参考３

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ＥＦＳＡ意見書

Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on a request from the Commission related to

Bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials

Question N° EFSA-Q-2003-191

Adopted on 23 June 2005 by written procedure

SUMMARY

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) has been asked to re-evaluate bis(2-ethylhexyl)phthalate (DEHP) for use in the manufacture of food contact materials.

Previously, a Tolerable Daily Intake (TDI) of 0.05 mg/kg bw was set by the Scientific Committee for Food (SCF), based on the endpoint of peroxisome proliferation in rodent liver. There is now a scientific consensus that liver peroxisome proliferation in rodents is not relevant for human risk assessment. The critical effects of DEHP relate to reproduction. From the several studies available, the critical observations were as follows.

A recent well conducted 2-generation reproduction study of DEHP in rats has documented effects on reproductive performance and fertility in the F0 and F1 parental animals. Substance-induced signs of adverse developmental toxicity were noted in the progeny of the F0 and F1 parents from 340 mg/kg bw/day onwards. The No Observed Adverse Effect Level (NOAEL) for reproductive performance and fertility was 340 mg/kg bw/day and for developmental toxicity 113 mg/kg bw/day, respectively.

A multigeneration reproductive assessment in which DEHP was administered to rats in the diet has also been recently performed. From this study, a NOAEL of 4.8 mg/kg bw/day for testicular toxicity and developmental toxicity can be derived.

Based on the current literature on DEHP testicular toxicity, the Panel allocated a TDI of 0.05 mg/kg bw, based on a NOAEL of 5 mg/kg bw/day, and making use of an uncertainty factor of 100.

The limited available data on DEHP concentration in foods and diets in UK and Denmark were used to provide an estimation of dietary exposure. In the UK, mean and high (97.5th percentile) intakes of DEHP from dietary sources were estimated to be respectively 0.15 and 0.3 mg/person/day in the adult population (equivalent to 2.5 and 5 µg/kg bw/day) considering a 60 kg adult.
In a Danish study, DEHP estimated mean exposure ranged from 0.19 to 0.3 mg/day, i.e. 2.7 to 4.3 µg/kg bw/day, considering a 70 kg adult. Based on the highest concentration of DEHP determined, exposure at high percentiles was estimated as 1.1 mg/day equivalent to 15.7 µg/kg bw/day.
In another Danish study, the main dietary sources of exposure were estimated to be leaf crops (53%), root crops (13%), milk (12%) and fish (10%). The total daily oral intake at the regional level (Denmark) was estimated to be 4.5 µg/kg bw/day in adults, 26 µg/kg bw/day in children aged 1 to 6 years, and 11 µg/kg bw/day in children aged 7 to 14 years.
Based on the detection limit, intake from infant formulae would be less than 10 µg/kg bw/day in infants of less than 6 months and 4 µg/kg bw/day in infants of more than 6 months. For infants of more than 6 months, ready-to-use baby foods were also taken into account and the exposure was therefore estimated to be 23.5 µg/kg bw/day.

The Panel noted that exposure to DEHP from food consumption is in the range of the TDI. There are, however, a number of other sources which contribute to the overall human exposure to DEHP. The Panel recommends that improved estimates of exposure to DEHP from all sources along with their relative importance should be provided in order to decide what proportion of the TDI can be allocated to food contact materials alone.

KEY WORDS :
Bis(2-ethylhexyl) phthalate, Di-(2-ethylhexyl) phthalate, DEHP, food contact materials, CAS n° 117-81-7, Ref N° 74640.

BACKGROUND

DEHP may be present in food, either due to migration from food contact materials containing DEHP or due to its widespread presence as an environmental contaminant which can be found in air, water, soil and food. DEHP was evaluated by the Scientific Committee for Food (SCF) in 1994 when a Tolerable Daily Intake (TDI) for use in food contact materials was established based on the then most sensitive end-point of peroxisome proliferation in rodent liver. There is a scientific consensus that liver peroxisome proliferation in rodents is not a relevant endpoint for human risk assessment (IARC, 1995). The Panel has therefore been asked to re-evaluate DEHP for use in food contact materials.

TERMS OF REFERENCE

The Commission asks EFSA to re-evaluate di-(2-ethylhexyl) phthalate (DEHP) for use in the manufacture of food contact materials.

ASSESSMENT

1. Chemistry

Identification of the substance

CAS-No.: 117-81-7

EINECS-No.: 204-211-0

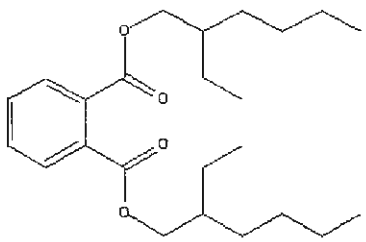
IUPAC name: Bis(2-ethylhexyl)phthalate

FCM Ref N°74640

Synonyms: 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester, Bis(2-ethylhexyl) 1,2-benzenedicarboxylate, Bis(2-ethylhexyl) o-phthalate, Bis(2-ethylhexyl) phthalate, DEHP, Di(2-ethylhexyl) phthalate

Molecular formula $C_{24}H_{38}O_4$

Structural formula:



Molecular weight: 390.6

Purity/impurities, additives

Purity: ≥ 99% (w/w)

Impurity: mainly other phthalates

Additives: none

Physico-chemical properties

Physical state: oily liquid

Melting point: - 55 °C

Boiling point: 230°C at 1013 hPa

Relative density: 0.980-0.985 g/cm^3 at 20 °C

Vapour pressure: 3.4x 10^{-5} kPa at 20 °C

Water solubility: 0.003 mg/l at 22 °C

Partition coefficient n-octanol/water: log K_{ow} 7.5

2. Use

DEHP is present in a large number of end products some of which are available for consumer use. The vast majority of DEHP use, more than 80%, goes to plasticizing of PVC or other polymers. The DEHP plasticized polymeric material has consumer and industrial uses such as flooring, sealants, and paints.

