphenol binding showed there were no significant differences in concentration or affinity for bisphenol-A of oestrogen receptor in vaginal tissue.

Overall, no effect on vaginal epithelium was seen in Sprague Dawley rats in this study. This suggests that there may be a significant difference in the oestrogenic activity of bisphenol-A between F344 and Sprague Dawley rats.

In a study in Sprague Dawley rats, which was reported in abstract form only, ovariectomised rats received a single i.p. injection of 0, 25, 50 or 100 mg/kg bisphenol-A (Bond et al., 1980). Oestrogenic activity was measured by changes in uterine water content; oestrogens can cause a fluid distension of the uterus. A statistically significant increase in % uterine water content was reported in the 50 and 100 mg/kg dose groups at 18 hours post-dosing. No further details are available.

In a study reported in abstract form only (Cummings, 1997), bisphenol-A was investigated in the delayed implanting model. In this study, female Holtzman rats identified as sperm positive (day 0) were hypophysectomised two days later. Removal of the pituitary prior to implantation permits the blastocysts to remain viable but unattached in the uterus. Oestrogenic activity is then detected as the ability to induce implantation. Animals received progesterone at 2 mg/rat on days 0-6 and 4 mg/rat on days 7-8. On day 7, rats were injected (s.c.) with 25-200 mg/kg bisphenol-A or 1 μ g of the positive control, estrone. On day 9, dye was administered to animals by intra-hepatic infusion, under anaesthesia, and the implantation sites were observed as blue bands after 10 minutes. All treated rats had implantations following administration of bisphenol-A at

200 mg/kg, 78% at 100 mg/kg, 50% at 50 mg/kg and 30% at 25 mg/kg. Implantation was observed in all animals treated with the positive control. The results of this study indicate that bisphenol-A administered by the s.c. route can induce implantation of the blastocysts in hypophysectomised Holtzman rats, a reflection of oestrogenic activity.

In an early study (Bitman and Cecil, 1970), the ability of bisphenol-A to increase glycogen content in the uterus was investigated as a measure of its oestrogenic activity. Immature (21-23-day-old) Wistar rats were injected subcutaneously with unstated doses of bisphenol-A. The authors report that in this assay, the minimum effective dose was 0.25 mg/bisphenol-A/animal (using the default body weight value in Table 4.24, this corresponds to a dose of 1.4 mg/kg bisphenol-A). No further details are available.

In a well reported study, the oestrogenic activity of bisphenol-A was investigated *in vivo* in MtT/E-2 cells implanted in rats (Maruyama et al., 1999). The authors state that this cell type will develop into tumours in response to oestrogen. Groups of 5-6 ovariectomised F344 rats were inoculated at two sites in the subcutaneous fat pads with MtT/E-2 cells. Animals then received 0, 0.1, 1 or 10 mg bisphenol-A by the i.p. route (assuming a rat weighs 0.175 kg, this is equivalent to 0, 0.6, 5.7 and 57 mg/kg), three times a week, starting on the day the fat pads were injected. Oestrogen (0.5 mg), given by s.c. injection, served as the positive control. Test sites were examined twice a week and the diameters of any tumours measured. Animals were sacrificed when the tumours reached 1-2 cm in diameter. At necropsy, the pituitary and uterus were removed and weighed, and serum prolactin levels measured.

The tumour results were briefly reported. The time to appearance of “visible” tumours occurred in a dose-dependent manner in bisphenol-A-treated animals, on days 25, 32 and 36 at 57, 5.7 and 0.6 mg/kg respectively. Tumours were seen in control and oestrogen-treated animals at 41 and 22 days, respectively. Compared to controls, no significant increase in serum prolactin levels or pituitary and uterus weights were seen in bisphenol-A-treated animals. A statistically significant increase in prolactin (697%), and pituitary (102%) and uterus (545%) weights was observed with oestrogen. The results of this study suggest that bisphenol-A administered by the ip route expressed oestrogenic activity towards inoculated MtT/E-2 cells in F344 rats. However, the limited reporting of the results mean no reliable conclusions can be reached from this study. Furthermore, the toxicological significance of this experimental approach is unclear.

Mice

In a well reported uterotrophic assay in CFLP mice (Coldham et al., 1997), prepubertal 18-day-old females received 0, 0.05, 0.5 or 5 mg bisphenol-A daily by s.c. injection for 3 days. Mice were sacrificed 24 hours after the final injection and uterine wet weight determined. The oestrogenic activity of bisphenol-A was expressed as the potency relative to 17β -oestradiol, by determining the molar concentration required to produce similar increases in the ratio of uterine wet weight/body weight. The authors report that treatment with 5 mg bisphenol-A was ceased due to symptoms of acute toxicity (the nature of which was not specified). At 0.05 and 0.5 mg bisphenol-A, uterine wet weights were similar to controls. Compared to controls, a statistically significant dose-related increase in uterine wet weight (77-471%) was observed with 5-100 ng 17β -oestradiol. Therefore, bisphenol-A showed no oestrogenic activity in this uterotrophic assay in mice.

An exploratory assay examining the oestrogenic activity of xenoestrogens by determining their effect on uterine vascular permeability was conducted by Milligan et al. (1998). In this generally well reported study, 12 and 6 ovariectomised Swiss mice were administered 0 or 10^{-5} mol (equivalent to 23 mg) bisphenol-A by s.c. injection, respectively. (125 I)-Radiolabelled albumin

was then administered by i.v injection 3.5 hours later. A blood sample was taken 0.5 hours later and animals were immediately sacrificed. The uteri and a thigh muscle sample were removed, and the radioactivity in these samples and in the plasma was determined. The ratio of the (^{125}I) counts per minute per milligram of tissue to (^{125}I) counts per minute per microliter of plasma was used as an index of tissue vascular permeability. Uterine vascular permeability was observed to increase in bisphenol-A treated animals compared to controls. Various concentrations of 17β -oestradiol (10^{-12} , 10^{-10} and 10^{-9} mol) were also tested in this experiment. Compared to controls, uterine vascular permeability was seen to increase at 10^{-10} and 10^{-9} mol 17β -oestradiol. The muscle vascular permeability in both bisphenol-A and 17β -oestradiol treated animals was similar to controls. The results suggest that s.c. administration of 23 mg bisphenol-A produced an increase in uterine vascular permeability, which (using the default values in **Table 4.24**) corresponds to a dose of approximately 900 mg/kg. The toxicological significance to human health of increased uterine vascular permeability is unclear.

Prolactin release

A study was conducted by Steinmetz et al. (1997) to determine the influence of bisphenol-A on prolactin release, as the study authors reported that *in vivo*, oestrogens can affect the neuroendocrine axis and thus affect prolactin release, by acting directly on the pituitary lactotroph (an oestrogen responsive cell) or indirectly via hypothalamo-pituitary factors that regulate lactotrophs, such as prolactin regulating factor (PRF). Groups of 12 F344 and 8 Sprague Dawley ovariectomised rats were implanted subcutaneously with capsules containing crystalline bisphenol-A or 17β -oestradiol. Controls received empty capsules. The release of bisphenol-A and 17β -oestradiol was estimated from an *in vitro* experiment to be approximately 40-45 $\mu\text{g}/\text{day}$ and 1.2-1.5 $\mu\text{g}/\text{day}$, respectively. Using default weight values (see **Table 4.24**) this corresponds to about 0.25 mg/kg bisphenol-A and 0.01 mg/kg oestradiol. Animals were killed after 3 days, serum analysed for prolactin and the anterior pituitaries removed and weighed. Compared to controls, bisphenol-A and 17β -oestradiol increased basal prolactin levels 7 to 8 fold and 10 fold in F344 rats, respectively. However, in Sprague Dawley rats only 17β -oestradiol produced an increase in prolactin levels (3-fold). Bisphenol-A did not alter anterior pituitary weights in either rat strain. 17β -Oestradiol doubled anterior pituitary weights in F344 rats but produced no significant increase in Sprague Dawley rats. The results indicate a marked strain difference in the influence of bisphenol-A on prolactin release in rats.

