

Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment



Preface

The Committee on Mutagenicity (COM) provides advice on potential mutagenic activity of specific chemicals at the request of UK Government Departments and Agencies. Such requests generally relate to chemicals for which there are incomplete, non-standard or controversial data sets for which independent authoritative advice on potential mutagenic hazards and risks is required. Frequently recommendations for further studies are made.

During 2003, the Committee provided advice on a wide range of chemicals which included DEET (an insect repellent), malathion (an insecticide), Flunixin (in the form of its meglumine salt) and malachite green (both used as veterinary medicines), 2-phenylphenol (a biocide) and a number of contaminants such as 1,3-dichloropropan-2-ol and polycyclic aromatic hydrocarbons (PAHs).

The Committee has an ongoing responsibility to provide Government Department's and Regulatory Authorities with advice on developments in procedures for the evaluation and risk assessment of mutagens. In this regard the Committee provided advice on the conduct of *in-vitro* mammalian cell mutation assays, the investigation of germ cell mutagens and a strategy for the assessment of the significance of *in-vivo* mutagenicity seen at high doses in bone-marrow tests. This latter statement is particularly recommended for regulatory agencies who may have to assess such data.

During 2003, the Committee also said farewell to three highly respected members. Professors Ashby, Cooper and Tweats have provided valuable advice to the Committee over many years. I wish to record my thanks for their scientifically excellent contribution and their commitment to public health during their terms of office with COM.

Professor P B Farmer Chair
MA DPhil CChem FRSC

1,3-Dichloropropan-2-ol (1,3-DCP) : new *in vivo* mutagenicity studies

- 2.1 1,3-Dichloropropan-2-ol (1,3-DCP) is a member of a group of chemicals called chloropropanols, which also includes 3-chloro-1,2-propanediol (3-MCPD) and 2,3 dichloropropan-1-ol (2,3-DCP). Chloropropanols are contaminants of some foodstuffs and of polyamine flocculants used in the treatment of drinking water. The COM considered the mutagenicity of 1,3-DCP in 2001 and noted that the *in-vitro* data indicated that it had mutagenic potential. In the absence of any *in-vivo* data in mammals, it was concluded that it would be prudent to assume that 1,3-DCP was potentially genotoxic *in-vivo* and agreed it should be tested *in-vivo* using the approach set out in the COM guidelines.
- 2.2 Two studies, a bone marrow micronucleus assay and an unscheduled DNA synthesis (UDS) assay had been carried out to investigate the *in-vivo* genotoxicity of 1,3-DCP. Members discussed the results of the two new studies and considered that both assays had been conducted to relevant guidelines and that the results were clearly negative. In the micronucleus assay, Members noted that the PCE:NCE ratio was very variable amongst controls and that the top and bottom ends of the range was outside the range for historical controls. These findings were not considered to invalidate the study but Members requested that the contract laboratory be asked to comment on the reason for the variability.
- 2.3 Members considered that it would be appropriate to consider these studies provided evidence that 1,3-DCP was not an *in-vivo* mutagen.
- 2.4 A statement is appended at the end of this report.

DEET (Diethyl-m-toluamide)

- 2.5 The COM considered the available mutagenicity data on DEET in April 2002 and concluded that whilst there were apparently no concerns regarding mutagenicity, there was a need to consider the full reports of the two unpublished studies (carried out for the DEET Joint Venture Group), namely a metaphase assay for clastogenicity in CHO cells and an assay for UDS in rat hepatocytes. The COM considered these additional reports.
- 2.6 Members noted that DEET appeared to be highly toxic *in-vitro* to mammalian cells in both CHO cells and rat hepatocytes and hence adequate results were only available from a relatively narrow concentration test range in both assays.
- 2.7 The COM concluded that there were limitations in all of the submitted studies. In particular, the COM considered that the exogenous metabolising fraction used in the *in-vitro* chromosome aberration assay had not produced satisfactory results with cyclophosphamide. However, overall the results of the *in-vitro* cytogenetics assay in CHO cells and an *in-vitro* UDS assay in hepatocytes were negative. This information together with the information previously reviewed by the Committee (lack of structural alerts with DEET, negative Ames tests and negative carcinogenicity studies) suggest there is no concern with regard to the mutagenicity of DEET.

- 2.8 An amendment to the published statement to include this conclusion was made (<http://www.doh.gov.uk/pdfs/deetstatement.pdf>.)

Flunixin, meglumine and Flunixin-meglumine

- 2.9 Flunixin in the form of the meglumine salt is a non-steroidal anti-inflammatory (NSAID) drug and a non-narcotic analgesic drug with antipyretic activities. It is used in veterinary medicine (including food-producing animals) but it is not used in human medicine. Flunixin-meglumine dissociates *in-vivo* to Flunixin and meglumine. In 1997, the European Medicine Evaluation Agency's (EMA) Committee on Veterinary Medicinal Products (CVMP) considered the safety of Flunixin-meglumine as part of its review of old veterinary medicinal products, operating under Council Regulation 2377/90, which covers the marketing authorisation of pharmacologically active substances used in veterinary medicines. The Food Standards Agency was concerned about the possible mutagenicity of Flunixin, meglumine and Flunixin-meglumine and asked for an opinion from the COM.
- 2.10 The COM considered the mutagenicity data on these compounds at meetings in 2001/2 and heard a presentation from the data holder (Schering-Plough) at the February 2003 meeting.
- 2.11 Most of the mutagenicity data were relatively old and had limitations. Members agreed there was no evidence to suggest that flunixin itself had mutagenic potential *in-vivo* but the *in-vitro* data were inadequate. There was limited evidence to conclude that flunixin meglumine was an *in-vitro* mutagen, which suggested that it was the meglumine component that was responsible for the mutagenic activity. There was a negative *in-vivo* bone marrow micronucleus test with flunixin meglumine, but this study was considered too limited to allow conclusions to be drawn. Meglumine was not considered to have any structural alerts. A positive result had been reported with meglumine itself in a bone marrow micronucleus test. In this study involving administration of 2 doses 24 hours apart with harvest at 6 and 24 hours later, a positive result was produced only at the 6 hour sampling point. Negative results were also obtained in a second assay using a similar dosing schedule, but with harvest only after 24 and 48 hours.
- 2.12 New *in-vivo* micronucleus data was considered at a further meeting and agreed to be inconclusive. One study did indicate activity 6 hours after the 2 doses spaced 24 hours apart. No activity was seen at 24 hours after the second dose. The Committee noted that there was much variability in the results with individual animals, and the effects may possibly be due to toxicity. Members agreed that on the basis of the available data, the situation regarding the *in-vivo* activity of meglumine was not completely resolved.
- 2.13 The COM considered that further studies were needed before it could be concluded that the mutagenic activity seen *in-vitro* with flunixin meglumine (believed to be due to the meglumine component) could be discounted.

2.14 The presentation from the data holder and subsequent COM discussion focused on the most appropriate testing strategy given the complex set of mutagenicity data on these compounds. The data holder accepted the proposed strategy which is outlined in para 2.15 below.

2.15 The COM concluded:

- i. The mutagenicity data on Flunixin, Flunixin-meglumine and meglumine are relatively old and the studies have not been conducted to contemporary standards.
- ii. It is therefore difficult to draw any definite conclusions on the mutagenicity of these chemicals. A number of prudent conclusions were agreed:
 - a. For Flunixin (a non-steroidal anti inflammatory veterinary medicine) there was limited evidence that Flunixin was mutagenic *in-vitro* but there was no evidence to suggest that flunixin had mutagenic potential *in-vivo*. Members felt the *in-vitro* mutagenicity data on this compound were inadequate and considered this should be raised with the CVMP.
 - b. For Flunixin-meglumine (Flunixin in the form of the meglumine salt), there is limited evidence for a mutagenic effect *in-vitro*. In addition the inconclusive *in-vivo* data on meglumine suggest that a definite conclusion regarding flunixin-meglumine cannot be reached.
 - c. For meglumine, the available *in-vivo* mutagenicity data are inconsistent with some positive and negative results. It is recommended that additional *in-vitro* mutagenicity tests conducted to modern standards are undertaken with meglumine to assess whether meglumine has any mutagenic potential using the approach recommended in the COM guidance. Negative results are available for a gene mutation assay in *Salmonella*. Therefore these additional tests should comprise:
 - i. An *in-vitro* chromosomal aberration test in mammalian cells.
 - ii. An *in-vitro* mouse lymphoma assay.

2.16 A statement is appended at the end of this report.

Malachite Green

2.17 Malachite green is a cationic triphenylmethane dyestuff used in a number of industries, including fish farming. It is used in freshwater fisheries for the treatment of external fungal and other infections, its main use being to stop fungal growth on the eggs. The COT has asked for advice on the mutagenicity of malachite green and the lipophilic metabolite leucomalachite green.

- 2.18 The COM last considered the mutagenicity of malachite green and leucomalachite green in 1999. A statement had been forwarded to the Committee on Toxicity (<http://www.doh.gov.uk/com/malachit.htm>). The COM had concluded that, on the basis of the limited data available, and specifically a ³²P-post-labelling study, both compounds should be considered as potential *in-vivo* mutagens. Further results were now available from the NTP studies on the mutagenicity and carcinogenicity of leucomalachite green. These new data included results of *lacI* mutations in the livers of BigBlue® rats fed leucomalachite green at levels of up to 543 ppm in the diet for 4, 16 and 32 weeks and results of DNA adducts measurements in the livers of rats fed leucomalachite green for 4 weeks using ³²P-postlabelling assays. (Culp SJ *et al*, *Mutation Research* vol 506-507, 55-63, 2002).
- 2.19 The Food Standards Agency had asked the COM for advice on these new data. The mutagenicity data were from studies on leucomalachite green fed to BigBlue® rats. An approximate 3-fold increase in *lacI* mutations was reported in the liver only at the top dose level at the 16-week time interval. An apparent dose-related increase in DNA adducts using the ³²P-post-labelling assay was reported.
- 2.20 Members agreed that the results for *lacI* mutations were unusual in that the claimed positive response seen at 16 weeks feeding had disappeared after 32 weeks of feeding leucomalachite green. It was noted that the inter-animal variation in mutation frequency was high and the apparent positive result might relate to one only out of the five animals fed leucomalachite green for 16 weeks. The Committee was not convinced that a clear positive response had been obtained in this mutagenicity study. A reported increase in DNA adducts in the liver of female BigBlue® rats did correlate with the increasing dose of leucomalachite green. However, it was noted that adduct levels produced were very low; the highest level of DNA adducts found equated to approximately 1 adduct per 10⁸ nucleotides (this was considered to be a difficult adduct formation frequency to detect). The author's explanation was that the reported increase in mutation frequency at 16 weeks may have been due to a proliferative response of background mutations rather than induced by leucomalachite green. The Committee did not find this particular argument convincing and agreed that overall no conclusions could be reached from the investigations reported in this published paper.
- 2.21 Members heard that additional information from an NTP bioassay in rats with leucomalachite green would be forthcoming and that it was possible that additional *in-vivo* mutagenicity studies using BigBlue mice would be reported.
- 2.22 In answer to the questions posed by the FSA, members agreed that no conclusions could be reached from the new mutagenicity data on leucomalachite green summarised in the paper by Culp SJ *et al* (*Mutation Research* vol 506-507, 55-63, 2002).
- 2.23 The Committee agreed there was no need to alter its previous advice on malachite green and leucomalachite green, but that the situation should be reviewed when full reports of the carcinogenicity bioassay became available.