3. Exposure via food

Contamination of food by DEHP can occur during processing, handling, transportation and packaging of food and via "secondary" food storage articles. During processing, food may be contaminated from PVC tubing and other process equipment containing DEHP. For example, transfer of DEHP from tubes to milk during different operations in dairies may occur (Wildbrett, 1977, Castle *et al.*, 1990). DEHP may be used in lubricants in the food processing industry e.g. in slaughter-houses. Contamination of food products with DEHP can occur via polymer and non-polymer components of food packaging materials, for instance printing ink used for flexible food packaging, glues used for paper and plastics, in aluminium foil-paper laminates and closure seal in bottles (MAFF, 1996). According to MAFF (1995), DEHP is often present in paper and board packaging and at generally low concentrations (typically less than 10 mg/kg) in foods packaged in paper and board.

No data on the levels of DEHP in food in the EU attributable to migration from food contact materials have been submitted by the industry. Exposure assessment based on analytical determination of the total concentration of DEHP in samples of foods or diets allow to assess overall dietary exposure, from packaging material and other sources.

An assessment of exposure to phthalates was performed based on the analysis of stored samples from the Total Diet Study conducted in the UK in 1993. Concentration data were combined with food consumption data from the National Diet and Nutrition Study of British Adults. Since phthalates are fat soluble, only those 10 food groups which make a major contribution to dietary fat intakes were selected for analysis. Among these, carcass meat, eggs, poultry and milk were analysed for individual concentrations of phthalates since these four food groups accounted for approximately 85% of the estimated dietary intake of total phthalates. Mean and high (97.5 percentile) intakes of DEHP from these sources were estimated to be respectively 0.15 and 0.3 mg/person/day in the adult population equivalent to 2.5 and 5 μg/kg bw/day for a 60 kg adult (MAFF, 1996).

In a Danish study (Petersen and Breindahl, 2000), DEHP was analysed in 29 different meals collected by test persons during 24 hours. Results were normalised to a daily diet of 10 MJ of energy and to a body weight of 70 kg in order to estimate total daily exposure. Among the 29 samples, 11 were above the limit of determination. The estimated mean concentration of DEHP in the diet varied according to the values assigned to the 18 samples which were under the limit of determination. Estimated mean exposure therefore ranged from 0.19 to 0.3 mg/day, i.e. 2.7 to 4.3 μg/kg bw/day considering a 70 kg adult. Based on the highest concentration of DEHP determined, exposure at high percentiles was estimated as 1.1 mg/day equivalent to 15.7 μg/kg bw/day.

A further Danish assessment of DEHP total dietary exposure based on estimated and measured concentrations in environmental compartments using the European Union System for the Evaluation of Substances (EUSES, a computer modeling program) was reported recently (Müller et al, 2003). The main dietary sources of exposure were estimated to be leaf crops (53%), root crops (13%), milk (12%) and fish (10%). These high contributions of vegetables to oral DEHP exposure may indicate that the exposure estimates for the UK, as given above, might be underestimates of the actual exposure because the UK figures were only based on food from animal sources.
For Denmark, the total daily oral intake at the regional level was estimated to be 4.5 μg/kg bw/day in adults, 26 μg/kg bw/day in children aged 1 to 6 years, and 11 μg/kg bw/day in children aged 7 to 14 years.

The highest local daily oral intake was estimated as 20 μg/kg bw/day in adults, 133 μg/kg bw/day in children aged 1-6 years, and 40 μg/kg bw/day in children aged 7-14 years. It must be underlined that more than 80% of these maximum exposure values derive from the highest estimated value of exposure via the local environment and consequently are not related to the diet itself. The contribution from dermal and inhalation exposure was negligible. Furthermore, EUSES, the computer modeling program which has been used for these intake estimates is a conservative one and the obtained values, especially for the local daily intakes, are not representative of the possible exposure via food contact materials.
In the same study (Müller *et al*, 2003), exposure from infant formulae was estimated based on two scenarios: an infant of less than 6 months weighing 5.5 kg and ingesting 900 g/day of formulae and an infant of more than 6 months weighing 8 kg and ingesting 525 g/day of formulae. Based on the maximum measured concentration among 11 commercial products (0.06 mg/kg of wet weight), this would lead to an exposure of 9.8 μg/kg bw/day in the infant of less than 6 months and 3.9 μg/kg bw/day in the infant of more than 6 months. For infants of more than 6 months, exposure from ready-to-use baby foods were also taken into account, considering the daily consumption of one jar of 250 g containing 0.63 mg DEHP/kg of wet weight (the maximum measured concentration among 11 commercial products). Total exposure from infant formulae and ready to use baby foods in infants aged more than 6 months was therefore estimated as 23.5 μg/kg bw/day.

Calculation of daily exposure to DEHP by the use of biomarkers in human urine was reported recently (Koch et al, 2003, 2004). However due to large differences with the previous results obtained with a similar methodology (Anderson *et al.*, 2001), the biomarker approach for the estimation of the overall intake of DEHP in the general population is under discussion (CSTEE opinion, 2004).

4. Toxicological evaluation

Introduction

The Panel did not carry out a new extensive risk assessment but took cognisance of the previous evaluations by the SCF and in particular considered the more recent DEHP Risk Assessment Report (RAR), prepared for the European Union Existing Substances Regulation, 793/93, 2004 (Annex 1), and the comments of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) on this RAR (Annex 2), in order to determine the most significant toxicological end-point for risk assessment. Based on this information, the Panel focused on the most sensitive toxicological end-points for the evaluation of DEHP, taken from the reproduction/developmental toxicity studies with this substance.