Effects on mammary gland

The influence of bisphenol-A on the normal growth and development of the mammary gland of rats was investigated by Colerangle and Roy (1997). An indication of changes in mitotic activity in the female mammary gland is considered to be a reflection of oestrogenic action. The authors stated that Noble rats were used in this study as they are particularly sensitive to oestrogenic activity; oestrogen treatment of female Noble rats for 11-12 months induced an 80-90% incidence of mammary tumours. Groups of rats (6 per group) were implanted subcutaneously with osmotic minipumps containing bisphenol-A administering a daily dose of 0, 0.1 or 54 mg/kg bisphenol-A. DES (0.1 mg/kg), administered by the same means served as a positive control. Animals were killed 11 days after implantation of the minipumps and mammary glands removed. Mammary gland growth was assessed by counting the number of mammary structures (terminal ducts (TDs), terminal end buds (TEBs) and lobules) and cells in 16 mm areas of the mammary gland.

In the mammary gland, the conversion of immature structures to mature structures was significantly increased with exposure to bisphenol-A. The average number of combined TDs and

TEBs was seen to decrease (controls 53%; low dose 38%; high dose 22%), and average number of lobules was seen to increase (47%, 62% and 78%) at both dose levels of bisphenol-A. The low- and high-dose bisphenol-A groups induced a 1.4- and 2.2-fold increase in cell numbers over controls, respectively. DES produced a 600% increase in cell numbers over controls.

However, there are concerns about the conduct of this study; Ashby and Odum (1998) draw attention to the fact that the same positive control (DES) data used in a 1996 study also appears in two other reports by Colerangle and Roy (1995 and 1997), and that the vehicle control data has also been duplicated. This raises uncertainties as to whether the control data were generated concurrently with the bisphenol-A data and raises questions about the validity of this study.

Summary of studies investigating endocrine modulating activity

Bisphenol-A has been shown to have endocrine modulating activity in a number of *in vitro* and *in vivo* screening assays. The potency of this activity in these assays generally ranged from 3 to 5 orders of magnitude less than that of oestradiol. No significant oestrogenic activity has been observed with bisphenol-A glucuronide *in vitro*. The available data also indicate that there is a marked strain difference in the response to bisphenol-A in rats. However, there are no data to indicate the underlying reasons for such differences.

It should be noted that these studies investigating endocrine modulating activity are essentially screening tests and many of them employ experimental protocols, which have not undergone any international validation. However, the first phase of the validation of the uterotrophic assay in OECD indicates that this model is robust and reproducible across laboratories (Kanno et al., 2001). Whilst this assay can be used to identify oestrogenic activity and can be an early screening test, its use for risk characterisation purposes is still a matter for discussion. In addition, many of the available *in vivo* studies have used parenteral routes of exposure, the relevance of which are uncertain with respect to relevant routes of human exposure.

4.1.2.9.2 Effects on fertility

One-generation studies

In a study that was well reported but not conducted to current regulatory guidelines (General Electric, 1976c), CD rats (10 per sex per group) were fed bisphenol-A in the diet for 10 weeks and then mated, 1 male to 1 female. The dietary levels used, 0, 1,000, 3,000 or 9,000 ppm corresponded to mean doses of approximately 0, 70, 200 and 650 mg/kg in males, and 0, 100, 300 and 950 mg/kg in females. Dosing was maintained throughout gestation and weaning of the F₁ generation. Following weaning, parental (F₀) animals were weighed, and then sacrificed and discarded.

At sacrifice, there was evidence of general toxicity in parental animals only at the top dose: a statistically significant reduction in body weight gain of 18% in males and 12% in females. Food consumption was not affected at any dose. No effects were observed on fertility index, litter size or post natal survival rate. At day 21 post partum, there was a statistically significant decrease in pup body weight gain relative to controls: 7% in the mid dose and 12% in the high dose (there was no effect at the low dose). No other parameters were investigated in the pups, which were used in a 90-day feeding study (see Effects on development section). The results of this one-generation study indicate that bisphenol-A did not produce any adverse effect on fertility up to dose levels at which parental toxicity was observed: 650 mg/kg in males and 950 mg/kg in females.

Using the same method, the study was repeated with 0, 100, 250, 500, 750 or 1,000 ppm bisphenol-A in the diet, which corresponded to mean dose levels of approximately 0, 5, 15, 30, 50 and 60 mg/kg in males, and 0, 10, 25, 50, 75 and 100 mg/kg in females, respectively (General Electric, 1978). Prior to mating, vaginal smears were taken for 21 consecutive days to determine the effect of bisphenol-A on the oestrous cycle.

At sacrifice, no effect was observed on body weight gain in the parental animals. No effects were observed on the oestrous cycle or on fertility index, litter size, post natal survival or pup body weight. Thus, in this additional one-generation study, in the absence of parental toxicity, no effect was observed on fertility or the offspring at the dose levels employed.

Two-generation studies

The effect of bisphenol-A on fertility was evaluated in an extensive oral two generation reproduction toxicity study in Crj;CD (SD) IGS rats (Chemical Compound Safety Research Institute, 2000). The F₀ generation consisted of groups of 25 rats per sex per group administered 0, 0.2, 2, 20 and 200 µg/kg/day bisphenol-A by gavage during a pre-mating period of 10 weeks for males and 2 weeks for females and a 2-week mating period. Males and females from each group were randomly paired and co-habited for 2 weeks. Females were also administered the test material during gestation and lactation. F₀ males and females were sacrificed after the mating period and weaning of F₁ pups, respectively. Twenty-five male and female F₁ generation offspring from each group were retained after weaning for assessment of their reproductive capacity. F₁ animals were administered bisphenol-A for a 10-week pre-mating period and a 3-week mating period (see below). Again, females received the test material during gestation and lactation, and male and female parental animals were sacrificed at the same times used for the F₀ generation. Twenty-five male and female F₂ generation offspring from each group were retained after weaning for assessment of sexual maturation. Males and females were administered the test material until they were sacrificed at the age of 7 and 14 weeks, respectively.

For all F₀ and all reared F₁ and F₂ animals, observations and weighings were performed regularly. In addition to determining reproductive capacity, various other parameters were assessed. Learning tests were conducted using a water filled multiple T-maze with 6 male and 6 female F₁ animals per dose group at 5-6 weeks of age. Several reflex assessments were determined in 1 rat per sex per litter until successfully completed. Sexual maturation (vaginal opening and preputial separation) was determined in F₁ and F₂ parent animals, along with anogenital distance (AGD). After sacrifice, all F₀ and F₁ parent animals were subjected to a thorough macroscopic and microscopic examination. In males, this included examination and weighing of the epididymis, prostate and seminal vesicles (including the coagulating gland). Serum testosterone, oestradiol, prolactin, LH, FSH, T₃, T₄ and TSH concentrations were also determined in 6 animals per sex per group from the F₀ and F₁ generations. Seminal vesicles and coagulating gland were weighed and subjected to histological examination. The motility and morphology of sperm in the epididymis was also determined in F₀ and F₁ males. All pups that were not selected for further assessment were sacrificed and also underwent histopathological examination.

In parental animals, no clinical signs of toxicity, nor any effects on body weight gain, food intake or treatment-related deaths were observed in any generation. No effect on behaviour (i.e. performance in learning tests) was observed in F₁ animals. Oestrus cycle, fertility index and the number of implantations in F₀ and F₁ females were not affected by treatment with bisphenol-A. (The mating period for F₁ animals was extended for a week, as at the end of the first week mating was confirmed in only 19/25 females administered 0.2 µg/kg/day, compared to 24/25,

22/25, 23/25 and 21/24 at 0, 2, 20 and 200 µg/kg/day respectively. At the end of the 3-week mating period no significant effect on the fertility index was observed between treated and control animals). No significant differences were observed between bisphenol-A and control animals for the time to preputial separation or vaginal opening. Compared to controls, a statistically significant decrease ($\leq 5\%$) in AGD was seen in F₁ males at 0.2, 20 and 200 µg/kg/day, F₁ females at 20 and 200 µg/kg/day and F₂ males and females at 20 and 200 µg/kg/day. These decreases were not statistically significant when the ratio of the AGD to body weight was determined (the AGD is correlated with body weight). No changes in the motility and morphology of sperm were observed in F₀ and F₁ treated males. No treatment-related changes were observed in any of the serum hormone levels measured. Bisphenol-A had no effect on sexual maturation or the oestrus cycle in F₂ animals and F₂ females, respectively. At necropsy, no treatment-related macroscopic findings or organ weight changes were observed in F₀ and F₁ parental animals.