Malathion

- 2.24 Malathion is an organophosphorus insecticide. It has been marketed in the UK for use in agriculture and horticulture since 1956. There were three products with approvals for use in agriculture and horticulture, home garden and use in pigeon lofts at the time when this review was initiated in January 2002. A number of products containing malathion are also licensed as human medicines for use in the control of head lice.
- 2.25 The Advisory Committee on Pesticides reviewed the available toxicological information on malathion as part of its ongoing review of organophosphorus compounds. The ACP asked for advice from COM and COC on mutagenicity and carcinogenicity at its 289th meeting on 17 January 2002. The Chairs of COM and COC agreed that a joint statement was required in view of the need for a full review of all mutagenicity and carcinogenicity data.
- 2.26 The COM undertook an initial consideration of the in-confidence mutagenicity data provided by the pesticide data holder and the available published information provided by the data holder at its 25 April 2002 meeting. A number of additional published papers on malathion and impurities present in technical grade malathion were also considered at this meeting. At its meeting of 10 October 2002 the COM considered some additional information provided in-confidence by the pesticide data holder (a report of one additional *in-vivo* study and information on the potential for variation in impurities between different sources of malathion) together with a number of published studies not previously reviewed.
- 2.27 The Committee agreed a number of conclusions, which were forwarded to the pesticide data holder. Additional data on the conduct and results of the *in-vivo* oral rat liver UDS assay were submitted by the pesticide data holder and were considered at the February 2003 meeting of COM. The Committee was informed that the data holder had responded to the 1st draft statement and there had been an exchange of views between the COM and the data holder particularly with regard to the adequacy of the *in-vivo* rat liver UDS assay. A revised draft COM statement containing additional comments in response to the data holder's submission was agreed.
- 2.28 Malathion was also considered at the COC meeting of 27 June 2002. A full statement from COC and COM is appended at the end of this report.

Phenol

- 2.29 The COM had previously considered phenol on a number of occasions (1994, 1995 & 2000) (<http://www.doh.gov.uk/hydphen.htm>). The *in-vitro* data on this compound were poor. The committee had decided that phenol should be regarded as an *in-vivo* somatic cell mutagen based on positive results at high doses in the bone marrow assays for clastogenicity. Negative results were obtained in carcinogenicity bioassays in rats and mice. The committee agreed that a threshold for mutagenicity could be assumed for the oral route because there was evidence to show that any phenol active metabolites formed *in-vivo* were rapidly detoxified by multiple pathways following ingestion. But a threshold for mutagenicity for exposure by other routes, such as inhalation or dermal, could not be assumed.

- 2.30 Since then, data have become available to provide a plausible mechanism to support the view that positive results in the bone marrow assays were not due to a direct mutagenic effect of phenol, but were due to a secondary threshold toxic effect, namely hypothermia occurring at dose levels associated with positive results in the micronucleus assays.
- 2.31 The Committee was asked to consider whether these new unpublished data together with the absence of any other positive *in-vivo* mutagenicity data and the negative results in the carcinogenicity bioassays indicated that phenol did not produce clastogenic effects at doses below those that produced hypothermia. These new data indicate that phenol can produce hypothermia in mice at doses that result in micronuclei formation. An intraperitoneal dose of 300 mg/kg phenol produced significant and prolonged hypothermia with a drop in body temperature of up to 7 degrees centigrade. No effects on body temperature were seen at doses below 300 mg/kg, and single doses of 400 and 500 mg/kg reduced body temperature and cause marked lethality. To investigate micronucleus induction single intraperitoneal doses of phenol were administered at 0, 30, 100 or 300 mg/kg and bone marrow harvested at 24 and 48 hours post dose (cyclophosphamide was used as a positive control and produced micronuclei). An increase in micronuclei was seen only at 300 mg/kg phenol, which was associated with significant and prolonged hypothermia. The investigators argued that the induction of micronuclei by phenol at the maximum tolerated dose is threshold related and may be causally related to hypothermia.
- 2.32 The COM considered that the new data provided a plausible mechanism. Members agreed the function of spindle fibres could be inhibited at low body temperatures, which could result in adverse chromosome effects, such as aneuploidy. The Committee was also aware of other data that indicated that hyperthermia can also induce chromosome damage both *in-vitro* and *in-vivo*, and that high body temperature induces micronuclei in mouse bone marrow. Members agreed that inhibition of the spindle function and disturbance of the mitotic apparatus was also a possible mechanism for this effect.
- 2.33 Members agreed that before definite conclusions could be drawn on the significance of these new data they would like to see a peer reviewed published report of this study. Members requested further data on the dose-response of hypothermia induced by phenol. It was also agreed that strong evidence to support this hypothesis would be provided if micronuclei were not induced by phenol in a separate group of animals maintained at normal body temperature (e.g. by the use of heated plates and warm beds). If such information could be provided members agreed that phenol could be regarded as having no significant *in-vivo* mutagenic potential at dose levels that do not produce any significant toxic effects (hypothermia).

2-Phenylphenol

- 2.34 2-Phenylphenol and its sodium salt are broad spectrum fungicides that are approved in the UK under the Control of Pesticides Regulations (COPR 1986 amended) for use as wood preservatives. They are also used as surface biocides in a number of areas. The COM have advised on the mutagenicity of these compounds, specifically in the context of the mechanism of the bladder tumours seen in male

rats fed high doses of these compounds, on a number of occasions, the most recent being in 1997. Significant new relevant data are now available. Furthermore an EU review of the use of 2-phenylphenol and its sodium salt in wood preservation is shortly to be initiated under the Biocidal Products Directive (98/8/EC). It would thus be timely to update the COM view on the mutagenicity of these compounds. This information would be helpful in developing the UK position with regard to the EU in the context of the Biocidal Products Directive.

- 2.35 At high dose levels 2-phenylphenol induces bladder tumours in the rat and it is particularly important to be able to exclude a genotoxic mechanism being involved. In 1992 the COM noted that although negative results were obtained in bone marrow assays and germ cell assays for mutagenicity *in-vivo*, some conflicting results were obtained in *in-vivo* assays for effects on the DNA in bladder epithelium. The COM recommended that data from an *in-vivo* study to investigate DNA adduct formation in bladder epithelium should be carried out using more sensitive methods, to provide definitive information regarding the absence of a genotoxic mechanism. At that time the Committee felt that there was insufficient concern to recommend a departure from a risk assessment approach based on the use of uncertainty factors to estimate safe levels of exposure (i.e. a threshold approach was adopted). In 1997 the Committee considered data from an *in-vivo* ³²P- post-labelling study on DNA adducts in the bladder but they had concerns at the limitations of the method used and recommended that this be further investigated. This recommendation was not made into a regulatory request because of other data on carcinogenicity provided to the ACP who felt that the post review regulatory requirements had been provided, and that a threshold based risk assessment was appropriate.
- 2.36 Considerable additional data has now been published relating to the metabolism and mutagenicity of 2-phenylphenol, including further *in-vivo* DNA adduct work. The Committee agreed that the new Accelerator Mass Spectrometry study (AMS) study provided good evidence for the lack of covalent DNA binding in the male rat bladder. The method was a particularly sensitive technique and the study had been well performed. Protein but not DNA binding had been clearly shown. Members agreed that the weight of evidence with regard to the DNA binding studies in the rat bladder was now sufficient to conclude that significant DNA binding was unlikely to occur.
- 2.37 The Committee agreed the following overall conclusions regarding the mutagenicity of 2-phenylphenol and its sodium salt, after consideration of all the new data:
- Data from several assays to investigate the ability of 2-phenylphenol or its sodium salt to produce gene mutation in *Salmonella typhimurium* were consistently negative.
 - Positive results were obtained in *in-vitro* metaphase analysis studies in CHO cells and also in mouse lymphoma assays in both cases in the presence of an exogenous metabolic activation system. The induction of small colonies in the latter assay is consistent with the compound having clastogenic potential.

- iii. Phenylhydroquinone (PHQ) and phenylbenzoquinone (PBQ), which are metabolites of 2-phenylphenol, (PHQ usually being present as conjugates), have been shown to produce oxidative DNA damage and single strand DNA breaks *in-vitro* using HL60 and V-79 cells.
- iv. Negative results were obtained in bone marrow assays for clastogenicity *in-vivo* and also in germ cells (dominant lethal assay), suggesting that any possible clastogenic potential was not expressed in the whole mammal.
- v. The weight of evidence from *in-vivo* studies to investigate 2-phenylphenol binding to DNA in the male rat bladder was negative: a recent study using a highly sensitive AMS techniques was particularly important in this regard. However the possibility of prolonged high level exposure producing DNA adducts cannot be entirely discounted.
- vi. Although a contributory role of oxidative DNA damage cannot be excluded when considering the mechanisms of bladder tumour induction in the male rat, this would not be expected to occur at low dose levels.
- vii. The Committee concluded that it would be reasonable to adopt a threshold based risk assessment for 2-phenylphenol and its sodium salt.

2.38 A statement is appended at the end of this report.

Polycyclic Aromatic Hydrocarbons in air pollution: A discussion of approaches to risk assessment

- 2.39 The COC has recently evaluated published carcinogenicity data on dibenzo(a,l)pyrene (DB(a,l)P) and had agreed that this compound was between 10-100 times more potent than benzo(a)pyrene depending on the test system used (see section 3.1 of the COC Annual Report). Thus a paper had been drafted which considered the potential impact of DB(a,l)P and other high carcinogenic potency PAHs on the existing approaches to risk assessment of PAHs in air pollution.
- 2.40 Three approaches to carcinogenic risk assessment have been advocated: the use of Potency Equivalency Factors (PEFs) (equivalent to Toxicity Equivalent Factors, TEFs, for general toxicity); the complete mixture method; and use of B(a)P as a surrogate carcinogen for all PAHs. It was possible that the B(a)P surrogate approach which is currently used to monitor for compliance with UK air pollution standard, might not be appropriate if high potency PAHs were present in air pollution and if the concentrations of these compounds varied significantly when compared to B(a)P. A review of the possible approaches to risk assessment has been undertaken, taking into account available data on carcinogenicity, including a discussion of kinetics of PAH uptake via the lung and self-induction of PAH metabolism. COM advice was particularly sought on the proposals for use of DNA adducts or mutations as surrogate end-points for carcinogenic potency.

- 2.41 Members agreed that the majority of these compounds were activated *in-vivo* to diol-epoxides. It was possible to make informed predictions regarding the pathways of metabolism and reaction of diol-epoxides with DNA from the structure of PAHs. Members agreed that PAHs could be considered as members of a single group of genotoxic carcinogens whose mechanism of activation, formation of DNA adducts and metabolism were all broadly similar. There were some exceptions where PAHs were also metabolised by another minor route also leading to activation and DNA binding, for example the bis-diol-epoxide formed by dibenz(a,h)anthracene (DB(a,h)A). It was agreed that PAHs activated by routes other than via a diol-epoxide would need to be considered carefully. Members noted the carcinogenic process leading to PAH induced tumourigenicity was complex and included factors such as tumour promotion and cell proliferation which would also affect carcinogenic potency. PAHs also had other molecular targets (e.g. oestrogen receptors) which might potentially impact on the carcinogenic process in target tissues.
- 2.42 The Committee reviewed the available data regarding use of DNA adducts as a surrogate marker for PAH carcinogenic potency. There was evidence that DNA adducts may be an adequate endpoint for this group of genotoxic carcinogens from two research groups using different experimental approaches. Dermal application of PAHs to the skin of mice showed a good correlation for DNA adducts in skin and lung with applied skin dose. Studies using intraperitoneal administration of PAHs to A/J mice showed a good correlation between total DNA adducts (Time Integrated DNA Adduct Levels; TIDAL) in lung and administered PAH dose. Both research groups had documented a good correlation between DNA adducts and tumourigenicity. It was suggested that DNA adducts could serve as a pragmatic marker (it is argued for DNA adduct formation from reactive diol-epoxides of PAHs) that correlates with tumourigenicity. It was noted that there was also data from studies in A/J mice using administration of mixtures of PAHs that suggested that any departure from additivity was limited.
- 2.43 Members noted the evidence for a lack of correlation between PAH induced mutation frequency in transgenic mice given oral doses of PAHs and target organ for carcinogenicity. Members felt that it would be important to evaluate dose-response for PAH induced mutation frequency in cancer target tissues in a similar way to the DNA adduct data before conclusions regarding the utility of mutations as a surrogate marker for PAH induced carcinogenicity could be drawn.
- 2.44 The Committee considered the proposals for experiments to rank carcinogenic potency of PAHs. It was agreed that intratracheal instillation of small doses in rats, with measurement of total DNA adducts in lung, would be appropriate for inhalation of PAHs. Members also considered that measurement of DNA adducts in skin following topical treatment would also yield a similar ranking of PAHs. It was suggested that it might be possible to undertake DNA adduct measurements and determination of PAH induced mutation frequency in the same animals, although it was acknowledged this would represent a very large research project and that it was not a practical approach for ranking PAHs. Members briefly considered whether *in-vitro* approaches could yield relevant data but agreed that differences in metabolism between *in-vitro* cultures and whole animals would limit the value of *in-vitro* studies.