The SCF expressed its opinion on DEHP in December 1994 (SCF, 1997) as follows:

> "The Committee considered the suggestion that a safety factor of less than 100 might be applied in establishing a TDI for DEHP because of apparent differences in sensitivity with regard to peroxisomal enzyme induction and metabolism between subhuman primates, man and the laboratory rodents. It was not convinced, however, that the evidence was sufficiently weighty to accept this suggestion.
> The Committee therefore retained as a matter of prudence a safety factor of 100 in establishing a TDI of 0.05 mg/kg bw/day for DEHP based on the NOEL for peroxisomal proliferation."

Studies considered by the AFC Panel

Available data demonstrate that exposure to DEHP affects both fertility and reproduction in rodents of both sexes and also produces developmental effects in offspring. In males, DEHP induces severe testicular effects, including testicular atrophy. Developing male rats have been found to be more

sensitive to DEHP-induced testicular toxicity than sexually mature animals (Gray and Butterworth, 1980; Sjöberg *et al.*, 1985, 1986). The onset of the lesion in young animals is also more rapid. Irreversible effects occur in rats exposed prenatally and during suckling (Arcadi *et al.*, 1998).

The following studies on reproduction and development toxicities considered by the Panel for the determination of a NOAEL which could be used as a basis for a TDI calculation are summarized below. Further details on these studies are given in Annex 1 (Risk Assessment Report) and 2 (CSTEE opinion).

Testicular effects have been observed in several repeated dose toxicity studies in rats, mice, and ferrets, (Gray *et al.*, 1977; NTP, 1982; ICI, 1982; Gray *et al.*, 1982; CMA, 1984a,b; Lamb *et al.*, 1987; Ganning *et al.*, 1990; Eastman Kodak, 1992; Moore, 1996; Poon *et al.*, 1997; Moore, 1997; Wolfe and Layton, 2003). In addition, minor effects were observed in hamsters exposed to DEHP and more severe effects were induced by monoethylhexylphthalate (MEHP) (Gray *et al.*, 1982). In the available studies marmosets were not sensitive to DEHP (Kurata *et al.*, 1995; 1996; 1998). No studies on testicular effects in rabbits are available.

The lowest identified NOAEL for testicular effects in the diet corresponding to 3.7 mg/kg bw/day in rats, is based on a high incidence (7/9) of Sertoli cell vacuolation at the next higher dose level (500 mg/kg equivalent to 37.6 mg/kg bw/day) in a 13-week guideline study (Poon *et al.*, 1997).

A 2-generation reproduction study of DEHP in rats (Schilling *et al.*, 1999) has documented effects on reproductive performance and fertility in the F0 and F1 parental animals at 1088 mg/kg bw/day. Substance-induced signs of adverse developmental toxicity were noted in the progeny of the F0 and F1 parents from 340 mg/kg bw/day onwards. The NOAEL for reproductive performance and fertility was 340 mg/kg bw/day and for developmental toxicity 113 mg/kg bw/day, respectively.

Wolfe and Layton (2003), studied the multigenerational reproductive toxicity of DEHP in Sprague-Dawley rats. The conclusions of this study were as follows:

- the NOAEL for testicular toxicity was 100 mg/kg (equivalent to approximately 8 mg DEHP/kg bw/day in the F0 animals and approximately 5 mg DEHP/kg bw/day in the F1 and F2 animals).
- the LOAEL for testicular toxicity was set at 300 mg/kg (equivalent to approximately 23 mg DEHP/kg bw/day in the F0 animals and 14 mg DEHP/kg bw/day in the F1 and F2 animals).
- macroscopic pathological findings in male accessory sex organs other than testes (mentioned above) were also present at this dose level and at higher doses.
- the NOAEL for toxicity to fertility was 1000 mg/kg (equivalent to approximately 77 mg DEHP/kg bw/day in the F0 animals, and 48 and 46 mg DEHP/kg bw/day in the F1 and F2 animals respectively.
- the NOAEL for developmental toxicity was 100 mg/kg (equivalent to approximately 8 mg DEHP/kg bw/day in the F0 animals and approximately 5 mg DEHP/kg bw/day in the F1 and F2 animals)
- the NOAEL for effects not related to reproductive toxicity in adult animals was 300 mg/kg (equivalent to approximately 23 mg DEHP/kg bw/day in the F0 animals, and 14 mg DEHP/kg bw/day in the F1 and F2 animals) and was based on reductions in body weights.
- Sertoli cell vacuolation was observed in the control group as well as in the 1000 mg/kg and 7500 mg/kg F1 males. It was not observed in the 10000 mg/kg animals with diffuse seminiferous tubule atrophy. In the 7500 mg/kg males, Sertoli cell vacuolation was observed in seminiferous tubules without atrophy. This vacuolation was similar to that observed in the control group males. This observed vacuolation of the Sertoli cells resulted from distortion during fixation and processing of the tissues according to a pathology working group. This distortion could have obscured any minimal toxic effects that may have been present.

The methodology used in this study to a large extent complies with OECD Guideline 416. , This study appears to be more robust than those underpinning the previous NOAELs based on reproductive toxicity.
A NOAEL of 5 mg/kg bw/day for testicular toxicity and developmental toxicity can be derived from this study.