In the offspring (all live pups up to day 21), no clinical signs of toxicity or effects on body weight gain during lactation were observed in F₁ and F₂ pups. No treatment-related changes were seen in the litter size, survival, sex ratio, AGD and reflex ontogeny. At necropsy, no treatment-related macroscopic findings were observed in F₁ and F₂ pups. Compared to controls, a statistically significant decrease in the absolute (17%) and relative (20%) weight of seminal vesicles (including the coagulating gland) was observed in F₂ males only at 2 µg/kg/day. No other treatment-related changes in organ weight were observed in F₁ and F₂ pups.

The slight ($\leq 5\%$) changes seen in AGD in F₁ and F₂ parental animals are not considered to be toxicologically significant as they are not statistically significant once correlated to body weight. The decrease in seminal vesicle weight at 2 µg/kg/day is also considered not to be toxicologically significant as no statistically significant decrease was observed at 20 or 200 µg/kg/day, histopathological examination revealed no morphological changes in the seminal vesicles and there were no weight changes in associated organs (prostate gland, testis and epididymis). Therefore, in this two-generation study, no parental toxicity or effect on fertility was observed at the low-dose levels employed.

Multigeneration studies

The effects of bisphenol-A on fertility and reproductive performance have been investigated in a comprehensive, good-quality multigeneration study (Tyl et al., 2000). The overall design of this study was based on the OECD two-generation reproduction toxicity study guideline, with additional dose groups and an extension to include the production of an F₃ generation. Groups of 30 male and 30 female Sprague Dawley rats were exposed to bisphenol-A in the diet at concentrations of 0, 0.15, 0.3, 4.5, 75, 750 or 7,500 ppm, which equated to approximately 0 (control), 0.001, 0.02, 0.3, 5, 50 or 500 mg/kg/day, respectively.

Exposure to bisphenol-A began for the F₀ generation at about 7 weeks of age and continued throughout a 10-week pre-breed exposure period, a 2-week mating period (when F₀ animals were mated [one male and one female] within each dose group) and gestation. F₀ males were sacrificed after the F₁ delivery period. Exposure of F₀ females to bisphenol-A continued throughout lactation until weaning (post-natal day 21) when F₀ animals were sacrificed. Selected F₁ animals were similarly mated to produce the F₂ generation and selected F₂ animals were mated to produce the F₃ generation. The same exposure regime was used for F₁ and F₂ animals with direct exposure to bisphenol-A in the diet commencing approximately at post-natal day 21. Selected F₃ animals were exposed to bisphenol-A only for a 10-week period from weaning as they were not mated. For the F₀ generation and retained F₁, F₂ and F₃ animals, clinical signs of

toxicity, body weights and food consumption were reported. Oestrous cycles were monitored in the last 3 weeks of the pre-breed exposure period and during the mating period for all generations. At the necropsy of adult animals, sperm samples were taken for analysis of epididymal sperm number, motility (using a computer assisted sperm motion analysis system) and morphology, testicular-resistant spermatid head counts, daily sperm production, and efficiency of daily sperm production, a number of organs were weighed and selected organs were examined histopathologically. Parameters assessed in the young offspring included litter size, body weights, survival, gross appearance, anogenital distance (AGD) (in F₂ and F₃ offspring only), sexual development and, for animals killed at weaning, gross appearance of organs at necropsy with attention given to reproductive organs.

There was evidence of general toxicity in adults of all generations at 500 mg/kg/day, seen as a statistically significant reduction in body weight gain ($\geq 13\%$ in all generations). An increased incidence of renal tubular degeneration was also seen in F₀, F₁ and F₂ (but not F₃) females at 500 mg/kg. Chronic hepatic inflammation was also seen in both sexes and all generations. These aspects are described in greater detail in Section 4.1.2.6.1; the liver effects are not considered to be treatment-related.

Considering the reproduction-related parameters, there was no effect on mating. Compared to controls, a statistically significant decrease in the average number of live pups per litter was seen at 500 mg/kg/day in all generations on the day of birth (F₁: 11.5 compared to 14.3 for controls; F₂: 10.8 compared to 14.6; and F₃: 10.9 compared to 14.8). These decreases were observed with no effect on post implantation loss or the number of dead pups per litter. No further increase or decrease in the number of post-natal deaths between treated and control groups were seen on day 4. After standardisation of litter sizes on post-natal day 4 the number of pups per litter remained equivalent across all dose groups up to weaning. At necropsy, a statistically significant decrease in absolute (not relative) uterine weight in F₀, F₁ and F₂ parental animals (22-35%) was seen at 500 mg/kg/day. Statistically significant reductions in both absolute (16-34%) and relative (15-34%) paired ovarian weights were seen in F₀, F₁, F₂ and F₃ (absolute only) females at 500 mg/kg/day. A statistically significant increase in paired ovarian follicle count (43%) was seen in F₀ females only at 500 mg/kg/day. No treatment-related effects were observed in reproductive organs of adult animals. Compared to controls, the only changes in sperm endpoints were a statistically significant decrease in epididymal sperm count (18%) in the F₁ generation at 500 mg/kg/day and a statistically significant decrease in daily sperm production (19%) in the F₃ generation at 500 mg/kg/day (with no effect on efficiency of daily sperm production). The effects seen on ovarian follicle counts, epididymal sperm counts and sperm production at 500 mg/kg were not consistent (they were only observed in 1 generation) and are thus considered to be chance findings.

In the offspring, a statistically significant decrease in mean body weight per litter was seen at 500 mg/kg/day on post-natal days 7-21 in F₁, F₂ and F₃ males and females (12-27%) compared to controls. No treatment-related effect was seen on gestational indices, sex ratios, or nipple and/or areola retention in male pups. Compared to controls, statistically significant increases in AGD (which was only measured in F₂ and F₃ animals) were seen on the day of birth at 0.001 (by 3%), 0.02 (3%), 0.3 (3%) and 50 mg/kg/day (4%) in F₂ females only. At 500 mg/kg/day, a statistically significant delay in vaginal patency in females was observed in F₁ (day 33.0 compared to day 30.5 in controls), F₂ (day 34.5 compared to day 31.0) and F₃ (day 33.8 compared to day 31.3) animals. A statistically significant delay in preputial separation was seen in males in F₁ (day 45.8 compared to day 41.9 in controls), F₂ (day 47.9 compared to day 42.1) and F₃ (day 45.2 compared to day 42.1) animals. A statistically significant delay in preputial separation was also

seen at 50 mg/kg/day but only in F₁ males (day 43.6); given that this was seen in only one generation at this dose, it is considered not to be treatment-related, but to be a chance finding.