- 2.45 The Committee agreed the proposals in the draft paper. It was noted that the paper would be provided to COC for comment and then submitted to a peer review journal for publication. Members asked for detailed information regarding air levels of highly potent carcinogenic PAHs when available.

Review of Committee Procedures

Horizon Scanning

- 2.46 The Code of practice published by the Office for Science and Technology (OST) on Guidance for Scientific Advisory Committees (<http://www.ost.gov.uk/policy/advice/copsac/>) encouraged Committees to develop strategies for the early identification of issues. This included i) “new issues”, eg previously unidentified potential chemical mutagens/carcinogens which may represent a risk to public health and where advice is required, and ii) “new or unexpected developments in science..”
- 2.47 The COM considered a number of topics for potential future consideration. Members considered that future consideration of the potential for formation of mutagens during food processing/cooking (particularly frying) would be valuable. On a separate subject it was suggested that the significance of mitochondrial mutation and the possible genotoxicity of phytoestrogens could be possible topics for future COM consideration.
- 2.48 Regarding new methods of evaluation, members agreed there could be a review of changes in gene expression induced by chemical mutagens. Also further consideration could be given to the appropriate weight of evidence provided by negative *in-vivo* results in two tissues (as suggested by the COM guidance) when discounting positive results *in-vitro*.
- 2.49 It was agreed that members could raise new issues or developments in chemical induced mutagenicity at any time and that the committee should formally discuss issues relating to horizon scanning at least once per year.

Test Strategies and Evaluation

In-vitro Mammalian cell mutation assays

- 2.50 The COM guidance on a strategy for testing chemicals for mutagenicity (<http://www.doh.gov.uk/com/guidance.pdf>) published in 2000 includes the use of a mammalian cell mutation assay as the third of three *in-vitro* screening tests recommended in Stage 1 of the testing strategy. The COM concluded that the mouse lymphoma assay using L5178Y cells was the preferred test. The Committee was asked to review this conclusion in the light of an expert opinion submitted by one pesticide applicant which focused on the use of mammalian cell mutation assays to screen for gene mutagens.

- 2.51 The Committee agreed that there was no evidence that the mouse lymphoma assay had any higher inherent sensitivity for the detection of point mutations than other assays in mammalian cell such as the *hprt* assay in CHO cells, provided that adequate number of cells had been used in each assay. This presented, on statistical grounds, some practical difficulty with regard to the use of surface cultures of CHO cells, rather than with suspension cultures of mouse lymphoma cells. The number of cells needed is governed by the spontaneous mutation frequency of the cell system used. This is covered in some detail in the OECD test guideline in which it is stated that, as a general guide, the cell number should be at least 10 times the inverse of the spontaneous mutation frequency. The Committee endorsed this and indicated that, allowing for cytotoxicity, it would usually be necessary to use 10^7 cells or more in the *hprt* CHO cell assay.
- 2.52 Members also confirmed that another reason why the mouse lymphoma assay was preferred as part of Stage 1 in the COM strategy (*in-vitro* screening for potential mutagenicity) was that it detected a wider range of genetic endpoints, for example, chromosome deletions as well as gene mutations. This gives the mouse lymphoma assay added value as the second *in-vitro* mammalian cell assay in addition to cytogenetics in mammalian cells. Members accepted that the *hprt* CHO cell assay might have a role in mechanistic studies where it was an advantage to specifically measure gene mutation.

Strategy for investigating germ cell mutagens

- 2.53 The COM guidance on a strategy for testing chemicals for mutagenicity (<http://www.doh.gov.uk/com/guidance.pdf>) published in 2000 recommended that in the initial stages of investigating whether a chemical has *in-vivo* activity there was no need to screen for germ cell mutagens. This was because all germ cell mutagens had been shown to produce positive results in bone marrow assays, and there was no evidence for any germ cell specific mutagens. It was recognised that the reverse was not true, and not all somatic cell mutagens were germ cell mutagens.
- 2.54 The Committee was made aware of a recent published paper (Witt Kl et al, Environmental and Molecular Mutagenesis, volume 41, 111-120, 2003) where evidence had been reported that N-hydroxymethylacrylamide produced positive results in the mouse dominant lethal assay for germ cell mutagenicity, but gave negative results in the micronucleus test in both bone marrow and peripheral erythrocytes. This chemical was negative in a range of *in vivo* bone marrow assays (using acute, sub-acute and subchronic dosing regimes). Positive results, however, were obtained when an especially sensitive method involving flow cytometry was used. Positive results were obtained in the dominant lethal assay in mice after one week and 8 weeks dosing. At the latter time point negative results were obtained in a micronucleus test using peripheral blood. A single dose dominant lethal assay also gave negative results.
- 2.55 The Committee was asked whether the data warranted any modification to the COM strategy of testing as outlined in the 2000 guidelines.

- 2.56 Members agreed that there were a number of possible reasons why this new study on N-hydroxymethylacrylamide detected a positive mutagenic result in germ cells but not in the bone marrow. In relation to the analysis for micronuclei in peripheral erythrocytes, it was noted that the spleen could have removed damaged cells. Members felt that accumulation in the germ cells (positive results had only been detected after repeated administration) could also be significant in producing positive effects on germ cell DNA. Additionally, a certain stage of a developing germ cell could be more susceptible to N-hydroxymethylacrylamide genetic damage, and germ cells might stay in a susceptible stage for a longer period of time than the faster dividing bone marrow cells. Members also felt that some of the bone marrow micronucleus tests involving repeated administration were equivocal, rather than negative.
- 2.57 The Committee noted that if the COM guidance was followed, a second *in vivo* assay in a different somatic tissue was needed. After consideration of all the available information, members agreed that N-hydroxymethylacrylamide was not a germ cell specific mutagen.
- 2.58 Members considered that this one study by itself did not warrant any change to the COM strategy as outlined in the 2000 guidelines, but that this issue should be kept under review in case other similar examples were found in the future.

Significance of *in-vivo* mutagenicity at high doses

- 2.59 The COM has previously agreed that it is prudent to assume that there is no threshold for mutagenicity unless appropriate mechanistic data can be provided to identify a threshold related mechanism. *In-vivo* studies in the bone marrow provide key data in identifying compounds as *in-vivo* mutagens. In some cases the only data available to indicate such *in-vivo* activity is from mutagenicity studies using excessively high doses (by current guidelines) associated with severe toxicity/lethality, and there is no evidence from carcinogenicity bioassays to suggest that a compound is a genotoxic carcinogen. Due to the importance of these data in risk assessment, where positive results normally lead to a 'non-threshold' approach being adopted by regulatory agencies, it is important that the observed effects are not secondary to toxicity. The Committee considered a paper drafted by the Secretariat at its October 2002 meeting which presented "in-confidence" data from submissions received by regulatory authorities. These were to be used as examples in order to draft generic guidance which could be published.
- 2.60 The COM undertook further consideration of this subject at its February and May meetings in 2003. A number of potential mechanisms with a threshold of action (eg hypothermia and erythropoiesis) had been identified in the discussion papers as well as severe toxic effects. The COM placed considerable emphasis on considering all the available data on a case-by-case basis, including results from adequately conducted carcinogenicity bioassays. Due to the close association between mutagenicity and carcinogenicity, two members of the COC attended the February 2003 meeting for the discussion of this item to provide advice on the weight of evidence to be given to carcinogenicity studies.

- 2.61 Members of COM and COC agreed that consideration needed to be given to the whole data package, including chemical reactivity, toxicokinetics and whether the carcinogenicity data of a compound is consistent with the profile of a genotoxic carcinogen. Chemicals needed to be considered on a case-by-case basis. A negative carcinogenicity bioassay on its own could not discount a 'high dose' positive *in-vivo* mutagenicity result. Conversely a positive result in a carcinogenicity bioassay may not necessarily mean that a chemical was a mutagen, as there were 'non-genotoxic' mechanisms that could induce cancer.
- 2.62 The committee had previously agreed that *in-vivo* mutagenicity data from dose levels associated with lethality (ie clearly above the top dose recommended by OECD guidelines) could not be interpreted with any certainty due to the confounding effects of toxicity. If adequate information were not available from lower doses then further testing would be required with more appropriate lower doses. Members considered that mechanistic data were valuable in discounting positive mutagenicity results seen at toxic doses i.e. to demonstrate the response was secondary to toxicity.
- 2.63 It was agreed that adequately conducted carcinogenicity bioassays, if available, provided important information to help evaluate the significance of positive results from mutagenicity studies at 'high' doses. Adequate negative carcinogenicity bioassays, preferably in two species, could be used to help discount evidence of mutagenicity at lethal doses, and where there was some concern at the relevance of the *in-vivo* mutagenicity data. Members discussed the need to take into account the differences in the maximum dose levels in these studies. It was noted that consideration of the differences in maximum dose levels would be of limited value in view of the differences in duration of dosing (usually 1-2 days compared to 2 years). However consideration should always be given to available toxicokinetic data.
- 2.64 Overall the COM considered that a weight of evidence approach using the whole data package on a case-by-case basis had to be used when considering whether a 'high' dose positive *in-vivo* result could be discounted in somatic cells due to a biologically plausible mechanism.
- 2.65 The Committee agreed that the following points need to be considered when deciding whether positive *in-vivo* bone marrow clastogenicity data can be discounted with regard to considering a compound as an *in-vivo* mutagen.
- i. The totality of the relevant data relating to the specific compound in question needs to be considered, including any information on its chemical reactivity, toxicokinetics, its mutagenic profile (*in-vitro* and *in-vivo*), and also any available data from carcinogenicity bioassays to enable an assessment to be made as to whether the compound appears to be a genotoxic carcinogen.
 - ii. Positive bone marrow *in-vivo* mutagenicity data from dose levels that are associated with high levels of toxicity or lethality (ie above the maximum dose level recommended in the current OECD guidelines) cannot be interpreted with any certainty because of the confounding effects of toxicity. If adequate information is not available at lower, non-lethal dose levels then retesting is necessary.

- iii. It is also important to consider whether there is any evidence for a plausible mechanism to support the contention that the observed positive results in the bone marrow assays at high dose levels may be secondary to other non-genotoxic effects rather than being a mutagenic effect of the compound (or its metabolites). Examples of such mechanisms include (but are not limited to) hypothermia, hyperthermia, and erythropoiesis.
- iv. Data from adequately conducted carcinogenicity bioassays, if available, provide important information to help in the assessment of the significance of such high dose bone marrow mutagenicity results. Such data may indicate that the carcinogenic profile of the chemical is consistent with either a genotoxic or a non-genotoxic mechanism, or that the compound is not carcinogenic.
- v. Only generic advice can be given in this area and it should be emphasised that each compound needs to be considered in a case-by-case basis. However consideration of the above factors, with expert judgement, may provide sufficient evidence to conclude that the positive *in-vivo* bone marrow data at high dose levels was due to a non-genotoxic effect. A threshold based risk assessment may thus be appropriate.