CONCLUSIONS

Based on all the available toxicological evidence, the Panel concludes that effects on reproduction and development are the most sensitive end-points on which to base the risk assessment. The Panel considers also that the Wolfe and Layton study (2003) was more robust than those underpinning the previous NOAELs based on reproductive toxicity, and that a NOAEL of 5 mg/kg bw/day related to testicular toxicity can be derived from it.

Based on the above statement, the Panel allocated a TDI of 0.05 mg/kg bw, based on a NOAEL of 5 mg/kg bw/day and making use of an uncertainty factor of 100.

The limited available data on DEHP concentration in foods and diets in UK (1993) and Denmark (2003) were used to provide an estimation of dietary exposure. In the UK, mean and high (97.5th percentile) intakes of DEHP from dietary sources were estimated to be respectively 0.15 and 0.3 mg/person/day in the adult population (equivalent to 2.5 and 5 μg/kg bw/day considering a 60 kg adult).
In a Danish study, (Petersen and Breindahl, 2000), the DEHP estimated mean exposure ranged from 0.19 to 0.3 mg/day, i.e. 2.7 to 4.3 μg/kg bw/day, considering a 70 kg adult. Based on the highest concentration of DEHP determined, exposure at high percentiles was estimated as 1.1 mg/day equivalent to 15.7 μg/kg bw/day.
In another Danish study (Müller *et al*, 2003), the main dietary sources of exposure were estimated to be leaf crops (53%), root crops (13%), milk (12%) and fish (10%). The total daily oral intake at the regional level (Denmark) was estimated to be 4.5 μg/kg bw/day in adults, 26 μg/kg bw/day in children aged 1 to 6 years, and 11 μg/kg bw/day in children aged 7 to 14 years.
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The Panel noted that exposure to DEHP from food consumption is in the range of the TDI. There are, however, a number of other sources which contribute to the overall human exposure to DEHP. The Panel recommends that improved estimates of exposure to DEHP from all sources along with their relative importance should be provided in order to decide what proportion of the TDI can be allocated to food contact materials alone.

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ANNEX 1

Extracts from Risk Assessment Reports on DEHP

Consolidated Final Reports
(dated September 2001, October 2003, February and March 2004)

1.Mutagenicity and carcinogenicity

The possible genotoxic effect of DEHP has been thoroughly investigated in several different short-term tests. The major metabolites of DEHP, mono(ethylhexyl)phthalate (MEHP) and 2-ethylhexanol (2-EH), have also been examined. Most of the studies are performed according to GLP principles and are comparable to guideline studies.

The results have been negative in the majority of the *in vitro* and in *vivo* studies on DEHP, MEHP and 2-EH for detection of gene mutation, DNA damage, and chromosomal effects. The more conclusive positive results were obtained on cell transformation, induction of aneuploidy, and cell proliferation. These test systems are, however, also sensitive to several non-genotoxic substances such as tumour promoters and/or peroxisome proliferators. Taken together all the results, both negative and positive, DEHP and its major metabolites are considered to be non-mutagenic substances.

No relevant human data on the carcinogenicity of DEHP are available. In experimental animals, the only inhalation study available (Schmezer et al., 1988) is on hamsters, and is considered inadequate for risk assessment as only one dose of DEHP was used in the study. Also, the dose of DEHP used was very low and the Maximum Tolerable Dose (MTD) was not reached as no signs of any toxicological effects were reported.

Following oral exposure, the carcinogenicity of DEHP has been investigated in numerous animal studies. Four long-term studies (Moore 1996 & 1997; NTP, 1982a,b) performed in rats and mice are of good quality and are considered adequate for evaluation of carcinogenicity of DEHP in experimental animals. DEHP shows clear evidence of hepatocarcinogenicity in both sexes of rats and mice in the four different studies. The increase in tumour incidence in the liver was statistically significant and a dose-response relationship exists. In rats, an increase in the incidence of mononuclear cell leukaemia (MCL) was also observed, significant in males of the Moore study only. The lowest-observed-adverse-effect level (LOAEL) and the NOAEL for tumour induction in rats (both liver tumours and MCL) were established as 2500 ppm (147 mg/kg bw/day for males) and 500 ppm (29 mg/kg bw/day for males) DEHP in the diet, respectively (Moore 1996). In mice the LOAEL and the NOAEL for induction of liver tumour is 1500 ppm (292 mg/kg bw/day for males) and 500 ppm (98 mg/kg bw per day for males) DEHP in the diet, respectively (Moore 1997). However, these carcinogenic effects on rodent species are linked to peroxisome proliferation and are no longer considered as relevant to humans.

2.Toxicity to reproduction and development

Available data demonstrate that exposure to DEHP affects both fertility and reproduction in rodents of both sexes and also produces developmental effects in offspring. In males, DEHP induces severe testicular effects, including testicular atrophy. Developing male rats have been found to be more sensitive to DEHP-induced testicular toxicity than sexually mature animals (Gray and Butterworth, 1980; Sjöberg et al., 1985, 1986). The onset of the lesion in young animals is also more rapid. Irreversible effects occur in rats exposed prenatally and during suckling (Arcadi et al., 1998).

MEHP is believed to be the active metabolite of DEHP affecting testes and reproductive functions both *in vivo* and *in vitro*. The possible role of other metabolites is, however, not fully elucidated.

Testicular effects have been observed in several repeated dose toxicity studies in rats, mice, and ferrets, (Gray et al., 1977; NTP, 1982; ICI, 1982; Gray et al., 1982; CMA, 1984a,b; Lamb et al., 1987; Ganning et al., 1990; Eastman Kodak, 1992; Moore, 1996; Poon et al., 1997; Moore, 1997; Wolfe and Layton, 2003). In addition, minor effects were observed in hamsters exposed to DEHP and more severe effects were induced by MEHP (Gray et al., 1982). In the available studies marmosets were not sensitive to DEHP (Kurata et al., 1995; 1996; 1998). No studies on testicular effects in rabbits are available.