To summarise, an adverse effect on reproduction was observed in this study; a statistically significant decrease in the average number of live pups per litter at birth was seen in all generations at 500 mg/kg/day. Although this effect was only seen at a dose level at which there was also parental toxicity, it is not clear whether or not this finding could be a secondary consequence of this parental toxicity, or whether it represents a direct effect of bisphenol-A on fertility. A statistically significant decrease was also seen in adult ovarian weights and in mean pup body weights (males and females from postnatal day 7), along with a statistically significant delay in vaginal patency and preputial separation, in all generations at 500 mg/kg/day. However, acquisition of developmental landmarks is dependent on both age and weight (i.e. heavier animals acquire the landmark earlier and lighter animals later), and a statistically significant decrease in body weight gain was seen in males and females of all generations at 500 mg/kg. In addition, if these effects were related to the oestrogenic activity of bisphenol-A, a different pattern of results would have been expected. Thus, a chemical with oestrogenic activity would be expected to hasten the onset of vaginal patency in the offspring of exposed dams, whilst delaying preputial separation (Biegel et al., 1998). Thus, it is considered that the delays in vaginal patency and preputial separation are related to decreases in body weight, and not a direct developmental effect. In addition, all these effects on developmental landmarks were observed in the presence of parental systemic toxicity ($\geq 13\%$ decrease in body weight gain in both sexes along with renal tubule degeneration in the kidneys of females) and are considered a secondary consequence of parental toxicity. The observed statistically significant increase in AGD was neither dose-related nor consistent across generations, being observed only in F₂ females at 0.001, 0.02, 0.3 and 50 mg/kg/day. The magnitude of the increases (0.03 to 0.04 mm) was minimal and no correlating effects of any kind were observed. Thus, the changes in AGD are not considered to be of toxicological significance.

Overall, this study showed 500 mg/kg bisphenol-A causes a reduction in the number of pups per litter. Although this finding was observed in the presence of some parental toxicity, it is not clear whether or not this finding could be a secondary consequence of this parental toxicity, or whether it represents a direct effect of bisphenol-A on fertility. The no effect level for parental toxicity is 50 mg/kg/day (see Section 4.1.2.6.1). Thus, the NOAEL for both parental and reproductive toxicity is 50 mg/kg/day in this well conducted multigeneration study.

Continuous breeding study

The effects of bisphenol-A on fertility and reproductive performance have been extensively studied in CD-1 mice using the test system known as the "Fertility Assessment by Continuous Breeding" (NTP, 1985b). This system involves four successive tasks. Task 1 is a preliminary 14-day toxicity study, conducted so that appropriate dose levels for the subsequent tasks can be selected. Task 2, the continuous breeding phase, involves a 14-week cohabiting phase during which reproductive performance is monitored. In Task 3, an optional "cross-over" mating trial is conducted; control males are mated with high-dose females and high-dose males are mated with control females. This is to determine whether any adverse effect seen in Task 2 is mediated through males or females. In Task 4, the reproductive offspring taken from the Task 2 final litters is assessed. The test substance is administered continuously through Tasks 2, 3 and 4 (except during the Task 3 mating phase).

Bisphenol-A was administered in the diet. Groups of twenty males and females (F₀ generation) were continuously exposed to the test substance at concentrations of 0, 0.25, 0.5 or 1.0% (using default values -see **Table 4.24**, Section 4.1.2.6.1- daily intakes of bisphenol-A are estimated to

have been 0, 300, 600 and 1,200 mg/kg in males, and 0, 325, 650 and 1,300 mg/kg in females) during a one-week pre-mating period and a 14-week mating trial (Task 2). The dose levels were selected on the basis of a preliminary study (Task 1), in which statistically significant reductions in body weight gain (>10%) were seen at dietary bisphenol-A concentrations of 1.25% and above. The control group comprised of forty animals of each sex receiving the diet only. After the pre-mating period, males and females from each group were randomly paired and allowed to cohabit for 14 weeks. During the cohabiting period the reproductive performance was monitored by counting the number of F₁ generation litters produced by each breeding pair and recording on the day of birth the litter size, proportion of live pups, litter size and sex ratio of the pups; all pups were then immediately removed and discarded. All litters produced after the cohabiting period remained with their mothers until weaning on day 21 post partum. The twenty F₀ males and twenty F₀ females from the top dose group (1.0% bisphenol-A) were then mated with twenty control females and twenty control males, respectively. Bisphenol-A was discontinued in the diet during this 7-day cohabitation period and then reinstated for 21 days upon separation of the breeding pairs. A control group of twenty untreated breeding pairs was also included (Task 3). The same reproductive assessment as described for the continuous breeding phase was conducted. Parental animals were sacrificed within 1 week of delivery. A maximum of twenty male and twenty female F₁ generation offspring (from the final litters of the control and high-dose groups in the continuous breeding phase) were retained after weaning for assessment of their reproductive capacity (Task 4). After rearing to sexual maturity, each F₁ female was paired with a F₁ male from the same dose group for 7 days. The resulting litters were evaluated and discarded on the day of birth as described for the litters produced during the F₀ generation cohabitation phase. For all control and high-dose F₀ and all reared F₁ animals, liver, kidneys, adrenals and reproductive organs were weighed and subjected to histopathological examination. In males, sperm analysis (concentration, motility and morphology) was undertaken, and effects on the oestrous cycle assessed in females.

There were no clinical signs of toxicity among F₀ generation animals. In the continuous breeding phase, a statistically significant decrease in maternal body weight was observed after each litter (between 6 and 9%), at the top dose, on postnatal day 0 compared to controls. No effect was observed on maternal postnatal (day 0) body weight following the cross-over mating phase. However, at study termination, a small but statistically significant decrease in body weight (4%) was observed in treated females compared to controls. No adverse effects on body weight gain were observed in treated males. An adverse effect on fertility was observed in the continuous breeding experiment and cross-over mating experiment. In the continuous breeding phase, a statistically significant decrease compared to controls was observed in the number of litters produced per pair (4.5 and 4.7 compared to 5.0 for controls), litter size (6.5 and 9.8 compared to 12.2 for controls) and the number of live pups per litter (6.3 and 9.7 compared to 12.1 for controls) in the high and mid-dose group. The litter size reductions occurred across all matings and the magnitude of all these decreases were dose-related. No effects on fertility were observed in the low-dose group. A statistically significant decrease in litter size (controls: 11.4, treated males: 9.1, treated females: 5.9) and number of live pups per litter (controls: 11.3, treated males: 8.4, treated females: 5.5) were observed in the cross-over mating. In the continuous breeding phase, a statistically significant decrease in live pup weight (6%) on postnatal day 0 was observed in females at the top dose after adjustment for litter size, including live and still births. In the continuous breeding phase a small but statistically significant decrease in body weight gain (4%) was only observed in treated females at study termination. No effect was observed on the sex ratio in the F₁ generation.

In the F₁ litters used in the cross-over breeding experiment, post natal (day 0) pup weights were significantly increased in males (9-11%) and in females (8-10%) in the mid- and high-dose

groups compared to controls. These increases were no longer evident in either sex at 21 or 74 days of age. Deaths among F₁ generation were observed during lactation (day 0-21) and post weaning (day 21-74). At the top dose there were only 8 litters that had at least one male and one female for the mating phase, and therefore only 11 breeding pairs at the top dose compared to 19-20 breeding pairs in the control, low-dose and mid-dose groups. In those litters selected for mating deaths had been observed in 6%, 4%, 14% and 38% animals up to day 74 in the control, low-dose, mid-dose and high-dose groups, respectively. It is not known how many animals of this total died during lactation. However, it does raise the possibility that there may be potential effects on pups due to exposure to bisphenol-A via the milk. In the F₁ generation, bisphenol-A treatment had no effect on the fertility index, litter size, number of live pups per litter, sex ratio or mean pup weights at birth.