2.66 A statement is appended at the end of this report.

Ongoing reviews

Chromium picolinate

2.67 Chromium picolinate is a widely available food supplement. The adverse effects of chromium were recently reviewed by the Expert Group on Vitamins and Minerals (EVM). The reports of genotoxicity associated with chromium picolinate were noted and chromium picolinate was excluded from their recommendations for an upper safe level. Following the publication of the EVM report, the Food Standards Agency advised that consumers should use other forms of trivalent chromium supplements until more detailed advice is available. The COM undertook a preliminary discussion of available mutagenicity data at its October 2003 meeting. A request for additional information has been forwarded to the manufacturers who supply chromium picolinate to the food supplement industry.

Statements agreed by COM during 2003

1,3-Dichloropropanol

Flunixin, meglumine and Flunixin-meglumine

Joint statement on review of malathion

2-Phenylphenol

Significance of in-vivo mutagenicity at high doses

Statement on the mutagenicity of 1,3-dichloropropan-2-ol

Introduction

1. 1,3-dichloropropan-2-ol (1,3-DCP) is a member of a group of chemicals called chloropropanols, which includes 3-monochloropropane-1,2-diol (3-MCPD) and 2,3-dichloropropan-1-ol (2,3-DCP). 1,3-DCP and 3-MCPD can be present as process contaminants in some foodstuffs and as contaminants of polyamine flocculants in drinking water. 1,3-DCP was last considered by the COM and the COC in 2001. COM concluded that it would be prudent to consider 1,3-DCP as potentially genotoxic *in vivo* but agreed that it should be tested for genotoxicity *in vivo* using the approach set out in the COM guidelines.
2. 1,3-DCP has been considered by the COC, originally in 1991 and more recently in 2001. The COC noted that in a carcinogenicity study undertaken by Hercules Inc¹, 1,3-DCP was administered in the drinking water of 80 male and 80 female Wistar rats for 104 weeks. Statistically significant positive trends were observed for benign and malignant tumours (intermediate and high dose level) in the liver, kidney, tongue/oral cavity and thyroid. The COC concluded that “it was prudent to assume 1,3-DCP is a genotoxic carcinogen and that exposure to 1,3-DCP should be reduced to as low as technologically feasible”².

COM evaluation: 2001

3. Members agreed that the metabolism of 1,3-DCP was likely to produce a reactive epoxide intermediate that could damage DNA. Members were aware that 1,3-DCP had been found to be mutagenic to *Salmonella typhimurium* strains TA1535 and or TA 100³⁻¹⁰. Studies with mammalian cells have produced increased frequencies of sister chromatid exchanges and chromosome aberrations^{11,12}. A positive result has been obtained in a mouse lymphoma assay^{13,14}. 1,3-DCP was negative in the wing spot test in *Drosophila melanogaster* (a somatic mutation and recombination test)¹⁵. No *in-vivo* mammalian studies had been carried out.
4. The Committee concluded that it would be prudent to regard 1,3-DCP as potentially genotoxic *in-vivo* and agreed that it should be tested for genotoxicity *in-vivo* using the approach set out in the COM guidelines.

COM evaluation: 2003

5. The Committee considered two new *in-vivo* genotoxicity studies at its May meeting. These comprised a rat bone-marrow micronucleus test and a rat liver unscheduled DNA synthesis (UDS) assay, both of which are widely used to assess genotoxicity *in vivo*.

Rat in-vivo bone-marrow micronucleus test¹⁶

6. The assay followed the current OECD guideline (No. 474). The highest dose used in the study was selected so that it would produce some signs of toxicity, but not severe effects, based on the results of a range-finding study. In the main study, 1,3-DCP was administered once daily for two consecutive days to groups of six male Han Wistar rats at doses of 25, 50 and 100 mg/kg. Bone marrow was harvested 24 hours after the final dose. A single sex study was considered adequate because no substantial difference in toxicity was observed between males and females in the range-finder.
7. Weight loss (25 mg/kg), piloerection and weight loss (50 mg/kg) and piloerection, weight loss and lethargy (100 mg/kg) were observed in treated animals, indicating that the test was conducted at adequate doses. The Committee noted that the ratios of polychromatic to normochromatic erythrocytes (PCE/NCE) were variable amongst individual animals of the control group. However, the mean value was within the range of historical control group mean data. Rats treated with 1,3-DCP at all doses exhibited group mean PCE/NCE ratios that were similar to those of the control group and within the normal range. Therefore toxicity to the bone marrow was not demonstrated, although 1,3-DCP clearly caused systemic toxicity.
8. There were no statistically significant increases in micronucleus frequency at any dose of 1,3-DCP. The positive control agent, cyclophosphamide, produced a clear increase in micronuclei.

Rat liver in-vivo UDS assay¹⁷

9. The UDS assay protocol conformed to the current OECD guideline (No. 486). Based on the results of the range-finder for the micronucleus study, the study was conducted in male rats and the highest dose was 100 mg/kg. Single doses of 40 and 100 mg/kg were administered to groups of four male Han Wistar rats. Hepatocytes were isolated for analysis for UDS by the autoradiographic technique after 12-14 hours in the first study (3 rats per dose group) and at 2-4 hours in the second study (3 rats per dose group).
10. In the 2-4 hour experiment, piloerection was observed in all 1,3-DCP treated rats and lethargy was observed at 100 mg/kg. There were no clinical signs following dosing in the 12-14 hour experiment. There was no evidence for any increase in UDS at either dose level or time point. The positive control compounds 2-AAF and DMN both gave clear positive results.

COM discussion

11. Members agreed that the two new studies met the previously stated requirement that 1,3-DCP should be tested for genotoxicity *in-vivo* using the approach set out in the COM guidelines. The studies were adequately conducted and gave clear negative results, and therefore Members considered that these studies provided evidence that 1,3-DCP was not an *in-vivo* mutagen. Members then gave consideration as to possible mechanisms whereby mutagenic activity observed *in vitro* was not expressed *in vivo*.

12. The role of metabolism in the *in-vitro* mutagenicity is unclear. Most bacterial studies have shown mutagenicity both in the presence and absence of metabolic activation. Two studies indicated that metabolism increased the mutagenicity in TA100 and/or TA1535^{7,9}, whereas one reported a decrease in TA100 revertants in the presence of metabolic activation⁸. Activation was found to result in reduced frequency of sister chromatid exchanges in V79 cells¹¹.
13. It has been postulated that metabolism to epichlorhydrin could be responsible for the mutagenicity of 1,3-DCP¹⁸. There is some evidence that bacteria can convert 1,3-DCP to epichlorhydrin¹⁹, which could account for the direct activity seen in the bacterial mutagenicity tests, although no data are available with respect to *Salmonella typhimurium*. It has been suggested that epichlorhydrin may be formed non-enzymically during the pre-incubation stage of the SOS chromotest assay with *Escherichia coli* strain GC4798⁶.
14. An alternative active metabolite is 1,3-dichloroacetone. It has been postulated that this may be formed from 1,3-DCP by action of alcohol dehydrogenase²⁰ or CYP2E1²¹. Glutathione conjugation is believed to be a detoxification pathway since 1,3-DCP depletes glutathione both *in vitro*²² and *in vivo*²³, and glutathione depletion has been shown to potentiate the toxicity of 1,3-DCP to rat hepatocytes²².
15. One known route of 1,3-DCP metabolism in rats involves hydroxylation to 3-monochloropropane-1,2-diol (3-MCPD), accounting for 1 to 14% of a 10 mg subcutaneous dose in rats²⁴. The COM has concluded that 3-MCPD can be regarded as having no significant genotoxic potential *in vivo*²⁵.
16. The COM considered that the metabolism of 1,3-DCP had not been fully elucidated. Metabolic activation *in vivo* to two active metabolites had been postulated. In both cases the compound formed would be expected to be rapidly de-activated *in vivo* by glutathione. In one case deactivation would also occur by the action of epoxide hydrolase. Thus once formed, the active metabolite is rapidly detoxified and hence 1,3-DCP would be unlikely to have significant activity *in vivo*. This is supported by the negative results obtained in the two new *in-vivo* mutagenicity assays.

Conclusions

17. The Committee concluded that both the rat bone-marrow micronucleus test and the rat liver UDS test had been carried out to an acceptable standard and were negative. Thus the additional information recommended by the COM as being necessary to provide adequate reassurance that the mutagenic activity seen *in vitro* was not expressed *in vivo* had now been provided.
18. The Committee noted the uncertainties with regard to routes of metabolic activation of 1,3-DCP and agreed that the two new mutagenicity studies supported the view that reactive metabolites, if formed, did not produce genotoxicity *in vivo* in the tissues assessed.
19. The Committee concluded that 1,3-DCP can be regarded as having no significant genotoxic potential *in vivo*.

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Statement on flunixin, meglumine and flunixin meglumine

Background to COM review

1. Flunixin in the form of the meglumine salt is a non-steroidal anti-inflammatory (NSAID) drug and a non-narcotic analgesic drug with antipyretic activities. It is used in veterinary medicine (including food-producing animals) but it is not used in human medicine. Flunixin-meglumine dissociates *in-vivo* to flunixin and meglumine. In 1997, the European Medicine Evaluation Agency's (EMEA) Committee on Veterinary Medicinal Products (CVMP) considered the safety of Flunixin-meglumine as part of its review of old veterinary medicinal products, operating under Council Regulation 2377/90, which covers the marketing authorisation of pharmacologically active substances used in veterinary medicines.
2. With regard to meglumine, the CVMP considered that it could be regarded as an excipient that had the purpose of increasing the solubility of flunixin. The CVMP concluded that "meglumine used as an excipient at up to 1.5 mg/kg bw does not fall within the scope of Council Regulation (EEC) number 2377/90".
3. The Food Standards Agency is concerned about the possible mutagenicity of Flunixin-meglumine, meglumine and Flunixin. The Food Standards Agency considered that it would be useful to seek the opinion of the COM.

Assessment of Mutagenicity data

4. As a general comment Members noted that relatively high levels of micronuclei had been documented in all of the *in-vivo* bone-marrow micronuclei studies submitted on these chemicals which had all been undertaken in the early 1980s by one particular contract laboratory.

Flunixin

5. The available mutagenicity data were relatively old and of limited value. Negative results were reported in *Salmonella typhimurium*² and in an *in-vivo* micronucleus assay in B6 mice using intraperitoneal administration of two doses separated by 24 hours (200 mg/kg bw reduced to 150 mg/kg bw) and harvested 6 hours after the last dose³. The DNA damage assay in *Escherichia coli* p3478 repair deficient strain showed an increase in DNA damage at one interim concentration, but this finding was not seen in a repeat test⁴. A positive result was claimed in a mitotic gene conversion assay in *Saccharomyces cerevisiae*⁵.
6. Overall Members agreed that there was limited evidence that Flunixin was mutagenic *in-vitro* but there was no evidence to suggest that Flunixin had mutagenic potential *in-vivo*. Members felt the *in-vitro* mutagenicity data on this compound were inadequate and considered this should be raised with the CVMP.