The lowest identified NOAEL for testicular effects in the diet corresponding to 3.7 mg/kg bw in rats, is based on a high incidence (7/9) of Sertoli cell vacuolation at the next higher dose level (500 ppm equivalent to 37.6 mg/kg body weight) in a 13-week guideline study (Poon et al., 1997). At the highest dose level (5 000 ppm equivalent to 375.2 mg/kg body weight) also a high incidence of atrophy of the seminiferous tubules with complete loss of spermatogenesis was found in addition to a higher incidence of cytoplasmic Sertoli cell vacuolation (9/10).

Both *in vivo* and *in vitro* experiments have demonstrated that the Sertoli cell is one of the the main targets of DEHP/metabolite-induced testicular toxicity, producing subsequent germ cell depletion (Poon et al., 1997, Arcadi et al., 1998, Li et al., 1998). Sertoli cells provide both physical support as well as secreting factors that are required for germ cell differentiation and survival and may also influence the signal transduction mechanism between these cells. Findings from an *in* vitro study have also shown that phthalate-induced changes in germ cell-Sertoli cell adhesion may occur during early postnatal development in rats.
The Sertoli cells are also the principal testicular site for the action of follicle stimulating hormone (FSH), a hormone which is essential for initiation and maintenance of spermatogenesis. In pubertal animals FSH is more important than in adults due to the initiation of spermatogenesis. The relatively rapid onset of phthalate-induced testicular injury suggests a specific mechanism of action on Sertoli cells. The four distinct mechanistic hypotheses which have been proposed to explain testicular injury implicate zinc-dependent enzyme activity, hormonal status, metabolic interactions, and FSH-dependent pathways. Some research results suggest that the FSH-stimulation of Sertoli cells is decreased by DEHP (Davis et al, 1994).

Wolfe and Layton (2003), studied the multigenerational reproductive toxicity of DEHP in Sprague-Dawley rats. The methodology used in this study to a large extent complies with OECD Guideline 416. At the time being, this study appears to be more robust than those underpinning the previous NOAELs based on reproductive toxicity. The conclusions of this study were as follows: The NOAEL for testicular toxicity was 100 ppm (equivalent to approximately 8 mg DEHP/kg bw/day in the F0 animals and approximately 5 mg DEHP/kg bw/day in the F1 and F2 animals) and was based on decreased absolute and/or relative testis weights noted at 7500 (F1, F2 and F3 males) and 10000 ppm (F0 and F1 males), macroscopic pathological findings (small or aplastic testes) noted at 300 (3/45 non-mating F1 males, 1/21 non-mating F2 males), 1000 (3/25 non-mating F2 males), 7500 (7/10 mating F1 males, 10/30 non-mating F1 males, 9/10 mating F2 males, 11/20 non-mating F2 males) and 10000 ppm (2 or 3 of 10 F0 males, 10/10 mating F1 males, 21/21 non-mating F1 males), and microscopic pathological findings (testis seminiferous tubular atrophy) noted at 300 (1/10 F1 males), 7500 (all F1 and F2 males) and 10000 ppm (all F1 males, 2 or 3 of 10 F0 males).
Microscopic and/or macroscopic pathological findings and organ weight changes (absolute and/or relative) were also noted in the epididymis, seminal vesicles and prostate. Thus, macroscopically small and/or aplastic epididymis were noted at 300 (2/45 non-mating F1 males, 1/21 non-mating F2 male), 1000 (3/25 non-mating F2 males), 7500 (1 or 2 of 10 mating F1 males, 9/10 mating F2 males, 7/20 non-mating F2 males) and 10000 ppm (21/21 non-mating F1 males). Small seminal vesicles were noted at 300 (1/45 non-mating F1 males) and 7500 ppm (1/10 mating F1 males), and small prostate was noted at 1000 (3 or 4 of 43 F1 non-mating males), 7500 (1/10 F0 mating males, 1/10 F1 mating males, 1/30 non-mating F1 males) and 10000 ppm (1 or 2 of 21 non-mating F1 males). Microscopic

pathological changes in the epididymis including sloughed epithelial cells/residual bodies and aspermia/oligospermia were found in F0 and F1 males at 7500 and 10000 ppm. Organ weight changes were noted in the epididymis (F1 and F2 males at 7500 ppm; F0 and F1 males at 10000 ppm), seminal vesicles (F2 males at 7500 ppm; F1 males at 10000 ppm) and prostate (F1 males at 7500 and 10000 ppm). At 7500 ppm changes in epididymis and prostate weights were also noted in F3 males.

The LOAEL for testicular toxicity was set at 300 ppm (equivalent to approximately 23 mg DEHP/kg bw/day in the F0 animals and 14 mg DEHP/kg bw/day in the F1 and F2 animals). At this dose level macroscopic pathological findings in testes (aplastic and/or small) were noted in animals of both generations (F1 and F2), and microscopic pathological findings in testes (seminiferous tubular atrophy) were noted in 1/10 F1 males.

Macroscopic pathological findings in male accessory sex organs other than testes (mentioned above) were also present at this dose level and at higher doses. Atrophy of seminiferous tubules in the testis was also observed at 100 ppm. However, this effect on the testis at 100 ppm was only noted in one animal in one generation (F1) and in the absence of any accompanying findings. At 300 ppm additional parameters and several generations of animals were affected. Effects on male accessory sex organs other than the testis could also be taken into consideration at this dose level. Therefore the LOAEL was set at 300 ppm.