At necropsy of the F₀ generation (controls and top dose group only), treatment-related effects were seen at the highest dose level; for both sexes relative liver weight was increased about 28% and relative combined kidney/adrenal weight increased 10-16% compared to controls, and relative seminal vesicle weight and proportion of motile sperm were decreased 19% and 39% compared to controls, respectively. Histopathological changes were reported in the liver and kidney of Task 3 (F₀) animals. Minimal multifocal necrosis of the liver was observed in 4/38 control males and 11/38 control females. Slight multifocal necrosis was observed in one control female. In treated animals, minimal multifocal necrosis was observed in 3/19 males and 3/19 females, slight necrosis in 9/19 males and 5/19 females and moderate necrosis in 3/19 males and 3/19 females. Multinucleated giant hepatocytes (slight to moderately severe/high) were observed only in treated animals: 11/19 males and 4/19 females. Centrilobular hepatomegaly in the liver was observed only in treated males; the severity was minimal, slight, moderate and moderately severe/high in 1/19, 7/19, 3/19 and 5/19 animals, respectively. Tubular cell 'nuclear variability' (slight to moderate) was observed in the kidney of treated animals only; 6/19 males and 12/19 females. Large microcalculi in the kidney were observed in females only; minimal in 1/38 controls and minimal, slight and moderate in 1/19, 4/19 and 2/19 treated animals, respectively. No histological change that was graded severe/high was seen in the liver or kidney of any animal. An amplification of spontaneous tubular and interstitial lesions normally observed in these mice was also observed in bisphenol-A treated animals. No histological changes were observed in male and female reproductive organs and no effect was observed on the oestrous cycle. Overall, the signs of general systemic toxicity were not marked in this study and therefore the effects on fertility are not considered to be a consequence of parental toxicity.

At necropsy of the F₁ generation, treatment-related effects of similar magnitude were generally observed in males and females; compared to controls, increased relative liver weights (6-29%) and kidney/adrenal weights (13-20%) were observed in all treated groups. In males, a statistically significant decrease in relative right epididymis weight (11%, 16% and 18%) was observed in all treated groups, compared to controls. Left testis/epididymis weights were significantly decreased by 10% at the mid dose and 9% at the high dose, and seminal vesicle weight was significantly decreased by 28% at the top dose. A statistically significant decrease in sperm motility in the mid-dose group only was not considered treatment-related, but a chance finding. Similar histopathological changes to those observed in the F₀ generation were also observed in males and females of the F₁ generation; the presence or increased incidence of multifocal necrosis and multinucleated giant hepatocytes in the liver and cortical tubular dilatation and tubular casts in the kidney at the low dose and above compared to controls. At the top dose; multifocal "mineralization" of hepatic cells was observed in females, along with microcalculi and "mineralization" of renal cells in both males and females. No histological changes were observed in the male and female reproductive organs.

In this study, adverse effects on fertility, namely a reduction in litter size and number of live pups per litter, were observed in each litter from the F₀ generation in the continuous breeding experiment at approximately 600 mg/kg and above, and at the only dose level tested in the crossover breeding experiment, approximately 1,200 mg/kg. A treatment-related decrease in the number of litters produced was also observed at 1,200 mg/kg during the continuous breeding phase. These effects were observed in the absence of significant parental toxicity. No effect on fertility was observed at 300 mg/kg, though no histopathology was conducted on these animals. In the F₁ generation at 300 mg/kg the only effect observed was a statistically significant decrease in epididymis weight of 11%. Histological examination was conducted on all F₁ animals, and the only effects observed were toxicity to the liver and kidney at all doses. No adverse effect on fertility was observed in the F₁ generation up to approximately 1,200 mg/kg, which might have been expected in view of the observed effects on fertility in the F₀ generation. Nevertheless, the absence of effects following the single F₁ mating does not detract from the reproducible results across the 4-5 litters produced by each F₀ generation pair. Therefore, overall, an adverse effect on fertility has been observed with bisphenol-A at approximately 600mg/kg and above. At 300 mg/kg no adverse effects on fertility were observed, though a decrease was seen in F₁ epididymis weight. This effect is considered treatment-related as the magnitude of the decrease was dose-related. Although this was the only effect observed on reproductive organs at 300 mg/kg, the health significance of this finding is not clear. Therefore, taking a cautious approach a no effect level could not be identified in this study due to the epididymis weight change observed at 300 mg/kg.

In a study which was briefly reported (Bolon et al., 1997), the results being presented in summary form only, the effect of bisphenol-A treatment on ovarian follicle count was determined in stored ovaries of the CD-1 mice from the NTP (1985b) continuous breeding study presented above. One ovary from each of 10 females per dose group was examined in F₀ mice from Task 3 and F₁ mice from Task 4 (see above). Each ovary was embedded in a paraffin block, sliced to produce approximately 400 sections and every 10th section was stained and the number of "small", "growing" and "antral" follicles determined. No significant differences were observed in the mean number of small, growing or antral follicles in ovaries from F₀ females administered 1,300 mg/kg bisphenol-A (Task 3), and F₁ females administered 325, 650 and 1,300 mg/kg (Task 4), compared to controls. Thus, at dose levels up to 1,300 mg/kg bisphenol-A did not affect ovarian follicle count.

Continuous breeding study

The effects of bisphenol-A on fertility and reproductive performance in CD-1 mice when administered by subcutaneous implant were also investigated in the 'Fertility Assessment by Continuous Breeding' test system (NTP, 1984). The protocol for this 4-task study was identical to that described previously (see above).

Bisphenol-A was administered via subcutaneous implant. Groups of twenty males and females (F₀ generation) were continuously exposed to the test substance at concentrations of 0, 25, 50 or 100 mg during a one-week pre-mating period and 17-week mating trial (Task 2). Dose levels could not be varied with body weight and due to difficulties experienced in preparing accurate bisphenol-A dose levels and release from the implants, implant doses of 0, 25, 50 or 100 mg gave a total release of approximately 0, 10, 20 and 40 mg bisphenol-A over 18 weeks, respectively. The dose levels were selected on the basis of a preliminary study (Task 1), in which an increase in mean reproductive tract weight in females was observed from 6.25 mg (corresponding to a total release of 1.6 mg bisphenol-A over 2 weeks). The control group comprised of forty animals of each sex. Task 3 and Task 4 were not conducted as the study was

terminated on the completion of Task 2 due to the technical problems experienced with the subcutaneous implants. All control and high-dose animals were sacrificed at the end of the continuous breeding experiment and liver, brain, pituitary and reproductive organs were weighed. No detailed histopathological examination was conducted.

There were no clinical signs of toxicity or adverse effects on body weight gain in parental animals. However, several animals in each treatment group expelled their implants through cutaneous lesions that developed directly over the implants or at the site of incision. When this occurred, animals were re-implanted with the original dose; several animals received new implants on three different occasions. No effect was observed on the fertility index, number of live pups per litter, sex ratio and the group mean live pup weights. At necropsy, no effect was observed on parental organ weights.

Although technical problems with the method of administration were encountered, no effects were observed on fertility. However, no parental toxicity was observed and no histological examination was conducted, limiting the value of this study.

Other related studies

The effect of bisphenol-A on preimplantation was investigated in mouse embryos (Takai et al., 2001). Superovulation was induced in female B6C3F1 mice, which were then mated with males of the same strain. Two-cell embryos were obtained 40 hours after induction of superovulation by flushing the oviducts. Embryos were then cultured for 48 hours in 0, 1nM or 100 μ M bisphenol-A. Seven embryos, now blastocytes, were transferred to each uterine horn of recipient ICR mice on day 3 of pseudopregnancy, which was induced by mating ovarian hyperstimulated females with vasectomised ICR males in a parallel procedure. The presence of a vaginal plug was used to identify day 1 of pseudopregnancy. Pups (randomly culled to six per litter where appropriate) were delivered and weaned by the recipient mother. Pups were weighed on postnatal day 21.

The rate of *in vitro* development of two-cell embryos to blastocytes was 62.1% (272/438) in control cultures after 48 hours. Compared to controls, a statistically significant increase in development was seen at 1 nM bisphenol-A (16%) with a decrease at 100 μ M (46%). A statistically significant increase in degenerated embryos (not quantified) was also seen at 100 μ M. Blastocytes that developed in the presence of bisphenol-A appeared to be morphologically normal, and no significant difference was seen in the number of cells per blastocyst. Compared to controls, no significant effect was seen on the number of pups per litter, sex ratio or pup body weight at birth, following pre-implantation treatment with bisphenol-A. A statistically significant increase in pup body weight was observed on post-natal day 21 at 1 nM (39%) and 100 μ M (34%).

Overall, in view of the nature of the exposure in this study, which is not relevant to humans, it does not add significantly to the overall database and no conclusions can be drawn from it.