Meglumine

7. Negative results were reported in an old plate incorporation assay in a limited number of *Salmonella typhimurium* strains⁶. A positive result had been reported in a bone-marrow micronucleus assay in B6 mice using intraperitoneal administration of 500 or 1000 mg/kg bw suspended in 0.25% methylcellulose (two doses given 24 hours apart and harvest 6 hours after last dose)⁷. A repeat test using 1000 mg/kg bw was also positive. Members queried how the repeat test could have been undertaken on the same day as the initial test. It was noted that negative results were obtained in a separate *in-vivo* micronucleus assay using intraperitoneal administration of two doses given 24 hours apart at up to 600 mg/kg bw of meglumine to CD1 mice⁸. Members noted that a clear positive control response had only been seen in males and not females in this latter assay and that the sampling regimen (24 and 48 hours after last dose) differed from the study in B6 mice.
8. A number of *in-vivo* bone marrow micronucleus assays were recently undertaken for the Committee using a variety of single/double intraperitoneal dosing regimens at dose levels of 500 mg/kg bw and 1000 mg/kg bw and sampling for micronuclei at 6 or 24 h after the last dose⁹. There was some evidence for an effect 6 h after the second dose in one study using Alpk:ApfCD-1 mice, but no definite conclusions could be reached as the result was not repeatable, there was considerable individual animal variation and the observed effect could have been complicated by toxicity.
9. The Committee agreed that there was a need to further evaluate the potential mutagenicity of meglumine. This is discussed in the COM evaluation section below.

Flunixin meglumine

10. Members agreed that the mutagenicity data on Flunixin-meglumine (ie the mixture used in veterinary medicines) was difficult to assess. Negative results were reported in an old plate incorporation assay using a limited number of *Salmonella typhimurium* strains¹⁰. Negative results were also reported in an *in-vitro* UDS assay using rat hepatocytes¹¹. Positive results were documented in a mitotic gene conversion assay in *Saccharomyces cerevisiae*^{12,13}. There was also evidence for DNA damage in two separate tests in *Escherichia coli* p3478 repair deficient strain but not in a third test^{14,15,16}. However there was limited evidence for a mutagenic effect in three separate mouse lymphoma assays^{17,18,19} and evidence for an equivocal response in a cytogenetics test in Chinese Hamster Ovary cells²⁰.
11. Overall the Committee agreed that there was limited evidence that Flunixin-meglumine was an *in-vitro* mutagen. Members noted that negative results had been documented in an *in-vivo* micronucleus assay in B6 mice using intraperitoneal administration of two doses (up to 80 mg/kg bw) separated by 24 h and sampling 6 h after the last dose. Members were concerned with regard to the adequacy of the selection of dose levels²¹. In addition the inconclusive *in-vivo* data on meglumine suggest that a definite conclusion regarding Flunixin-meglumine cannot be reached.

COM Evaluation

12. The Committee noted the poor quality of mutagenicity data on Flunixin, meglumine and Flunixin-meglumine. Members noted that while negative results obtained in carcinogenicity bioassays with Flunixin-meglumine in the rat and the mouse provided some reassurance with regard to Flunixin, they were not informative with regard to meglumine.
13. The Committee was aware of several *in-vivo* bone-marrow micronucleus assays using meglumine but noted that the results were inconsistent. Thus there was evidence in two separate studies (in B61 and Alpk:ApfCD-1 mice) for a mutagenic effect following intraperitoneal dosing of 1000 mg/kg bw following a treatment regime of two doses of meglumine (24 hour apart) with a sampling time of 6 hours after the second dose (ie 30 hours after the first dose)^{7,9}. One of these studies had been undertaken by a Committee member. However this result was not repeated in two recent bone marrow micronucleus assays in mice which had been conducted for the Committee which attempted to repeat these findings using an equivalent treatment regime⁹.
14. The Committee considered a number of options for further investigating the mutagenicity of meglumine. The Committee agreed the most appropriate way forward would be to request additional *in-vitro* assays with meglumine to properly assess whether meglumine had mutagenic potential *in-vitro* before considering the need for any further *in-vivo* mutagenicity studies with meglumine. Thus data were required from an *in-vitro* chromosomal aberration assay and a mouse lymphoma assay, which together with the data currently available for a test in bacteria using Salmonella, would complete the *in-vitro* package to modern standards given in the COM guidance. In this case the weak and inconsistent induction of micronuclei in mice after two treatments with meglumine 24 hours apart with sampling 6 hours after the last treatment would be disregarded as strain-or-system specific effects of no genotoxic relevance if these two additional *in-vitro* tests were negative.
15. Thus negative data were available for a test in bacteria using Salmonella, but data were required from an *in-vitro* chromosomal aberration assay in mammalian cells and a mouse lymphoma assay to complete the *in-vitro* package to modern standards given in the COM Guidance²². If these were negative, there would be no concerns regarding meglumine.

Consideration of presentation by Schering-Plough on mutagenicity testing strategy (6 February 2003)

16. Schering-Plough (referred to as the data holder in this statement) suggested that two further *in-vivo* mutagenicity studies be carried out with meglumine, namely a bone-marrow chromosome aberration assay in rats and a bone-marrow micronucleus test in rats.
17. Members considered the presentation and concluded that provision of two adequately conducted *in-vitro* tests as outlined in COM Guidance with negative results would obviate the need for any further *in-vivo* testing. The purpose of these assays would be initial screening of meglumine and its metabolites *in-vitro*. The data holder accepted this proposal.

18. COC Members considered some additional data²³ provided by the data holder on the conduct of the carcinogenicity studies in the rat and mouse using Flunixin-meglumine and drew conclusions with regard to the adequacy of these studies for the assessment of Flunixin-meglumine and meglumine (see paragraph 12 above). The COM noted comments from the data holder on the rapid dissociation of Flunixin-meglumine into Flunixin and meglumine and the suggested fast metabolism of meglumine. The COM agreed that it would be useful for the data holder to consider undertaking appropriate ADME studies with meglumine using radiolabelled material.

COM conclusion and recommendations

19. The COM concluded:
- i. The mutagenicity data on Flunixin, Flunixin-meglumine and meglumine are relatively old and the studies have not been conducted to contemporary standards.
 - ii. It is therefore difficult to draw any definite conclusions on the mutagenicity of these chemicals. A number of prudent conclusions were agreed:
 - a. For Flunixin (a non-steroidal anti inflammatory veterinary medicine) there was limited evidence that Flunixin was mutagenic *in-vitro* but there was no evidence to suggest that flunixin had mutagenic potential *in-vivo*. Members felt the *in-vitro* mutagenicity data on this compound were inadequate and considered this should be raised with the CVMP.
 - b. For Flunixin-meglumine, (Flunixin in the form of the meglumine salt) there is limited evidence for a mutagenic effect *in-vitro*. In addition the inconclusive *in-vivo* data on meglumine suggest that a definite conclusion regarding flunixin-meglumine cannot be reached.
 - c. For meglumine, the available *in-vivo* mutagenicity data are inconsistent with some positive and negative results. It is **recommended** that additional *in-vitro* mutagenicity tests conducted to modern standards are undertaken with meglumine to assess whether meglumine has any mutagenic potential using the approach recommended in the COM Guidance. Negative results are available for a gene mutation assay in *Salmonella*. Therefore these additional tests should comprise:
 - i. An *in-vitro* chromosomal aberration test in mammalian cells.
 - ii. An *in-vitro* mouse lymphoma assay.

COM/03/S4 March 2003

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4. DNA polymerase deficiency assay; *Escherichia coli*; flunixin NMG. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref D15344.
5. Mitotic gene conversion; *Saccharomyces cerevisiae* – flunixin. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref A15345.
6. Ames Test – *S. typhimurium*; N-methyl-D-glucamine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref D15346.
7. Micronucleus-N-methyl-D-glucamine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref 15347.
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9. Anon. Activity of methylglucamine in the mouse bone marrow micronucleus assay. Report submitted to April 2002 COM meeting.
10. Ames Test – *S. typhimurium*; flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref 15335.
11. Assessment of genotoxicity in an unscheduled DNA synthesis assay using adult rat hepatocyte primary cultures. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref A18272.
12. Mitotic gene conversion; *Saccharomyces cerevisiae* – flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref A15340.
13. Mitotic gene conversion; *Saccharomyces cerevisiae* – flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref A15341.

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14. DNA polymerase deficient assay; *Escherichia coli*; flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. D15338.
 15. Evaluation of flunixin meglumine in the bacterial DNA repair test. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. D15339.
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 17. Evaluation of SCH 14714 NMG for mutagenic potential using L51787 Tk +/- cells. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref D14318.
 18. Mouse lymphoma – flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref D 15336.
 19. Mouse lymphoma mutation assay – Banamine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref A18271.
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 21. Micronucleus-flunixin meglumine. Flunixin Meglumine MRL Submission Dossier Study Reports, Volume 13. Ref D 15337.
 22. Committee on Mutagenicity (2000). Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. Guidance on a Strategy for Testing of Chemicals for Mutagenicity.
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Joint statement on review of malathion

Background

1. Malathion is an organophosphorous insecticide. It has been marketed in the UK for use in agriculture and horticulture since 1956. There were three products with approvals for use in agriculture and horticulture, home garden and use in pigeon lofts at the time when this review was initiated in January 2002. A number of products containing malathion are also licensed as human medicines for use in the control of head lice.
2. The Advisory Committee on Pesticides is reviewing the available toxicological information on malathion as part of its ongoing review of organophosphorous compounds. The ACP asked for advice from COM and COC on mutagenicity and carcinogenicity at its 289th meeting on 17 January 2002. There are inconsistent results in mutagenicity studies (both *in-vitro* and *in-vivo*) and there is evidence for the mutagenic activity of some impurities which may be present in some batches of technical malathion. There is also some limited evidence for tumourigenicity in rats (in particular the occurrence of benign nasal tumours in animals given high oral doses of technical grade malathion in the diet).
3. The Chairs of COM and COC agreed that a joint statement was required in view of the need for a full review of all mutagenicity and carcinogenicity data.
4. The COM undertook an initial consideration of the in confidence mutagenicity data provided by the pesticide data holder¹⁻⁵ and available published information provided by the data holder⁶⁻⁵⁵ at its 25 April 2002 meeting. A number of additional published papers on malathion⁵⁶⁻⁶¹ and impurities present in technical grade malathion were also considered at this meeting⁶²⁻⁶⁵. The COC reviewed the available carcinogenicity data on malathion which included in confidence reports (provided by the pesticide data holder regarding two studies in rats one in mice)⁶⁶⁻⁶⁸ and published reports of long-term bioassays in rats^{69,70} and mice⁶⁹ at its 27 June 2002 meeting. Three long-term bioassays using malaoxon (the principle metabolite of malathion and also present in technical grade malathion as an impurity) were available which included two in rats^{71,72} and one in mice⁷¹. In addition the Committee also considered in confidence reports of Peer Reviews of the histology slides from the 1993-96 malathion bioassay in F344 rats^{73,74}, the 1992-94 bioassay in B6C3F1 mice⁷⁵ and some additional supplemental information for the 1992-1994 bioassay in B6C3F1 mice⁷⁶ and the 1993-1996 bioassay of malaoxon in F344 rats⁷⁷ provided by the Pesticide Data holder. A number of additional follow up reports from the contract laboratory concerning the 1993-96 bioassay of malathion in F344 rats were also reviewed⁷⁸⁻⁸¹. In addition the pesticide data holder submitted a response to questions from COC secretariat which provided an overall summary of the histology of the nasal tissue in animals with tumours and additional evaluation of the historical control data on nasal tumours in F344 rats and possible mechanisms for nasal tumours induced in F344 rats fed high doses of technical grade malathion⁸². A published Peer Review of a number of published carcinogenicity bioassays was also available⁸³. The COC also considered expert reports from the EPA⁸⁴ and a Scientific Advisory Panel⁸⁵ established by EPA to review malathion. At its meeting of 10 October 2002 the COM considered some additional information provided in confidence by the pesticide data holder (a report of one additional *in-vivo* study⁸⁶ and information on the potential for variation in impurities between different sources of malathion⁸⁷) together with number of published studies not previously reviewed⁸⁸⁻⁹². The in confidence reports provided by the pesticides data holder contained appropriate Good Laboratory Practice and Quality Assurance statements.

