COLLECTIVE EXPERT APPRAISAL: SUMMARY AND CONCLUSIONS

Related to the establishment of a Toxicity Reference Value based on the reprotoxic effects of di-n-butyl phthalate (CAS No. 84-74-2)

AFSSET Solicited Request No. 2003/AS03

Only the French language version of this document shall prevail.

Overview of the question

AFSSET's work programme on reprotoxic TRVs included organisation of a pilot phase to ensure implementation of the recommended development method. Reprotoxic TRVs were developed as part of the submissions for linuron, di-n-butyl phthalate (DnBP), benzyl butyl phthalate (BBP), nonylphenol, toluene and ethylene glycol ethyl ether (EGEE).
On 25 July 2007, the Directorate General for Health (DGS) requested that AFSSET validate these TRVs through a collective expert appraisal.

Organisation of the expert appraisal

AFSSET entrusted the validation of these TRVs to the Expert Committee (CES) for "Assessment of risks linked to chemical agents". This Committee mandated a *rapporteur* to conduct an expert appraisal of DnBP, which was carried out in accordance with the French Standard NF X 50-110 "Quality in Expertise Activities - General Requirements of Competence for Expert Appraisals".

Description of the working method

Based on the document *Construction d'une VTR reprotoxique pour le DnBP* [Development of a reprotoxic TRV for DnBP]" prepared by the toxicology laboratory team at the University of Western Brittany[1], the *rapporteur* assessed the compliance of the method used compared with the recommendations of the Working Group on the following points: i) information retrieval and ii) toxicity profile, in order to select the critical effect and source study to use. He then gave his opinion about the choices made with regard to available data.
The development of TRVs differs depending on the assumption made or data acquired on the substance's mechanism of toxic action. Currently, the default hypothesis is to consider a monotonic relationship between exposure, or dose, and effect, or response. On the basis of current knowledge and conventions, it is generally accepted that for reprotoxic effects, toxicity is expressed only above a threshold dose (with the exception of germ cell

[1] Annex 2 of the reference document for the development of a TRV based on reprotoxic effects

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mutagenicity). Nevertheless, this assumption may be questioned if warranted by the available data.
Mathematically, the development of a TRV is therefore defined as follows[2]:

$$TRV = \frac{\text{Critical dose}}{UF} \text{ where}$$

Critical dose = NOAEL, LOAEL or BMDL
UF = globally applied uncertainty factor

In practice, establishment of the TRV involves the following four steps:

- choice of the critical effect;
- choice of a good quality scientific study enabling establishment of a dose-response (or dose-effect) relationship;
- choice or establishment of a critical dose from experimental doses and/or epidemiological data;
- application of uncertainty factors to the critical dose to account for uncertainties.

This method is detailed in the reference document for the establishment of a TRV based on reprotoxic effects (AFSSET, December 2006), and development of the TRV for DnBP is based on this method.

As a result of its internal discussions, the CES has reached a decision about the choice of the critical dose and uncertainty factors. The CES emphasised the need to refer back to the supplemental studies which, while they are not directly used to identify the critical dose, are useful for choosing uncertainty factors (toxicokinetic studies, availability of other NOAELs or LOAELs, etc.).

Results of the collective expert appraisal

Summary of toxicity data

This part is based on the summary of work carried out by the submitter in 2006 as part of the pilot phase. For more information, the reader can refer to the "*Document de référence pour la construction d'une valeur toxicologique de référence fondée sur des effets reprotoxiques – annexe 2*" [Reference document for the development of a Toxic Reference Value based on reprotoxic effects – Annex 2]. Given the abundance of scientific literature on the toxic effects of DnBP, the report is based on monographs written previous to this work by national organisations and on literature published subsequent to these monographs.

DnBP is a plasticiser of the phthalate family. In Europe, it is used mostly in the production of resins and polymers such as polyvinyl chloride (PVC). It is also found in adhesives and food packaging. In the general population (non-specific use) primary exposure is via oral route. Furthermore, the available data were obtained mainly on the oral route. It was therefore considered appropriate, as part of this expert assessment, to develop a TRV for the oral route.
Since 2001 (the 28th Adaptation to Technical Progress [ATP]) DnBP has been classified by the European Union as a reprotoxic substance for humans, Category 2 for development (possible risk of harm to the unborn child) and Category 3 for reproduction (possible risk of impaired fertility).