In a poorly reported sperm abnormality study, which was available in abstract form only, male C₃H/He mice received 5 daily intraperitoneal injections of 0 or 85 mg/kg bisphenol-A (Bond et al., 1980). It was reported that no morphological changes were detected between the sperm of treated and control animals. No further details are available.

exposure period. A statistically significant decrease in body weight gain during gestation, an increase in the duration of gestation, and a decrease in litter size on days 0, 1 and 4 of lactation were observed in the positive control group. A slight decrease in sperm production and cauda epididymal sperm concentration was also observed in F₁ males in the positive control group. In conclusion, the effects of bisphenol-A previously reported by Sharpe et al. (1996) at 1.0 ppm were not reproduced in this study, and no effects were observed at the additional dose levels of 0.01, 0.1 and 10 ppm.

Since the Sharpe et al. (1996) study, this group has reported a temporal downwards shift in the normal range of testes weights in control animals in their laboratory (Sharpe et al., 1998). Sharpe et al. (1998) state that although the decrease in testis weight in controls over time is unexplained it followed a permanent change in water supply to the animal facility. While Sharpe remains confident in the validity of the original study he does state that "we now consider that biological factors, of which we are unaware and for which we have not controlled, have the potential to exert developmental effects on testis weight which are at least as great as the maximum effect that can be induced by the addition of a potent oestrogen (DES) to the mother's drinking water during pregnancy and lactation." On this basis, the inability of Cagen et al. (1999a) to reproduce the Sharpe et al. (1996) result suggests that the effect seen on testis weight in Sharpe et al. (1996) may not have been due to an effect of bisphenol-A but to uncontrolled factors.

In a well reported study (Kwon et al., 2000), the effects of bisphenol-A on pubertal development and reproductive functions were investigated. Groups of 8 time-mated Sprague Dawley rats were administered 0, 3.2, 32 or 320 mg/kg bisphenol-A in corn oil by gavage from day 11 of gestation to postnatal day 20. An additional dose group of 8 animals served as a positive control and received 15 µg/kg DES for the same period. Dams were sacrificed when pups were weaned (post-natal day 21) and selected organ weights determined. F₁ males were killed on post-natal day 180 and reproductive organ weights were determined (testes, epididymides, ventral and dorsolateral lobes of the prostate and seminal vesicles). For F₁ females, 1-3 pups/litter were sacrificed on post-natal day 10, the brains removed and the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) determined. The volume of the SDN-POA was determined as it has been reported by Nagao et al. (1999) that sexual behaviour has been closely associated with the size of the SDN-POA; reductions in volume cause loss in sexual behaviour. Pubertal development (day of vaginal opening and first ovulation) was determined in remaining F₁ females, along with oestrous cyclicity from approximately 4 months of age. At approximately 6 months of age the lordosis (curvature of the lumbar and cervical spine) behaviour was evaluated in 1-2 F₁ females/litter. Remaining F₁ females were sacrificed on post-natal day 180 and ovaries and uteri examined by light microscopy along with ventral prostates in males.

In treated dams, no effect was observed on the number of live pups per litter or body weight during pregnancy, lactation or at termination compared to controls. In F₁ females, no effect was observed on body weight, the volume of the SDN-POA, pubertal development, oestrous cyclicity or lordosis behaviour. In F₁ males, no effect was seen on body or reproductive organ weights. No treatment-related effects were observed in F₁ animals of either sex on microscopic examination. Compared to controls, the only effects observed with DES were a statistically significant increase in liver weight (17%) in dams at termination, a statistically significant increase in SDN-POA volume in F₁ females (approximately 39%), and irregular oestrous cyclicity in F₁ females. Therefore, no adverse effects on pubertal development or reproductive function were observed in this study at dose levels of 3.2, 32 or 320 mg/kg bisphenol-A.

In a poorly reported study, which is available in abstract form only, pregnant Sprague Dawley rats were administered 0, 0.005, 0.05, 0.5, 5 or 50 mg/l bisphenol-A in the drinking water from day 2 of gestation until pups were 21 days old (Gould et al., 1998a; Liaw et al., 1998). Assuming

that a female rat drinks 20 ml water a day and weighs 0.175 kg, daily intakes of bisphenol-A are estimated to have been 0, 0.0006, 0.006, 0.06, 0.6 and 6 mg/kg. DES was administered as a positive control at 0.05 mg/l. Dam body weights, organ weights and uterine implant sites were examined post nately on day 21. Female offspring were examined for adverse effects on puberty, oestrous cycle pattern, and hypothalamo-hypophyseal regulation of luteinizing hormone. The only effect reported in dams was an increase in relative kidney weight of unstated magnitude in females at the top dose. No effect was observed on litter size or sex ratio. In female offspring, no effect was observed on age or body weight at vaginal opening, age of first ovulation or subsequent oestrous cyclicity. For DES a decrease in body weight gain, relative ovary weight, food consumption, weight gain during gestation, and serum luteinizing hormone concentrations were observed in dams. In the offspring, vaginal opening occurred significantly earlier and at a lighter body weight. No effect was observed on ovulation. No further details are available. In conclusion, bisphenol-A did not exhibit developmental toxicity in this low-dose study.

In a well reported dietary study (General Electric, 1976c), pairs of mating F₀ CD rats were fed bisphenol-A in the diet at 0, 1,000, 3,000 and 9,000 ppm throughout their mating period and up to day 21 post partum. The F₁ animals (15 per sex per dose) were then used in a 90-day feeding study and fed bisphenol-A in the diet at identical parental dose levels. Bisphenol-A in the diet at 0, 1,000, 3,000 and 9,000 ppm, corresponded to mean dose levels of 0, 68, 206 and 671 mg/kg in F₁ males and 0, 75, 228 and 699 mg/kg in F₁ females. Routine haematology, biochemical and urinalysis were performed monthly. Following necropsy, microscopic examination was performed on animals in the top dose group only. Prior to commencement of the 90-day study, day 21 post partum, statistically significant decreases in F₁ body weight gain of 7 and 12% were observed relative to controls, at 3,000 and 9,000 ppm, respectively. No effect on body weight gain was observed at 1,000 ppm. At the end of the 90-day study, statistically significant reductions in mean body weight of 11% and 24% in males, and 17% and 22% in females, were observed in the mid- and high-dose groups, respectively, relative to controls. Food consumption was reduced by 14% in males at the top dose and $\geq 12\%$ in females from the mid-dose and high-dose in comparison to controls. No treatment-related changes were observed at necropsy or following microscopic examination, in males or females at the top dose. In conclusion, there were no effects at approximately 70 mg/kg in this study. At higher doses the only effect observed was an adverse effect in body weight gain, but it is not clear if this decrease is the result of palatability or a toxic effect.

Using the same protocol, the study was repeated with 0, 100, 250, 750 and 1,000 ppm bisphenol-A in the diet (General Electric, 1978). This resulted in mean doses of 0, 7, 17, 34, 51 and 70 mg/kg in males, and 0, 8, 20, 39, 60 and 82 mg/kg in females. No treatment-related effects were observed in body weight gain or food consumption. At necropsy, no treatment-related changes were observed. In conclusion, there were no adverse effects in animals at about 70 mg/kg.

In a drinking water study (Rubin et al., 2001), groups of 6 female Sprague Dawley dams received 0, 0.1 or 1.2 mg/kg bisphenol-A from day 6 of pregnancy to weaning. The offspring were sacrificed at various time points and "genital tracts" examined microscopically. The authors state that at all time points, offspring were selected from as many different litters as possible from each group. Male offspring were sacrificed at 3 (no. of pups = 12) and 5 months (no. of pups = 18) of age, and female offspring at 8 (no. of pups = 12) and 12-16 months (no. of pups = 34). Effects on anogenital distance were also determined in animals sacrificed during the neonatal period (12 males and 12 females). In female pups, vaginal cytology was also determined for 18 consecutive days at 4 and 6 months of age (in 23, 18 and 28 offspring from the

0, 0.1 and 1.2 mg/kg groups, respectively) and the day of vaginal opening recorded. In addition, 8 female offspring from each dose group were ovariectomised and killed 3 months later, at which time luteinizing hormone (LH) levels were determined.