Introduction to review

5. The Committees had access to sections of the draft risk assessment prepared by the Pesticides Safety Directorate (PSD) for the ACP. This include evaluations of the absorption, distribution, metabolism and excretion and toxicology data on malathion. Information on the manufacturing process used by the pesticide data holder and the impurities present in commercial technical material was presented.
6. The Committees noted that malathion is an organophosphorothiolate compound and required activation to malaoxon which inhibits cholinesterases in mammals. It is rapidly absorbed orally and is rapidly metabolised and excreted (mainly in the urine) as metabolites. Dermal absorption studies in human volunteers suggested a moderate absorption (6-15%) depending on formulation and malathion concentration. The available information from a wide range of toxicity studies (including acute, sub acute, sub chronic and chronic studies and reproduction studies in a range of species) demonstrated that inhibition of cholinesterase is the most sensitive toxicological effect in all available studies. The toxicity of malathion can be influenced by the presence of impurities in the technical material. Thus, for example, data presented in the PSD draft risk assessment showed that isomalathion and OOS-trimethyl phosphorothioate can inhibit the detoxification (via carboxyesterases and glutathione) of malaoxon resulting in a potentiation of cholinesterase effects of this compound. It was notable that the acute toxicity of technical grade malathion differed between two manufacturing sources. In answer to questions raised by the COM, the pesticide data holder reported that the levels of impurities would vary according to quality of raw materials, reaction parameters and purification steps. In addition it was also reported that the level of one impurity (isomalathion) could increase during storage of technical grade malathion. Thus the possibility that impurities may have mutagenic potential needed to be considered. The Committee agreed that the specification of the test materials used in mutagenicity studies undertaken by the pesticide data holder had been adequately demonstrated. The evidence provided by the Pesticide Data Holder suggested that the impurities present in technical grade malathion from other manufacturers would be qualitatively similar but it was not possible to draw any conclusions on the levels of the individual impurities in the test materials used in published mutagenicity studies.

COM review of mutagenicity

In-vitro data on malathion

7. It was agreed that the primary objective of the COM review was to consider the mutagenic potential of commercially supplied (e.g. technical grade) malathion. Technical grade malathion used in many of the reported mutagenicity tests had a purity of between 93-96%, whilst in a small number of published studies the authors reported using highly purified malathion ($\geq 99\%$)^{8,38,39,51}. The COM agreed that malathion was a DNA methylating agent⁵⁶. N7-Methyl guanine was the main identified adduct but others could not be excluded. There were also data to show that iso-malathion (an impurity formed during manufacture and storage) and OOO-trimethylphosphorothioate (a process impurity found in some batches of technical grade malathion) could alkylate nitro-benzyl-pyridine *in-vitro*⁶². The Committees noted the proposed metabolism pathway in the draft risk assessment document prepared by the Pesticides Safety Directorate (PSD) and agreed that there were a number of malathion metabolites identified in metabolism studies, which could theoretically methylate DNA.

8. The Committee agreed that there was no evidence for mutagenicity of technical grade malathion in bacteria. The results in a number of tests in *Salmonella typhimurium* and *Escherichia coli* were negative^{1,14,21,24,30,31}. The significance of enhanced SOS DNA repair activity in *Escherichia coli* was unclear⁵⁰. The COM noted that only brief details of the studies in yeast were available,^{21,31} but considered that further evaluation of the data would be of limited value in view of the clear evidence for in vitro mutagenicity in mammalian cells (see next para).
9. Regarding *in-vitro* assays in mammalian cells, the COM agreed that a cytogenetics assay with technical grade malathion conducted by the pesticide approval holder, was positive in the presence and absence of endogenous metabolic activation at the high dose (which was moderately cytotoxic)⁴. The Committee reviewed the available published literature and agreed that details of purity of malathion used was absent in several studies. However, the COM agreed that positive results had been obtained in published studies in human lymphocytes, where malathion was of technical grade or of higher purity ($\geq 99\%$)^{23,27,51}. Activity was reported both in the presence and absence of exogenous metabolic activation. Additionally, technical grade^{23,27} and purified malathion ($\geq 99\%$ pure)^{38,39} had been shown to induce Sister Chromatid Exchanges in mammalian cells (CHO cells) and human lymphocytes and fibroblasts. The COM also noted that evidence for a gene mutation in the *hprt* locus in human lymphocytes had been published⁴¹. The authors of this study noted that positive results had also been reported for different batches of technical grade malathion, which had contained slightly different levels of impurities. In a subsequent publication, these authors had provided limited information to suggest that malathion might induce specific mutations in the *hprt* gene in human lymphocytes. There was evidence for an increase in mutations at G:C base pairs and at GG dinucleotides in *hprt* gene in malathion treated cells compared to unexposed cells⁸⁹. The Committee agreed that the mouse lymphoma assay, recently conducted by the pesticide data holder using technical grade material, had also given positive results in the presence and absence of exogenous metabolic activation after a short 4-hour exposure period³. However, a second trial using a 24-hour exposure period was negative (COM Members commented that the control mutation frequency in this study was relatively high and this might mask a weak positive response).
10. The COM agreed that the UDS assay in isolated hepatocytes with technical grade material, which had been submitted by the pesticide data holder, was negative². The Committee noted that although the top dose level (0.16 $\mu\text{L}/\text{mL}$) had produced evidence of cytotoxicity, the dose might have been too low to detect any DNA damage induced by impurities rather than by malathion itself. The Committee noted that a recent COMET assay with malathion (98% pure) and using human peripheral blood lymphocytes had yielded negative results¹². However, no exogenous metabolic activation had been used and the exposure period (1hour) used in this study was shorter than expected for an adequate regulatory study (ca 3 or 24-h).
11. Overall the COM agreed that the available evidence was consistent with the conclusion that technical grade malathion (including its metabolites and impurities) induced the mutagenicity observed in *in-vitro* studies.

In-vitro data on malaoxon

12. The Committee reviewed the available *in-vitro* mutagenicity data on malaoxon, the principle metabolite of malathion. Malaoxon had mutagenic activity in two mouse lymphoma assays in the absence of exogenous metabolic activation, and induced SCEs in CHO cells^{64,65}. The test material used in these assays was approximately 94-96% pure. Malaoxon (98% pure) had also induced DNA damage in human peripheral blood lymphocytes in one assay⁶³.
13. The committee considered that the mutagenic activity seen *in-vitro* with technical grade malathion could, in part, be due to the metabolism of malathion to malaoxon.

In-vivo data on malathion

Studies submitted by the pesticide data holder

14. The Committee agreed that the oral rat bone marrow clastogenicity study, conducted by the pesticide data holder using high doses of technical grade material, had been adequately conducted and was negative⁵.
15. The Committee had reservations regarding the adequacy of the oral *in-vivo* liver UDS assay in rats submitted by the pesticide data holder⁸⁶. Members were concerned with regard to the apparent positive response in animals 24 and 25 in the assay. The Committee did not accept the rationale provided by the contract laboratory that these positive responses were caused by technical errors (staining errors, the timing of dosing relative to other rats and/or use of control rats sampled 2-4 hours after dosing as controls for treated rats sampled 12-16 hours after dosing). Members also considered it feasible that a positive control substance had been dosed to these animals by mistake or there had been a mislabelling of the animals. Whilst such explanations may be plausible, there was a lack of direct evidence linking the positive effects in the two rats to any experimental error. However, it was noted that no evidence of mutagenicity was found in a repeated 12-16 hour sampling time experiment. Overall the COM agreed that no weight could be attributed to the study and that it should be repeated. Negative results in such a repeat study would remove any concerns that technical grade malathion was mutagenic to rats when dosed orally.

Published Studies

16. The Committee was aware that there were a large number of published *in-vivo* studies which reported positive results for malathion. No definite conclusions could be drawn from the majority of these studies because the test material used was not of technical grade or the purity could not be determined and/or the methods used were inadequate by current standards^{6,8,18-20,28,33,42,57,90}. The Committee was aware that there were some studies which reported negative findings *in-vivo* with technical grade malathion^{17,46,52,53} but agreed that it was important to consider in detail the available studies where positive finding had been reported and where there was sufficient information reported to comment on the results^{18,57,90}.

Oral administration

17. Giri et al (2002)⁵⁷ documented limited evidence for an increase in chromosomal aberrations in bone-marrow of groups of 3 Swiss mice (sex not given) given either a single oral dose of 5 mg/kg bw or five daily doses of 2 mg/kg bw. A fixation time of 24 hours after the last dose was used. The test material used was a technical grade material manufactured in India⁹³. The Committee agreed that in view of the lack of evidence for carcinogenicity in the long-term feeding carcinogenicity bioassay in mice, that there was no need for a further oral study in mice at this juncture.

Dermal administration

18. The Committee considered the study by Dulout *et al.* (1982)⁹⁰ which had used both the dermal and intraperitoneal routes of dosing in bone-marrow micronucleus assays in mice. Members agreed it was difficult to explain why such a strong positive response had been documented following dermal application compared to intraperitoneal dosing. The test material (a technical grade material manufactured in Argentina) had been dissolved in corn oil which would have led to residual vehicle on the skin. Members noted that dermal absorption in human volunteers ranged from 5% to 15% depending on formulation and dilution with water. It was not possible to quantify the extent of dermal absorption in this study. There would have also been some oral ingestion arising by grooming of the animals, but this could not be quantified. Members noted that there was no evidence for a dose response following dermal administration in this study, but felt that the results could not be refuted and thus there was a need for a repeat study in mice.

Intraperitoneal administration

19. The Committee agreed there was evidence from several studies for a mutagenic effect of technical grade malathion in the bone-marrow of mice given the test material by intraperitoneal administration^{18,57,90}. The Committee agreed that these data suggested technical grade malathion had mutagenic activity *in-vivo* in mice and reinforced the need to evaluate dermal exposure to technical grade malathion adequately.
20. Overall the Committee concluded that malathion was mutagenic *in-vivo* in mice dosed by the intraperitoneal route, and possibly also by the oral and dermal routes. Conversely, there was no evidence of *in-vivo* mutagenicity in rats dosed orally. These data may indicate a species-specific *in-vivo* mutagenicity in mice.

Studies in humans exposed to malathion and other pesticides

21. The Committee discussed the available studies in humans exposed to malathion^{14,29,35,44,47,49,54}. It was noted that an increase in the incidence of micronuclei had been reported by the Californian Health Department, in a preliminary study of 13 applicators who used malathion in 1992^{14,54}. This result was not confirmed by a further study undertaken by the same group of researchers in 1993^{14,54}. Overall the Committee felt that no conclusions could be drawn from this study. The Committee considered that no conclusions could be derived from the other available published studies relating to monitoring of human exposure for mutagenic effects. The Committee concluded that appropriately designed studies of pesticide applicators using malathion, might be informative with regard to mutagenic activity in humans.

Response of data holder to COM Evaluation (19/11/02 and 2/01/03)

22. The data holder provided some additional data on the 19 November 2002 on the *in-vivo* liver UDS assay in rats⁸⁶, namely additional details of the conduct of the study and results in animals 24 and 25 and photomicrographs of slides from animal 24 and from a negative and positive control animal. It was noted that additional repeat investigations had been included in the study which had been triggered when technical difficulties concerning animals 24 and 25 had been noted. The data holder provided an additional *in-vivo* intraperitoneal micronucleus assay in the mouse⁹⁴. The data holder provided comments on the dermal *in-vivo* micronucleus assay in mice published by Dulout *et al*⁹⁰. Additional photomicrographs and comments on the conduct of the rat liver UDS assay were provided by the pesticide data holder on 2 January 2003.