The NOAEL for toxicity to fertility was 1000 ppm (equivalent to approximately 77 mg DEHP/kg bw/day in the F0 animals, and 48 and 46 mg DEHP/kg bw/day in the F1 and F2 animals respectively) and was based on impaired fertility and litter parameters noted at 7500 ppm and above, and various decreased sperm end-points noted at 7500 (F1-, F2-, F3 males) and 10000 ppm (F0-, F1 males). None of the F1 mating pairs produced offspring at 10000 ppm (this finding was correlated with no spermatids present in the testes of F1 males at 10000 ppm). At 7500 ppm statistically significant decreases in the pregnancy indices were noted for the F2 mating pairs (8/17 vs. 17/17). The total number of males per litter was decreased at 10000 ppm in the F1a litters (26%) and at 7500 ppm across all F1 litters combined (F1a+F1b+F1c) (approximately 20%). The total number of F1a pups per litter was decreased at 7500 ppm (22%) and at 10000 ppm (21%). The total number of pups per litter across all F1 (F1a+F1b+F1c) litters combined (18%) was also decreased at 7500 ppm. There was also an increase in the number of cumulative days to deliver the F1a litter for F0 animals at 10000 ppm.

The NOAEL for developmental toxicity was 100 ppm (equivalent to approximately 8 mg DEHP/kg bw/day in the F0 animals and approximately 5 mg DEHP/kg bw/day in the F1 and F2 animals) and was based on the fact that the testicular effects were much more severe in the F1 and F2 generations than in F0, indicating the developmental phases as sensitive to the testicular toxicity of DEHP.

The NOAEL for effects not related to reproductive toxicity in adult animals was 300 ppm (equivalent to approximately 23 mg DEHP/kg bw/day in the F0 animals, and 14 mg DEHP/kg bw/day in the F1 and F2 animals) and was based on reductions in bodyweights noted in both sexes at 7500 (F1, F2 animals) and 10000 ppm (F0, F1 animals), absolute and/or relative organ weight changes noted at 1000 ppm and above (increased liver: 1000 ppm and above; increased kidneys: 1000 ppm and above; increased adrenals: 10000 ppm; increased pituitary: 10000 ppm), and microscopic pathological findings noted at 1000 ppm and above (liver hypertrophy: 1000 ppm and above; cortex vacuolisation of the adrenals: 7500 ppm and above; dilation of the tubules and mineralization in the kidneys occasionally associated with chronic pyelonephritis: 1000 ppm and above). Microscopic pathological findings in the adrenal glands were also indicated in F1 animals at 1000 ppm (no further data).

Sertoli cell vacuolation was observed in the control group as well as in the 1000 ppm and 7500 ppm F1 males. It was not observed in the 10000 ppm animals with diffuse seminiferous tubule atrophy. In the 7500 ppm males, Sertoli cell vacuolation was observed in seminiferous tubules without atrophy. This vacuolation was similar to that observed in the control group males. This observed vacuolation of the Sertoli cells resulted from distortion during fixation and processing of the tissues according to a pathology working group. This distortion could have obscured any minimal toxic effects that may have been present.

Some other results have also shown that DEHP and MEHP may exert a direct effect on Leydig cell structure and function as determined by testosterone output and also that DEHP and MEHP produce similar changes both *in vivo* and *in vitro* both in Leydig cells and in Sertoli cells (Jones et al., 1993). It

is plausible that malfunction of Leydig cells affects the physiology of adjacent Sertoli cells. Findings also indicate that different phthalates may exert changes that are unique to one or common to both cell types.

Developing and prepubertal rats have been found to be much more sensitive to exposure to DEHP than adults (Gray and Butterworth, 1980; Sjöberg et al., 1985c, 1986b, Arcadi et al., 1998). The younger animals respond to a much lower dose or produce a more serious lesion with a comparable dose on a mg/kg bw/day basis. In some instances, the onset for the production of the lesion is also more rapid. Exposure of rats prenatally and during suckling has produced irreversible effects at dose levels inducing only minimal effects in adult animals at the same exposure levels (Arcadi et al., 1998). In the 90-day study conducted by Poon et al., (1997), rats were dosed at 32-37 days of age and reach sexual maturity at approximately 70 days. Since the rats were only immature for part of the dosing (33-38 of 90 days) and the study did not discern an age-dependent effect, the results of this study are considered relevant for both young and adult males. Furthermore, humans are exposed to DEHP for their whole lifetime, i.e prenatally to death, via the environment, consumer products and medical devices. In addition, occupational exposure may occur.

DEHP has been observed to decrease the levels of zinc in the testes and the levels of testosterone in rodents (e.g. Oishi & Hiraga, 1980; Oishi, 1986; Agarwal et al. 1986a,b). Zinc-deficient and low protein diets have been shown to enhance the susceptibility to the gonadotoxic effect in adult males (Agarwal et al., 1986a). Co-administration of zinc did not, however, prevent the atrophy (Oishi and Hiraga, 1983): DEHP may interfere with gastrointestinal absorption of zinc rather than causing a direct effect on the testes. Co-administration of testosterone or the vitamin B_{12} derivative adenosylcobalamin with DEHP to male rats appears to prevent testicular injury (Parmar et al., 1987; Oishi, 1994). A low protein diet has been shown to enhance the susceptibility to the gonadotoxic effect (Tandon et al., 1992).