[2] NOAEL: "no observed adverse effect level"; LOAEL: "lowest observed adverse effect level"; BMDL: "benchmark dose lower confidence level"

Toxicokinetics

In rodents, following oral absorption, DnBP is hydrolysed by esterases in the intestinal tissue to form the primary monoester derivative "monobutyl phthalate" or MBuP, and is rapidly and efficiently absorbed by the intestinal tract. MBuP may then undergo side-chain oxidation. Urinary metabolites are mainly the glucuronide-conjugated derivative of MBuP, free MBuP (approximately 66-70% of the metabolites) and oxidised derivatives of MBuP. There are differences between species related to excretion or non-excretion of conjugated metabolites. In humans, similar proportions of total MBuP in 24-hour urine have been recovered in volunteers exposed by oral route (approximately 64-73% of the metabolites). One study showed that conjugated MBuP (mainly glucuronide conjugate) accounted for 94% of total urinary MBuP in humans (Saillenfait and Laudet, 2005).

General toxicity

By oral route, the proliferation of liver peroxisomes observed in rats exposed to DnBP has long been regarded as the most sensitive adverse effect. Nevertheless, there is now consensus among scientists that this effect cannot be transposed to humans, since liver peroxisome proliferation occurs to a much lesser degree in humans than in rodents. Hepatic effects associated with this proliferation are thus not expected in humans at animal experimental doses. Reprotoxicity and developmental disorders from exposure to DnBP are therefore the two main toxic effects considered for humans.

Reprotoxic effects

Studies of DnBP toxicity have mainly shown developmental disorders of the male reproductive system. On the basis of current knowledge, these effects are considered as the critical effects for oral exposure to DnBP. These effects are described in rats exposed *in utero* via the maternal diet. Exposure takes place late in gestation, the key developmental period for the reproductive system. Among the critical effects reported, the most common are decreased anogenital distance, cryptorchidism, hypospadias, hypoplasia or agenesis of the epididymis, hypospermia, focal testicular dysgenesis and nipple retention. These effects were also reported in an epidemiological study published in 2005, the only human study identified to date (Swan *et al.* 2005). This study showed a significant relationship between urine concentrations of DnBP observed in the mother during pregnancy and abnormalities of the external genitalia in newborn boys (decreased anogenital distance, testicular migration disorders, scrotal hypoplasia).

From a review of the literature on the reprotoxicity of DnBP, and based on the latest data and the TRVs already available, three studies conducted in animals were selected. They are considered representative of the reprotoxic effects observed and are summarised below.
An initial study, on two generations (Wine, 1997), was conducted in Sprague-Dawley rats exposed continuously to feed containing 52, 256 and 509 mg/kg/day for males and 80, 385 and 794 mg/kg/d for females. The mating, gestation and fertility indices of the F1 generation were lower than those of the F0 at the highest dose. From 256 mg/kg/d in males and 385 mg/kg/d in females, numerous reproductive organ anomalies were observed (decreased organ weights, degenerative lesions of the testicular germinal epithelium, interstitial hyperplasia, absence or hypoplasia of the epididymis). Effects on the second generation (F2) were identical but more severe and appeared from the first doses tested, from 52 mg/kg/d for males and 80 mg/kg/d for females (in the absence of maternal toxicity for the first dose tested). No NOAEL could be identified for the F2 generation.

The second study was conducted in Sprague-Dawley rats exposed *in utero* by oral gavage of the dam from the 12th to the 21st gestation day to 0 – 0.5 – 50 – 100 and 600 mg/kg/d

(Mylchreest *et al.* 2000). In newborn males, numerous lesions of the male reproductive system were observed in the highest dose group. An absence of nipple regression was observed from 100 mg/kg/d. This effect would appear to be a representative marker of impaired sexual differentiation. The NOAEL was identified at 50 mg/kg/d.
The third study (Lee *et al.* 2004) was conducted in Sprague-Dawley rats exposed *in utero* and during lactation (exposure of dams by oral route from the 15th gestation day up to the 21st day after parturition) to doses of 0 – 2 – 24 – 234 and 1137 mg/kg/d. A reduction in testicular spermatocytes was observed at the 21st postnatal day for the first dose tested (2 mg/kg/d), as well as hypoplasia of the alveolar buds in females. The LOAEL was identified at 2 mg/kg/d on observation of spermatocyte reduction and nipple dysplasia in newborn males or females. No NOAEL could be identified.

Mechanism of action

The mechanism of action proposed for DnBP involves endocrine disruption with an anti-androgenic action linked to a significant reduction in testosterone secretion and damage to Sertoli cells.
Thus, in light of all the available data, it would appear that the reprotoxic and developmental effects observed in animals could not be excluded in humans.