COM comments

23. Regarding the new data on the rat liver UDS assay, COM members considered the additional data submitted on the 19/11/02 and on 2/01/03 and agreed that the assay was flawed. The arguments regarding the background staining of the slides did not match with the reported positive NNG values for animals 24 and 25. Overall members could not endorse this assay. The lack of evidence for a carcinogenic effect in livers of rats dosed with malathion is noted. However considering the clear mutagenicity of malathion *in-vitro* and in some other *in-vivo* assays, an adequate, technically defensible assay is considered appropriate.
24. Members noted the new intraperitoneal bone marrow micronucleus test which gave negative results and provided some reassurance, although the specification of the test material was uncertain and only 1000 PCEs had been scored⁹⁴.
25. COM members acknowledged there are considerable problems with the conduct of study reported by Dulout *et al.* 1982⁹⁰. However members felt that the pesticide data holder had not provided a rationale to suggest it was a false positive response and agreed that a repeat dermal assay was appropriate.

26. The COM considered other comments from the Data holder regarding the interpretation of human studies and the assessment of *in-vitro* studies with malathion. The Committee agreed that no alterations with regard to these areas of the statement were required.

COM conclusions

27. The Committee agreed the following conclusions:
- i. Technical grade malathion is mutagenic *in-vitro* in human lymphocytes and mammalian cells (mouse lymphoma cells). The observed mutagenic activity is due to malathion, its metabolites and its impurities.
 - ii. The weight of evidence suggests technical grade malathion is not mutagenic following oral administration to rats. There is a need for a further *in-vivo* oral rat liver UDS assay with technical grade material supplied in the UK. Negative results in such an assay would provide full reassurance with regard to oral exposure of rats to technical grade malathion. There is good evidence for a mutagenic effect of technical grade malathion given intraperitoneally to mice, and some limited evidence for mutagenicity in mice with oral or dermal administration. The Committee recommends that a further study using dermal administration in mice would be appropriate to adequately assess the potential for mutagenicity by this route of exposure as this is particularly relevant to the route of human exposure.
 - iii. There are a number of studies of humans exposed to malathion which have investigated potential mutagenic effects. There are limitations in all of these studies predominantly due to small numbers of subjects, study design and co-exposure to other chemicals which might have mutagenic effects. No conclusions could be drawn from the available data. Further monitoring of populations exposed to malathion might be informative with regard to potential mutagenic activity in humans.

COC review of carcinogenicity

28. The Committee reviewed the conduct, adequacy and results from the available carcinogenicity bioassays in rats and mice where malathion or malaoxon had been administered in the diet.⁶⁶⁻⁷² In reviewing these studies, the COC agreed that particular reference should be made to the evaluation of nasal tissue. The Committee commented that nasal tissue was generally not examined in carcinogenicity bioassays until around 1990. The Committee made the following comments on each of the long-term bioassays.

Malathion bioassays in rats

29. NCI 1978, *Osborne Mendel rat*⁶⁹. The study authors had reported that there was an increase in thyroid follicular cell carcinomas/adenomas in females in the high dose group. A peer review of pathology had concluded that there was no significant increase in thyroid tumours⁸³. The COC noted there was no examination of nasal tissue, non-neoplastic pathology was reported in females but not males, there were no full necropsies of decedents and the control groups were too small. Overall it was agreed that this study was inadequate by contemporary standards and no conclusions could be drawn.
30. FDRL 1980, *Sprague Dawley Rat*⁶⁶. The study authors had not reported any evidence for a treatment related carcinogenic effect. The committee noted that there was no examination of nasal tissue. With this limitation noted, the committee considered the study had in general been adequately undertaken.
31. NCI 1979, *Rat F344*⁷¹. There was an increase incidence in phaeochromocytomas in low dose but this was not found in high dose males, and the absence of adrenal medullary hyperplasia further questioned the significance of this finding. There was limited evidence for increased leukaemia in low dose males. It was agreed that this study provided no consistent evidence for a carcinogenic effect. The COC noted that examination of nasal tissue had not been undertaken in this study, but agreed that overall the study had in general been conducted adequately.
32. HLS 1993-1996, *Rat F344*⁷². The COC agreed that this bioassay was conducted to modern standards and was the critical carcinogenicity study in the rat. Dietary levels used were 100 ppm (reduced to 50 ppm at month 3), 500 ppm, 6000 ppm and 12000 ppm. (Averaged dose levels (mg/kg bw/day) in males and females were respectively; 4/5, 29/35, 359/415, 739/868 mg/kg bw/day). The COC considered that the top two dose levels in males exceeded the Maximum Tolerated Dose (MTD) level. This was based on reduced mean body weight (-16.8% at month 18 in the 12000 ppm group and -11.1% at 6000 ppm) and statistically increased mortality compared to control animals. In females the MTD was exceeded at 12000 ppm (based on reduced mean body weight at 24 months; -16.8% and significant mortality compared to controls) but was not exceeded at 6000 ppm (mean reduction in body weight < 5.1% compared to controls, 62% survival to termination). The study investigators had documented two nasal tumours in males (an olfactory epithelial adenoma in one animal at 6000 ppm and an olfactory epithelial carcinoma in one animal at 12000 ppm). The COC noted that an independent peer review of the nasal pathology had been undertaken⁷³. The COC concluded that the report of the peer review was satisfactory and agreed that the classification of tumours used in this review should be used in the evaluation of this cancer bioassay. Following the peer review of the pathology, a consensus agreement had been reached that there were three nasal respiratory adenomas (one male and one female) at 12000 ppm and one female at 6000 ppm) and an olfactory epithelial adenoma in one male at 6000 ppm. These tumours were associated with severe non-neoplastic pathology in the nasal tissue at 6000 and 12000 ppm (including epithelial degeneration, hyperplasia and inflammation). The peer review also documented four tumours of the oral palate. The data holder had provided a concise summary of the neoplastic pathology in animals with nasal tumours⁸². The COC observed that based on the relatively small laboratory historical control data, the incidence of nasal tumours was highly statistically significant. However it was evident from the EPA review and information supplied by the data holder that the background incidence of nasal tumours was unclear and depended upon the number of nasal sections used in bioassays^{82,84}. In modern bioassays this would comprise 5 sections

whilst only two sections were used in the bioassays in the laboratory historical control database. The historical control range from 20 bioassays undertaken as part of the NTP program was 0-2% in both males and females. The Committee considered that overall there was evidence of a tumourigenic response to malathion in the nasal tissue of the rat but the observed tumours were induced in tissue which was subject to severe ongoing nasal inflammation. The COC agreed there was an increase in liver tumours at the two highest dose levels and that this was likely to be due to an effect of treatment on the background pathology of ageing animals.

Malathion bioassays in mice

33. *NCI 1978, Mouse B6C3F1*⁶⁹. The COC observed that there was no examination of nasal tissue, there were no full necropsies of decedents and the control groups were too small. A statistically significant increase in combined hepatocellular carcinoma and neoplastic nodule were reported in males animals by trend analysis. No increase was reported when analysed by pair wise comparison or by life-time analysis. The Committee commented that the terminology used to describe histological lesions of the liver was now considered outdated and that there was a high incidence of hepatocellular carcinoma in historical control groups. The committee agreed that by contemporary standards this bioassay was inadequate. The Committee observed the evidence of coughing and wheezing in most high dose animals from week 72 which provided some evidence for an effect on the respiratory tract.
34. *HLS 1992-1994, Mouse B6C3F1*⁶⁸. The COC agreed that this bioassay had been conducted to modern standards. Dietary levels of 100 ppm, 800 ppm, 8,000 ppm, and 16000 ppm were used. (Averaged dose levels (mg/kg bw/day) in males and females were respectively; 17.4/20.8, 143/167, 1476/1707, 2978/3448 mg/kg bw/day) The COC agreed that the Maximum Tolerated Dose level was exceeded at 16 000 ppm in both males (-20 % reduction in mean body weight compared to controls at week 78) and in females (-16% reduction in mean body weight compared to controls at week 78). The MTD was exceeded in males at 8000 ppm (-14% reduction in mean body weight compared to controls at week 78). The COC considered that the 8000 ppm dose level in females equated to the MTD (reduction in mean body weight for females was -9.7%). The study investigators reported the finding of hepatocellular adenoma, hepatocellular hypertrophy and increase liver weight in most mice (male and female) at 8000 ppm and 16000 ppm. A Peer review of histology in male mice essentially confirmed the liver findings reported by the study investigators⁷⁵. The Peer review group also identified significant and excessive non neoplastic pathology of the nasal tissue similar to that reported in F344 rats. The Committee agreed that the liver tumours were treatment related.

Malaoxon bioassays in rats

35. *NCI 1979, Rat F344*⁷¹. A statistically significant increase in thyroid C-cell adenomas and carcinomas were reported in females at the top dose. A Peer review had been undertaken⁸³. The Committee agreed with the conclusion reached by the peer review group that the evidence for a carcinogenic effect in the thyroid was equivocal. The Committee noted that examination of nasal tissue had not been undertaken in this study. The Committee noted the high dietary level was 1000 ppm, but agreed that, based on the preliminary 13 week study (no effects on body weight or histology at 2000 ppm), a higher dose level might have been used.

36. *HLS 1993-1996, Rat F344*⁷². The COC agreed that this bioassay had been conducted to modern standards. Dietary levels of 20 ppm, 1000 ppm, and 2,000 ppm were used. (Averaged dose levels (mg/kg bw/day) in males and females were respectively; 1/1, 57/68, 114/141 mg/kg bw/day) The Committee considered that the Maximum Tolerated Dose level had not been exceeded in this study (-1.4% to -7.1% reduction in body weight compared to controls in males and -4.0% to -8.8% in females at termination). However a dose related increase in mortality was reported in males. A significant dose-related trend in mononuclear cell leukaemia was reported in males but no statistically significant increase was evident in pair wise comparisons with controls. The incidence of this tumour was within the historical control for the laboratory. A significant increase in testicular interstitial cell tumours was reported at 2000 ppm when the data were corrected for time to tumour identification. However the observed increase was within the historical control incidence. There was thus no evidence for any treatment related increase in neoplasia. The study investigators found significant non-neoplastic pathology of the nasal tissue and respiratory tract. This included significant purulent inflammation of the lungs. There was evidence of food particle deposition in many animals in this study.

Malaoxon bioassay in mice

37. *NCI 1979, Mouse B6C3F1*⁷¹. The COC noted that examination of nasal tissue had not been undertaken in this study. The Committee noted the high dietary level was 1000 ppm, but agreed that, based on the preliminary 13 week study (no effects on body weight at 2000 ppm), a higher dose level might have been used. No treatment related neoplasia were reported.

Overall malathion carcinogenicity evaluation

38. The COC agreed that the assessment of the significance of the observed nasal tumours in F344 rats in the modern study undertaken by HLS USA was the critical neoplasm for discussion. The Committee agreed that the finding of liver tumours in F344 rats and B6C3F1 mice in the modern studies should also be evaluated in detail. The findings reported in earlier studies undertaken in the 1970's and 80's contributed little weight of evidence to the assessment of malathion.