Based on the available data, which varies in both the study design and number of animals included, testicular effects have been demonstrated in both male rodents and non-rodents: rat (NOAEL = 3.7 mg/kg bw/day) mouse (NOAEL = 98.5 mg/kg b.w/day), and the ferret (LOAEL = 1200 mg/kg bw/day) (Poon et al., 1997, Moore, 1997; Lake et al., 1976). In addition, minor effects were observed in hamsters exposed to DEHP and more severe effects were induced by MEHP (Gray et al., 1982). In the available studies with marmosets testicular toxicity has not been observed after treatment with DEHP (Kurata et al., 1995; 1996; 1998, 2003). The reasons for the differences in study results have been suggested to concern toxicokinetic considerations and altered zinc homeostatsis. Moreover, other factors such as animal age, study design, animal model selection also have to be considered. For instance, marmosets which are new-world monkeys vary in their metabolic pathways and capacities and are not as closely related to humans as are cynomolgus and Rhesus monkeys (old-world monkeys) (Caldwell, 1979a,b). The use of marmoset monkeys rather than neonatal macaque apes was recommended because Sertoli cell replication is negligible neonatally in the latter species (Sharpe et al, 2000). Experimentally, by modulating Sertoli cell replication with a gonadotropin-releasing hormone antagonist, the authors also compared marmosets and rat (Wistar). They showed that marmosets and neonatal rats are similar. However, perinatal rats, unlike infantile and adult marmosets, lack replication. Although Sertoli cell replication seems to be more similar in man and marmosets, and the efficiency of spermatogenesis is poor in marmosets as well as in humans, there is, however, no evidence that the results obtained in prepubertal rats are not relevant for man or that use of adult marmosets should be preferred. Other mechanism(s) and/or factors that cause the observed differences in the DEHP-induced testicular toxicity have not, however, been fully substantiated. Based on the available animal data it is not possible to definitely conclude the relevance of these differences in humans. However, it is known from the limited toxicokinetic data in humans, that MEHP, the testicular toxicant, is formed following exposure to DEHP. Therefore, DEHP-induced testicular effects observed in animal studies are considered relevant for humans.

Effects on male fertility have been observed in mice and rats. In mice, DEHP adversely affects the number of fertile matings. In a continuous breeding study an oral NOAEL of 0.01% in the diet (20

mg/kg bw/day) was identified for fertility (Lamb et al, 1987). In rat, the oral NOAEL for body weight, testis, epididymis, and prostate weights and for endocrine and gonadal effects in male rats was considered to be 69 mg DEHP/kg bw/day in a 60 day study (Agarawal et al, 1986a,b). In a complementary crossover mating trial, females given 0.3% DEHP were more seriously affected than males. None of the females were able to produce pups: the fertility index was 0 (0/16) for females and 20% (4/20) for males compared to 90% for the control group (18/20).

There are indications that oral dosing of DEHP causes hypo-oestrogenic anovulatory and polycystic ovaries in adult female rats (Davies et al., 1994). There also are indications that DEHP treatment alters the oestrous cycle and causes concentration changes of testosterone and oestradiol as shown in ovary cell cultures with cells obtained from cycling female rats administered DEHP *in vivo*. No NOAEL or LOAEL has, however, been established for these effects.

Effects on developmental toxicity have been observed in several studies. The rat has been shown to be the most sensitive species to DEHP-induced malformations. Irreversible testicular damage in the absence of obvious effects on the dams was shown in male pups exposed *in utero* and during suckling at very low dose levels (LOAEL = 3.5 mg/kg bw/day) (Arcadi et al., 1998). Their mothers were exposed to DEHP in drinking water at doses from about 3 mg/kg bw/day during pregnancy and lactation. Alterations in kidneys tended to ameliorate with time; the testicular lesions did, however, not appear to reduce with growth. Histopathological changes were still observed at termination of the study, 8 weeks after delivery. The same levels of exposure did not produce similar effects in adult male rats.

In mice, DEHP is embryotoxic and teratogenic at oral dose levels below those producing observable evidence of toxicity to the dams:
In a continuous breeding study in mice, an oral NOAEL for maternal and developmental toxicity of 600 and 20 mg/kg bw/day respectively were identified, (Lamb et al., 1987). In a developmental toxicity study an oral NOAEL was identified as 44 mg/kg bw/day. The NOAEL for maternal toxicity was 91 mg/kg bw/day (NTIS, 1984; Tyl et al., 1988). In a dietary 2-generation study in mice, the maternal NOAEL was 0.05% DEHP (91 mg/kg bw/day) and the NOAEL for F1 offspring 0.025% (48 mg/kg bw/day) (NTIS 1988).

Both *in vivo* and *in vitro* study results indicate that DEHP can interfere with endocrine function and also influence sexual differentiation (e.g. Gray et al., 1999, Jones et al, 1993). Due to the effects on the Leydig cells as measured by a decreased testosterone output, it cannot be excluded that DEHP may exert an antiandrogen effect. The results of recently performed *in vivo* studies in rats exposed to DEHP or DBP support the hypothesis that exposure to phthalates may be provoked by an antiandrogen mechanism (Gray et al., 1999, Mylchreest and Foster, 1999). The present data in experimental animals are of concern for humans.

Because of uncertainties with regard to the actual dosing in the study by Arcadi et al, (1998), which has given the lowest effect level, the NOAEL of 4.8 mg/kg bw/day (Wolfe and Layton, 2003) is selected for risk characterisation in humans.

ANNEX 2

Extracts from CSTEE opinions on the results of the Risk Assessment of DEHP

1. CSTEE opinion on the results of the Risk Assessment of DEHP (09 January 2002)

Mutagenicity

DEHP has been studied extensively for its genotoxic effects in a wide range of test systems, both *in vitro* and *in vivo*. The majority of these studies did not reveal any activity. The CSTEE supports the RAR that the data on genotoxicity do not suggest a classification of DEHP.