Analysis and assessment of the choices for establishment of the TRV

Choice of the critical effect

The most relevant effects on development were observed in the male reproductive system. Evidence of impairment was both histological and functional. The critical effect chosen, namely **histological and functional impairment of the male reproductive system**, is consistent with all published reports and does not call for discussion *a priori*. This impairment varies depending on the studies: testicular lesions (migration disorders, degenerative lesions of the seminal vesicles, spermatocyte reduction, interstitial cell hyperplasia), lesions of the epididymis and seminal vesicles, decreased anogenital distance, increased rates of hypospadias and nipple retention. These effects indicate an endocrine disruption mechanism.

Pivotal study

The three studies chosen, described above, are considered of good quality and were also selected by internationally recognised bodies in the context of developing TRVs. The three animal studies were conducted according to strict protocols: the United States National Toxicology Program's RACB [Reproductive Assessment by Continuous Breeding] Protocol for the first two, and the Animal Care and Use Committee of the National Institute of Health Sciences (Japan) for the third. These studies were thus given a Klimisch rating of 1 and can be used for the development of a TRV. The two earlier studies were reviewed in the risk assessment report of the European Union. They address the problems surrounding the 'developmental impact' with different study protocols. Only the study by Lee (2004) takes into account an exposure period up to the end of lactation (postnatal day [PND] 21). The differences observed in effects on the testes between the study by Mylchreest (2000) and the study by Lee (2004) could be related to the use of tests that are not comparable. Similarly, in the study by Wine (1997), effects on the testis were not studied for doses below 250 to 400 mg/kg/d, which may explain the differences in results compared with those of Lee.

The final choice between the three studies was based essentially on:

- the quality of the study (Klimisch 1);

- the observation of effects indicating endocrine disruption in the absence of maternal toxicity;
- the use of a critical sensitivity period;
- post-treatment monitoring, including a comparison of reversibility of toxic effects ;
- identification of the lowest LOAEL, with equal criteria.

Thus, taking into account the uncertainty associated to changes observed in animals for predicting the long-term health impact for humans, and even though Lee's study does not enable a NOAEL to be identified, the CES chose the study by **Lee *et al.* (2004)** as the key study.
In this study, female Sprague-Dawley rats (8 animals per dose) were exposed via feed from the fifteenth gestation day (GD15) to the 21st day after parturition (PND21), which was the end of lactation. The feed was spiked with DnBP at concentrations equal to 0 – 20 – 200 – 2000 and 10,000 parts per million (ppm), corresponding to doses of 0 – 2 – 24 – 234 and 1137 mg/kg/d in body weight. A decrease in testicular spermatocytes was observed at the 21st day after birth from the concentration of 20 ppm (2 mg/kg/d) in 50% of males, as well as changes in the mammary glands in female neonates (hypoplasia of the alveolar buds). The effects chosen are **spermatocyte reduction in males ($p<0.05$) and nipple hypoplasia in females ($p<0.05$)**.

Choice of the critical dose

In the study chosen, the reduction in spermatocytes and dysplastic nipple lesions was detected at the lowest dose tested, corresponding to a **LOAEL of 2 mg/kg/d**. No NOAEL could thus be proposed. It can simply be specified that the NOAEL is between 0 and 2 mg/kg/d.
To determine this LOAEL, the authors used several analytical methods based on the data. Significance thresholds of 0.05 and 0.01 were chosen for interpreting results. The histopathological lesions were compared by Fisher's exact test and their severity was determined using a Mann-Whitney U test.
As part of the risk assessment of DnBP, the European Union proposed a LOAEL of 50 mg/kg/d based on the study by Wine (1997) (European Union, 2003). Other studies not referenced in the European report have been published since then (Mylchreest, 2000; Lee *et al.* 2004). As part of this work, taking recent evidence into account, a LOAEL of 2 mg/kg/d, which is 25 times lower, is proposed for the development of the TRV on the basis of the Lee study (2004).

During the pilot phase, AFSSET proposed developing a BMDL by considering the reduction in spermatocytes. The obtained response was dichotomous (effect/no effect). Several models were tested, including the gamma (stochastic) and Weibull (distribution) models that are applicable for dichotomous responses. A benchmark response level (BMR) of 10% in 'extra risk' was chosen (extra risk corresponds to the increased risk compared to the probability of not being affected in the control group, presumably the more conservative response). A BMD_{10} at 0.08 mg/kg/d and a $BMDL_{10}$ at $4.4.10^{-5}$ mg/kg/d were obtained (the results of the entire process are detailed in the 'Reference document for the development of a Toxic Reference Value based on reprotoxic effects', Annex 3).