A statistically significant increase in pup body weight gain was observed for combined male and female weights at 0.1 and 1.2 mg/kg on postnatal days 4, 7, 11 and 22, compared to controls. Animals were not weighed separately by sex on these days. On days 11 and 22, animals exposed to 0.1 mg/kg were heavier than those exposed to 1.2 mg/kg. Statistically significant increases in body weight gain were observed in females only, from day 28. On days 87 and 110, the increase in bodyweight gain in females at 0.1 mg/kg was statistically significant when compared to females at 0 and 1.2 mg/kg. These increases in bodyweight gain were not quantified.

Bisphenol-A did not affect the mean number of pups per litter, sex ratio, day of vaginal opening or anogenital distance. No macroscopic abnormalities were observed in genital tract tissues at any time during the study. Vaginal cytology demonstrated effects on the oestrus cycle in the offspring of animals exposed to 1.2 mg/kg; only 21% and 23% of these animals exhibited regular oestrus cycles at 4 and 6 months, respectively. The authors report that the pattern of non-regular oestrous cyclicity varied in individual females and was not easily defined. No effect on oestrus cycle was seen in the offspring of animals exposed to 0.1 mg/kg, compared with controls. In ovariectomised animals, a statistically significant decrease in LH levels (19%) compared to controls was observed only at 1.2 mg/kg.

In conclusion, bisphenol-A treatment produced an increase in pup body weight gain at 0.1 and 1.2 mg/kg. However, this increase was not dose-related and when sexes were examined separately, was statistically significant only in females. At 1.2 mg/kg, an adverse effect on the oestrus cycle was observed, although no distinct pattern in disruption was observed. A statistically significant decrease in LH levels in ovariectomised females was also seen at 1.2 mg/kg.

In a series of experiments investigating how sampling strategy can influence the outcome of studies investigating endocrine activity (Elswick et al., 2001a), time mated Sprague Dawley rats were administered bisphenol-A in the drinking water at 0, 0.005, 0.05, 0.5, 5 or 50 mg/l from day 2 of gestation to the end of weaning (post-natal day 21). The authors estimated daily intakes of bisphenol-A to have been approximately 0, 0.001, 0.01, 0.1, 1 or 10 mg/kg/day. This study was conducted in two blocks of animals separated by 4 months and resulted in 13-16 pregnant dams per dose group. Two males per litter from the initial block of animals, and 1 male per litter from the subsequent block of animals, were retained until 6 months of age. These males were then sacrificed and weights of organs of the reproductive tract were measured, including ventral prostate weight. A histopathological examination was also performed on ventral prostates.

In the initial block, no treatment-related effects were observed on ventral prostate weights. In the subsequent block, compared to concurrent controls, statistically significant increases of approximately 19, 15, and 8% at 0.01, 1 and 10 mg/kg/day bisphenol-A were observed. It is reported that these statistically significant increases remained when the data for both blocks were combined (data not shown). No treatment-related microscopic findings were observed in either block at necropsy. Although an increase in ventral prostate weight was observed in the second block, this was based on only 1 pup per litter and was not dose-related. Furthermore, the authors point out that the control mean prostate weight was considerably lower in the second block (0.387 g) compared to the first (0.517 g), and the standard error of the mean was approximately twice that of the first block (0.174 g compared to 0.092 g). These concerns over the different control mean values and large variability remained when the data for the two blocks were combined. The authors also point out that ventral prostate weight was not correlated with

terminal body weight. A historical control base for control ventral prostate weights was not available for the laboratory. No information was provided on other organ weights of the reproductive tract. The authors felt that the large intra-litter variability of ventral prostate weight affects the ability to interpret the results. Overall, this investigation of sampling strategy suggests that the number of pups sampled may influence the outcome of results. In view of this, and given the concerns raised by the authors in relation to the intra-litter variability of prostate weights, no reliable conclusions can be drawn from this study in relation to the effect of bisphenol-A.

In a dietary study, available as an abstract only (Fritz and Lamartiniere, 1999), the effect of bisphenol-A on the male reproductive tract was investigated. Male Sprague Dawley rats were exposed to 0, 2.2 and 23.1 $\mu\text{g}/\text{kg}/\text{day}$ bisphenol-A from “conception” to day 70 post partum. The authors report that “lifetime” exposure to bisphenol-A resulted in significantly reduced body weights from days 1-70 postpartum. Compared to controls, no significant effect was seen on the sex ratio, anogenital distance (in both sexes), age of testes descent, dorsolateral and ventral prostates and testes weights in bisphenol-A-treated animals. It was reported that a dose-related increase in seminal vesicles and epididymis weight was seen in bisphenol-A-treated animals. A dose-related decrease in sperm density and motility was reported with bisphenol-A, though this decrease was not significant. A significant increase in androgen receptor protein levels was reported at 23.1 $\mu\text{g}/\text{kg}/\text{day}$ bisphenol-A only. No further information is available. Due to the limited information provided no reliable conclusions can be drawn from this study.

In a study reported as an abstract only (Piersma et al., 1998), several chemicals including bisphenol-A were tested in a reproduction/developmental toxicity screening assay (OECD Guideline 421). In addition to the test guideline protocol, reproductive hormones were assessed and histological examinations performed on the reproductive organs of parents and pups. The authors report that all the tested chemicals showed one or several reproductive toxic effects; infertility, superovulation, preimplantation loss, resorptions, phenotypic feminization, reduced pup weight or testicular pathology. No further details are available. The limited details from this poorly reported study mean no reliable conclusions can be drawn from this data in relation to bisphenol-A.

A postnatal developmental study using the s.c route of administration is available (Nagao et al., 1999). Groups of 30-31 male and female Sprague Dawley pups were given s.c. injections of 0 or 300 mg/kg bisphenol-A in corn oil daily on postnatal days 1 to 5. On postnatal day 21, 5 male and 5 female control and treated pups were sacrificed and histopathological examination of the testes, epididymides, prostates, seminal vesicles, ovaries and uteri undertaken. At 12 weeks of age, males and females (number not reported) were mated with untreated animals to evaluate their reproductive function. Sperm-positive females were sacrificed on day 13 of gestation and the number of implants and live and dead embryos determined. Females that were not sperm-positive after 14 days cohabitation with males were sacrificed approximately 6 days later. Masculine sexual behaviour was evaluated in the treated males; animals were housed with an ovariectomised female (brought into sexual activity by treatment with estradiol benzoate) and the number of mounts, intromissions and ejaculations, and latency to first mount, intromission and ejaculation recorded over a one hour period. Five control, and five treated, males and females were then sacrificed and histopathological examination of the testes, epididymides, prostates, seminal vesicles, ovaries and uteri undertaken. A further five control males and five treated males were sacrificed, a histopathological examination of the brain conducted and the region of the SDN-POA identified and its volume calculated.

No clinical signs of toxicity were observed in the treated pups. All male and female rats treated with bisphenol-A showed normal reproductive function and the day of preputial separation and testicular descent in males, and day of vaginal opening in females, was comparable to controls.

these questions (Gupta, 2001) and has addressed all of the points raised. However, one key concern was the statistical analysis applied to the measurement of AGD in the offspring. Although clarification on this point was provided, there remain concerns that an inappropriate statistical analysis may have been applied to the data related to AGD.

In a study to investigate potential strain differences in the sensitivity of responses to oestrogens, Spearow et al. (1999) investigated the effects of exposure to 17 β -oestradiol in various strains of mice. Although not directly involving an investigation of bisphenol-A, this study has been included because of its potential relevance to the interpretation of the results of studies with bisphenol-A in different mice strains. Groups of male CD-1, C57BL/6J, C17/J1, S15/J1, E/J1 and CN/J1 mice (approx 16/dose) received silastic implants containing 0, 2.5, 10, 20 or 40 μ g 17 β -oestradiol at 22-23 days of age. Animals were killed when 43 days old, testes removed and weighed, and light microscopy undertaken on the testes to determine spermatogenic index and the percentage of seminiferous tubules showing sperm maturation to the elongated spermatid stage of development.