Carcinogenicity

DEHP has been shown to induce hepatocellular tumours in six experiments in rats and 2 experiments in mice after long-term dietary administration. However, the mechanism involved in DEHP carcinogenicity in rodents (activation of PPAR-α) is not relevant to humans (IARC, 1995). There are some indications that DEHP may induce Leydig cell tumours and mononuclear cell leukaemia in rats. However, there are deficiencies in the reporting (Leydig cell tumours) and the relevance for humans is not readily apparent (mononuclear cell leukaemia). Taken together, DEHP does not fulfil the criteria for classification as a carcinogen.

Repeated dose toxicity

Only repeated dose studies of DEHP by the oral route are adequate for risk assessment. Critical organs in laboratory animals are the liver, kidney and testis. Repeated dose treatment of rats and mice leads to hepatomegaly due to increased hepatocyte proliferation and peroxisome proliferation. These effects are mechanistically linked to the presence of the peroxisome proliferator-activated receptor alpha (PPAR-α) in the liver. There are marked species differences in the PPAR-mediated effects of DEHP, so that the hepatotoxic effects of DEHP noted in rodents are not judged to be relevant for humans. The RAR assigns a NOAEL for kidney toxicity of 28.9 mg/kg/d in males and 36.1 mg/kg/d in females from a 2-year study in rats (Moore, 1996) based on increased absolute and relative kidney weight. The CSTEE is in agreement with these values, although the critical effect for risk characterisation is testicular toxicity.

Testicular toxicity has been noted in a number of repeated-dose experiments in rats and mice. In a 90-day study in rats, dose-dependent Sertoli cell vacuolation was demonstrated with a NOAEL of 3.7 mg/kg/d (Poon et al., 1997).

The CSTEE has previously used this NOAEL value in its opinion of 26/27 November 1998 and recommends to use this value also in the present context.

The RAR on DEHP uses *i.a.* effects on the kidney and the testis observed in rats as critical endpoints for extrapolation to humans. Two studies point to the possibility that rodents may not be ideal models for these endpoints. In 12-15 month old marmosets exposed to DEHP at levels of 100, 500 and 2500 mg/kg bw/day for 13 weeks, no testicular or other effects were observed (Kurata *et al.*: Toxicol. Sci. 42, 49-56, 1998). However, the possibility exists that younger animals may be more sensitive towards testicular toxicity (as in rodents) than the animals used in this study. In knock-out mice lacking PPAR-α, kidney and testicular toxicity was less pronounced after feeding of a diet containing 12000 ppm

DEHP for 4, 8 and 24 weeks compared to wild-type mice (Ward *et al.*: Toxicol. Pathol. 26, 240-245, 1998), indicating the involvement of the receptor in part of the effects in kidney and testis.

Reproductive toxicity

A very recent well conducted and reported 2-generation reproduction study of DEHP in rats (Schilling *et al.*, 2001; CSTEE/2001/25-Add.3) has documented effects on reproductive performance and fertility in the F0 and F1 parental animals at 1088 mg/kg/d. Substance-induced signs of adverse developmental toxicity were noted in the progeny of the F0 and F1 parents from 340 mg/kg/d onwards. The NOAEL for reproductive performance and fertility was 340 mg/kg/d and for developmental toxicity 113 mg/kg/d, respectively.
In a continuous breeding study in mice, DEHP has been shown to decrease fertility both for males and females at 200 mg/kg/d and higher (Lamb *et al.*, 1987). A NOAEL of 20 mg/kg/d was identified in this study. . In a 90-day study in rats, dose-dependent Sertoli cell vacuolation was demonstrated with a NOAEL of 3.7 mg/kg/d (Poon et al., 1997).
As noted above, testicular toxicity has been seen in a number of studies in rats and mice. Developing males have been shown to be more sensitive towards DEHP-induced testicular toxicity than sexually mature animals. Especially the study of Arcadi *et al.* (1998) has noted serious and irreversible testicular effects after exposing rats pre- and post-natally to DEHP in drinking water. A LOAEL (a NOAEL was not identified, but preliminary experiments suggested that this was 5-fold lower) of approximately 3.5 mg/kg/d from this study has been used for risk characterisation for developmental toxicity. Although the results of this study clearly are of relevance, it is difficult to use this study as the critical one, since there are obvious limitations in the conduct and reporting. DEHP was diluted in the drinking water but no recording of water intake was performed. Also, there were no measurements of the DEHP concentrations in the drinking water administered to the animals assuring that the reported concentrations were correct. Further, the findings of the Arcadi *et al.* (1998) study are not supported by the findings of the very good 2-generation study of Schilling *et al.* (2001).
Thus, it is scientifically difficult to accept the Arcadi et al. (1998) experiment as a definite study for risk characterisation. Instead, the CSTEE supports the NOAEL of 20 mg/kg bw/day for developmental toxicity from the Lamb et al. (1987) study.

2. CSTEE opinion on the results of the Risk Assessment of DEHP (08 January 2004)

In a previous CSTEE opinion (CSTEE, 1998), testicular toxicity was identified as the critical end-point for DEHP from a 13-week dietary study in Sprague-Dawley rats, and a NOAEL was set at 3.7 mg/kg bw/day based on a mild Sertoli cell vacuolation (Poon et al, 1997).

The latest version of the Risk Assessment Report has used a new NOAEL of 4.8 mg/kg bw/day for testicular toxicity and developmental toxicity derived from a recent 3-generation reproductive study in rats (Wolfe and Layton, 2003).

The CSTEE supports this since the results seen in this newer study are more robust than those underpinning the previous NOAEL of 3.7 mg/kg bw/day.