The LOAEL had been set at 2 mg/kg//d. This LOAEL corresponds to an incidence of response (for reducing spermatocyte development) of 50%, which is very high. It is therefore logical that the BMD_{10} is much lower than this LOAEL (10% response). The BMDL is also very different from the BMD, due to the fact that the number of points and the number of animals tested were low, and the observed effect level was high and different from the BMR used (10%). These methodological limitations prevent the BMDL from being selected as the critical dose.

Choice of uncertainty factors

- UF_A: inter-species variability: the factor chosen is the maximum factor of 10. This is justified by the results of the epidemiological study by Swan *et al.* (2005) which seem to confirm that the anti-androgenic effects demonstrated in animals can also occur in humans. While the glucuronide conjugation of monobutyl phthalate appears higher in humans than in rats, it may be that the substance is more rapidly detoxified in humans. However, in the absence of more precise quantitative data, it is still prudent to consider an inter-species factor by default.
- UF_H: intra-species variability: the factor 10 is chosen by default when using studies conducted in animals, to take into account the greater variability within the human species.
- UF_L: use of a LOAEL: AFSSET recommends a factor of 3 or 10 depending on the case. In the case of DnBP, two arguments are put forward:
    - one is in favour of applying a factor of 3, justified by the use of the study that identified a very low LOAEL compared to the other studies (in the other studies, the NOAELs can be up to 25 times higher), suggesting that the chosen effects are very sensitive;
    - the other is in favour of applying a factor of 10, justified by wide variability within the study as evidenced by the difference observed between the BMD and BMDL values, and lack of a NOAEL. Furthermore, the first dose tested already caused spermatocyte reductions in 50% of the animals and nipple dysplasia in 100% of animals.

  The CES chose the application of a UF_L factor of 10, in accordance with AFSSET's method of developing reprotoxic TRVs.

The Expert Committee (CES) for "Assessment of risks linked to chemical agents" accepted the results of the collective expert appraisal at its meeting on 10 July 2008 and informed the Directorate General of AFSSET.

Conclusions of the collective expert appraisal

- DnBP metabolism in humans and animals is generally similar, involving production of a common monoester (MBuP). However, there are quantitative differences in glucuronide conjugation of monoester metabolites of DnBP in rats and humans. The CES draws attention to the lack of knowledge about the impact of these differences and their potential implications for variations in toxicity between species;
- The mechanism put forward (anti-androgenic effect linked to a marked decrease in testosterone secretion primarily affecting the Sertoli cells) is plausible in humans.
- The effects observed in animals are effects on development of the male reproductive system such as lesions of the testes or epididymis, decreased anogenital distance, increased rate of hypospadias, and nipple retention;
- Effects observed in animals appear to be relevant for humans. This hypothesis is supported by a recent study in humans that highlighted a relationship between exposure to phthalates in mothers during pregnancy and attenuation of androgen action in their male babies.

The CES thus proposes developing a TRV specifically for effects on development during gestation, for sub-chronic exposure.

-- Di-n-butyl phthalate (DnBP) CAS No. 84-74-2 --			
Critical effect	**Critical dose[3]**	**UF**	**TRV**
Reduction of spermatocytes and nipple dysplasia observed in the offspring Prenatal oral toxicity study in Sprague-Dawley rats (GD15-PND21) Lee *et al.* 2004	**LOAEL = 2 mg/kg/d** No NOAEL BMDL little relevance	**1000** UF_A 10 UF_H 10 UF_L 10	**TRV = 0.002 mg/kg/d** **Confidence level** Data collection: high Study: average (+ uncertainty) Critical dose: average (no NOAEL) TRV: average

The TRV is established on the basis of current knowledge. However, the level of knowledge on the toxicity of phthalates, of which the best known have a developmental toxicity related to their endocrine disrupting activity, differs significantly from one substance to another. Since toxicity studies were not conducted using the same protocols for all phthalates, the LOAELs and NOAELs resulting from these studies cannot be compared and the TRVs proposed

[3]Time conversion factors, allometric coefficients: NIL. The CES did not wish to apply an allometric adjustment to the critical dose because this type of adjustment has not yet undergone extensive study in France and the reference document for developing the TRVs based on reprotoxic effects has not yet addressed this issue. The need for a better understanding of animal-human transposition and the UF_A uncertainty factor led to the recommendation that further consideration be given to this aspect.

cannot directly reflect the toxicity of these substances. Thus, even though benzyl butyl phthalate (BBP) and DnBP likely have similar toxicity, the TRV proposed for DnBP is lower than that of BBP.

Maisons-Alfort, 25 September 2008.

On behalf of the Expert Committee (CES) for
“Assessment of risks linked to chemical agents”,

Chairman of the CES

M. Michel Guerbet