Statistically significant differences in testes weight in control animals were seen between strains, heavier testes weights being observed in CD-1 and S15/J1 mice. Compared to controls, testes weight decreased in all strains of mice, though testes weight was affected by strain and dose. Strain accounted for more variation in testes weight than dose of 17 β -oestradiol. C57BL/6J mice were the most sensitive (2.5 μ g 17 β -oestradiol produced a 60% decrease) and CD-1 mice the most resistant (40 μ g produced a 30% decrease). Testicular histology also revealed strain differences. Low to moderate doses of 17 β -oestradiol were reported to have "obliterated" spermatogenesis. In contrast, very little inhibition of spermatogenesis was seen in CD-1 mice with increasing doses of 17 β -oestradiol. Compared to controls, statistically significant decreases were also seen in the % of seminiferous tubules with elongated spermatids. Again strain differences were evident. Gamete maturation to the elongated spermatid stage of development was completely eliminated in C57BL/6J mice at 10 μ g. In contrast, an abundance of normally maturing spermatids was found in the testes of all CD-1 mice at 20 μ g. The results of this study show marked genetic differences in sensitivity to the effects of 17 β -oestradiol on the male reproductive tract.

4.1.2.9.4 Summary of toxicity to reproduction

No human data are available. Bisphenol-A has been shown to have endocrine modulating activity in a number of *in vitro* and *in vivo* screening assays. The potency of this activity in these assays generally ranged from 3 to 5 orders of magnitude less than that of oestradiol. No significant oestrogenic activity has been observed with bisphenol-A glucuronide *in vitro*. The available data also indicate that there is a marked strain difference in the response to bisphenol-A in rats. However, there are no data to indicate the underlying reasons for such differences.

It should be noted that these studies investigating endocrine modulating activity are essentially screening tests and many of them employ experimental protocols, which have not undergone any international validation. However, the first phase of the validation of the uterotrophic assay in OECD indicates that this model is robust and reproducible across laboratories (Kanno et al., 2001). Whilst this assay can be used to identify oestrogenic activity and can be an early screening test, its use for risk characterisation purposes is still a matter for discussion. In addition, many of the available *in vivo* studies have used parenteral routes of exposure, the relevance of which are uncertain with respect to relevant routes of human exposure.

The effects of bisphenol-A on fertility and reproductive performance have been investigated in three good quality studies: two generation and multigeneration studies in the rat, and a continuous breeding study in the mouse. Although no effect on fertility was seen in the rat two-generation study, low-dose levels were employed (0.2-200 µg/kg/day). In the multigeneration study, an effect on fertility (reduction in litter size) was seen in all three generations at the top dose of 500 mg/kg. Although this effect was seen only at a dose level causing parental toxicity (a reduction in body weight gain (>13%) in both sexes and renal tubule degeneration in females only), it is not clear whether or not the finding could be a secondary consequence of parental toxicity, or a direct effect of bisphenol-A. In the light of this uncertainty, and given that an adverse effect on fertility has been seen in the mouse, it is prudent to assume that bisphenol-A may be having a direct effect on fertility in this study. No effects on fertility were seen at 50 mg/kg. The continuous breeding study in the mouse provides some evidence that bisphenol-A can cause adverse effects on fertility. In the F₀ generation, no effects on fertility were seen at 300 mg/kg/day, but at dose levels of approximately 600 mg/kg/day and above, reductions in the numbers of litters produced, litter size and numbers of live pups per litter were observed in each of the 4-5 litters produced. These effects were observed in the absence of significant parental toxicity. In contrast, no adverse effects on fertility were observed in the single litter tested at each dose level from the F₁ generation. A statistically significant and dose-related decrease in epididymal weight was seen at all doses in the F₁ generation. However, the significance of this finding is uncertain given that there was no effect on fertility in this generation, and where an adverse effect on fertility was seen (in the F₀ generation); there was no effect on epididymal weight. In spite of the uncertainty, the epididymis is associated with sperm transport and storage, and any reduction in the weight of this organ would be of concern. Although no effects were seen in the two-generation rat study, it is not considered suitable for use in the risk characterisation due to the low-dose levels employed (0.2-200 µg/kg/day). However, this data combined with that for the multigeneration study does provide a comprehensive dose-response range for effects on fertility in the rat. In addition, comparing the rat and mouse data it can be seen that similar toxicological profiles were observed for effects on fertility; effects were seen in both species at approximately the same dose level (i.e. reductions in litter size at 500 mg/kg/day in the rat and at 600 mg/kg/day in the mouse). Consequently, it is considered that the NOAEL of 50 mg/kg/day identified in the rat multigeneration study is also likely to produce no adverse effects in mice for which there is only a LOAEL of 300 mg/kg/day (for a small but statistically significant decrease in epididymal weight in F₁ males only). Therefore, the NOAEL of 50 mg/kg/day identified from the multigeneration study will be used for risk characterisation purposes, in relation to effects on fertility.

No evidence that bisphenol-A is a developmental toxicant was observed in standard development studies in rats and mice. In rats, a maternal LOAEL and foetal NOAEL of 160 and 640 mg/kg/day, respectively, were identified. In mice, maternal and foetal NOAELs were 250 and 1,000 mg/kg/day, respectively. In a rat multigeneration study, a statistically significant decrease in mean pup body weight gain, with concomitant delays in the acquisition of developmental landmarks (vaginal patency and preputial separation) was observed at 500 mg/kg on post-natal days 7-21 in males and females of all generations (F₁-F₃). These decreases in pup body weight gain and delays in development were seen in the presence of maternal toxicity. No maternal toxicity and no treatment-related effects were reported in the offspring of animals exposed to 50 mg/kg. However, additionally, some studies have investigated the potential of bisphenol-A to affect male reproductive tract development in rats and mice. Conflicting results have been reported in these studies, in both species. In mice, adverse effects on male reproductive tract development (an increase in prostate weight in two studies and a reduction in epididymis weight in one study) have been reported at dose levels in the range 2-50 µg/kg.

However, these results have not been reproducible in two other studies, one of which included additional dose levels, and using larger group sizes compared with those used in either of the two studies showing effects. It is noted that in contrast to the studies showing effects on the male reproductive tract, the studies that did not find an effect of bisphenol-A also did not show any effects of DES. Furthermore, no functional changes in reproductive parameters or reproductive organ development were observed in a recent rat two-generation study using similar dose levels. The reasons for the differences in these results are unclear. Recent evidence from one study suggests that there are differences in the sensitivity of different mice strains to the effects of oestrogens, which may be related to the selection of strains for large litter size. This difference in sensitivity may in part explain some of the differences in the current database, although the relevance of these rodent strain differences in relation to human health remains unclear.

Overall, in standard developmental studies in rodents, there is no convincing evidence that bisphenol-A is a developmental toxicant. However, the available and apparently conflicting data from studies conducted using low doses (in the $\mu\text{g}/\text{kg}$ range) do raise uncertainties. Overall, the majority of EU member states felt that the studies reporting effects at low doses could not be dismissed. However, the member states disagreed on how these studies should be used, if at all, in the risk characterisation for this endpoint. The disagreements were based on differing views about the uncertainties surrounding the reproducibility of the findings and their biological significance, if any, to human health.

This issue was referred to the Competent Authorities in June 2001. It was agreed unanimously by the Competent Authorities that further work was required to resolve the uncertainties surrounding the potential for bisphenol-A to produce adverse effects on development at low doses. In addition, it was agreed that a provisional NOAEL of 50 mg/kg/day for developmental effects, derived from the rat multi-generation study, should be used in the risk characterisation in the interim, whilst awaiting the outcome of further testing, with the aim of identifying those scenarios which are clearly of concern irrespective of the outcome of the further testing.