Nasal tumours in F344 rats

39. The COC noted that tumours of the nasal tissue in F344 rats were rare and that there were difficulties in assessment particularly with regard to the adequacy of historical control data from the laboratory. The COC noted the additional sectioning and microscopic evaluation of additional slides from the HLS USA study suggested that the US NTP historical control data for F344 rats might be more appropriate. Overall it was agreed that a tumourigenic response had been documented in this study. The COC observed that the observed tumours were all benign and included an olfactory epithelial adenoma arising from the Bowmans' gland in a male fed 6000 ppm and a respiratory epithelial adenoma in a male fed 12000 ppm. In females one animal at 6000 ppm and one at 12000 ppm had respiratory epithelial adenomas. All tumours were well defined and there was no evidence of pleomorphism or atypia⁸². The Committee considered that the significance of these tumours had to be assessed against a background of severe inflammation, which exceeded that found in inhalation carcinogenicity studies with chemicals that were directly irritant to the nasal tissue. It was unclear from the HLS malathion

study in F344 rats to what extent inhalation of food particles contributed to the induction of this inflammatory response in this study. The Committee agreed that direct irritant effects on the nasal passages was possible when animals were fed powdered diets. It was noted that the food pots used in the malathion study had some degree of covering which might have limited inhalation of food particles. It was noted that there was clear evidence to show the effect of food particles in the induction of localised inflammation of the nasal passages and the lungs in the malaoxon bioassay in F344 rats undertaken at the same laboratory as the critical malathion F344 rat bioassay. The Committee considered that the proposal from the data holder that de-esterification of malathion in nasal tissue to form acids was also potentially plausible. It was noted that nasal tissue would have appropriate metabolic capacity to metabolise malathion to its corresponding diacid metabolites. The Committee discussed the possibility of a genotoxic mechanism in the induction of nasal tumours. However the weight of evidence including the formation of tumours only at excessive doses, the evidence for severe prolonged localised inflammation in the target tissue, and the lack of multi-organ response suggested that a non-genotoxic mechanism was probable in this instance.

Liver tumours in F344 rats and B6C3F1 mice

40. The Peer review pathology report of the Huntingdon Life Sciences bioassay in F344 rats conducted between 1993 and 1996 reported a consensus incidence of 5/70 (7.1%) hepatocellular adenomas in females at a dietary level of 12000 ppm (ca 868 mg/kg bw/day)⁷⁴. The historical control incidence for the laboratory was 0-5.4% (n= 254). The Peer review report noted that there were a number of non-neoplastic microscopic findings in the five animals with tumours, which included hypertrophy, congestion and vacuolation. The COC agreed that the time of death and liver pathology seen in these animals would appear to be generally consistent with ageing pathology in this strain of rat. There was a significant increase in mortality in the female high dose group from month 18 to 24 (above that seen in controls) resulting in limited numbers of animals available at terminal sacrifice and thus the possibility existed that there might be some animals which could have developed liver tumours being missed. However there was no evidence of a dose response in females and only one hepatocellular adenoma was found in treated males (in a high dose male). All the available pathology evidence suggested that these tumours are most likely to be an effect of malathion on the background pathology of ageing F344 rats and are unlikely to be of significance for human health.
41. The COC commented that the increased incidence of hepatocellular adenomas in male and female B6C3F1 mice was associated with clearly overt increases in liver weight and hepatocellular hypertrophy and are unlikely to be significant to humans. It was suggested that, given the evidence for hepatocellular hypertrophy induced at doses exceeding the MTD, that it was highly likely that these tumours were induced through a non-genotoxic mechanism of technical grade malathion.

Overall evaluation of malaoxon

42. The Committee concluded that there was no evidence for a carcinogenic effect of malaoxon, the main metabolite of malathion.

COC conclusion

43. The COC drew the following overall conclusion:

- i. The COC agreed that technical grade malathion had been tested in four long-term dietary bioassays in rats and two long-term dietary bioassays in mice. The most recent studies undertaken in F344 rats (1993-96) and in B6C3F1 mice (1992-94) were adequate for the evaluation of carcinogenicity. There is evidence for tumourigenicity in the nasal tissue and liver (females only) of F344 to rats fed malathion. The nasal tumours were associated with severe ongoing inflammation, which is most likely involved in the mechanism of tumourigenesis. There was evidence for liver tumours in female F344 rats and male and female B6C3F1 mice. The weight of evidence suggested that these liver tumours were induced through a non-genotoxic mechanism and were not relevant to human health.

Overall conclusion: Mutagenicity and Carcinogenicity of Malathion

44. The Committees agreed the following overall conclusion:

- i. Technical grade malathion is mutagenic *in-vitro*. The observed activity is due to malathion, its metabolites and impurities.
- ii. The evidence from mutagenicity studies suggests technical grade malathion is not mutagenic in rats by the oral route. There is a need for a further *in-vivo* oral rat liver UDS assay with technical grade material supplied in the UK. Negative results in such an assay would provide full reassurance with regard to oral exposure of rats to technical grade malathion.
- iii. There is good evidence from published studies that technical grade malathion is mutagenic in mice following intraperitoneal administration and some limited evidence for mutagenicity in mice following oral and dermal administration. These data may indicate a species-specific *in-vivo* mutagenicity in mice. The Committee recommends that a further study using dermal administration in mice is appropriate to adequately assess the potential for mutagenicity by this route of exposure as this is particularly relevant to humans.
- iv. Technical grade malathion has been shown to induce benign tumours at high oral doses which were above the Maximum Tolerated Dose in rats (nasal tissue and liver (females only) and in mice (liver) following administration via the diet in long term bioassays. The nasal tumours were associated with severe ongoing inflammation, which is most likely involved in the mechanism of tumourigenesis. The weight of evidence suggested that the liver tumours were not relevant to human health. The Committees concluded that the nasal and liver tumours were induced by non-genotoxic mechanisms.

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Statement on 2-phenylphenol and its sodium salt

Introduction

1. 2-Phenylphenol and its sodium salt are broad spectrum fungicides that are approved in the UK for use as wood preservatives. They are also used as surface biocides in a number of areas. The COM have advised on the mutagenicity of these compounds, specifically in the context of the mechanism of the bladder tumours seen in male rats fed high doses of these compounds, on a number of occasions, the most recent being in 1997. Significant new data are now available. Furthermore an EU review of the use of 2-phenylphenol and its sodium salt in wood preservation is shortly to be initiated under the Biocidal Products Directive (98/8/EC). It would thus be timely to update the COM view on the mutagenicity of these compounds. This information would be helpful in developing the UK position with regard to the EU in the context of the Biocidal Products Directive.
2. The advice of the COM was thus sought on the mutagenicity of 2-phenylphenol and its sodium salt, and specifically whether the induction of bladder tumours at high dose levels in male rats in the chronic bioassay is likely to have arisen from a genotoxic mechanism. If this were the case the risk assessment would need to adopt a non-threshold approach.

Background

3. The COM gave detailed consideration to a comprehensive review of the mutagenicity of 2-phenylphenol in 1992 and agreed the following conclusions:
 - i. Data from several assays to investigate the ability of 2-phenylphenol or its sodium salt to produce gene mutation in *Salmonella typhimurium* were consistently negative.
 - ii. Positive results were obtained in *in-vitro* metaphase analysis studies in CHO cells*. Mouse lymphoma assays in the presence of exogenous metabolic activation also gave positive results in the form of small colonies. These results suggested that a metabolite of 2-phenylphenol had clastogenic potential. These findings conflicted with an earlier *in-vitro* metaphase analysis in a single study.
 - iii. Negative results were obtained in bone marrow assays for clastogenicity *in vivo* and also in germ cells (dominant lethal assay), indicating that any possible clastogenic potential was not expressed in the whole mammal.
 - iv. Conflicting results appeared to be obtained in *in-vivo* assays for effects on DNA in bladder epithelium, the target tissue of concern. The Committee therefore wished to see data from a more sensitive *in-vivo* method to investigate adduct formation. There was insufficient evidence to recommend a departure from the use of the safety factor approach for regulation of this compound at the present time.

*Not included in the 1992 conclusions. The positive results seen in the *in vitro* metaphase study in CHO cells were in the presence of an exogenous metabolic activation system.

4. In the light of this advice the Advisory Committee on Pesticides (ACP) requested further data relating to DNA adduct formation in the urinary bladder epithelium of the male rat with particular emphasis on the dose response relationship at levels known to produce hyperplastic and neoplastic changes in male rats.
5. In 1997 industry provided these data to the registration authority (HSE) which was referred to COM for an opinion. The following conclusions were drawn by the COM:

The Committee expressed concern regarding the limitations of methodology used in the study entitled “³²P-post-labelling study of technical grade 2-phenylphenol to examine the potential for the formation of DNA adducts in the urinary bladder of the male rat.” The Committee requested additional analyses of the bladder epithelial samples from this study using an appropriate sensitive adduct enrichment method for the detection ³²P-postlabelled adducts (namely both nuclease P₁ and butanol extraction) and appropriate control experiments to evaluate the fate of 2-phenylphenol DNA adducts during the extraction and enrichment procedures.

6. The ACP considered the COM opinion, together with additional data from specialist feeding studies/carcinogenicity bioassays in 1998 and concluded that the post-review data requirements had been met and confirmed that a threshold based approach remained relevant for risk assessment purposes.

Consideration of new data published since the comprehensive review in 1992

7. A number of papers relating to the metabolism of 2-phenylphenol and its sodium salt and the production of reactive species were considered together with DNA adduct studies in rat bladder epithelium, mutagenicity data on the quinone metabolites and other data relevant to the mutagenicity of 2-phenylphenol. This new information is summarised below.

Metabolism

8. The metabolic profile of 2-phenylphenol is now well established^{1, 2, 3}. Recent data on the metabolism of 2-phenylphenol in the rat and the mouse confirms that at relatively low dose levels (around 20mg/kg bw/day) the compound is metabolised and excreted in the urine as sulphate, or to a lesser extent, the glucuronide conjugate¹. At higher doses increased levels of conjugates of PHQ were present, but very little non-conjugated PHQ or PBQ. Studies on the comparative metabolism in the rat and the mouse indicate only minor differences². It is difficult to see how these could explain the marked species difference in the induction of bladder tumours in the male rat and not the mouse. It is possible that localised deconjugation of PHQ glucuronide (or sulphate) may play a role at high dose levels in the male rat².

Formation of DNA adducts in male rat bladder epithelium

9. Further data are now available on the ability of 2-phenylphenol or its sodium salt to induce DNA adducts in the rat bladder *in vivo*. The earlier studies had given conflicting results, with negative results using radioactivity detection following a very high acute exposure with ^{14}C -2-phenylphenol, but positive results using ^{32}P -postlabelling in a subchronic dietary study using a single high dose level (2%) and examining total bladder DNA (rather than epithelial DNA)^{4,5}.
10. The study considered by the COM in 1997, and criticised because of its insensitivity, has now been published, and a rationale for the chosen methodology was given⁶. The Committee did not agree with this and maintained their view that an appropriate sensitive enrichment method for the detection of ^{32}P -post-labelled adducts should have been used. However a study to investigate both DNA and protein binding in the liver, kidney and bladder of male F344 rats has now been published using the highly sensitive AMS technique⁷. An extensive dose response was investigated, (6 single doses by gavage over the range 15-1000mg/kg 2-phenylphenol). A clear dose-related and essentially linear response was seen with protein binding in the liver and kidney, but protein binding was seen in the bladder only at high dose levels (about 500mg/kg and above). There was no evidence for DNA binding at any dose level in bladder tissue.
11. The Committee agreed that the weight of evidence from *in-vivo* studies is now sufficient to conclude that 2-phenylphenol does not produce significant DNA binding in the male rat bladder. However most of the available data were from short term studies and the one, limited, subchronic study had provided some evidence of adduct formation in bladder DNA. The Committee thus felt that the possibility of prolonged high level exposure producing DNA adducts could not be entirely discounted.

Genotoxicity of PHQ and PBQ

12. A number of studies have investigated the ability of the PHQ or PBQ metabolites to induce chromosome damage, micronuclei or DNA damage in HL60 or V79 cells^{8,9,10}. There was also some evidence for the induction of aneuploidy⁸. Positive results were obtained, sometimes only in the presence of arachidonic acid supplementation. Members noted that these cells were readily stimulated to generate an oxidative environment. The relevance of positive data with high concentrations of metabolites rather than the parent compound, was felt to be questionable. Members felt that it was not possible to exclude oxidative damage arising from PBQ or PHQ contributing to the induction of bladder tumours at high dose levels, but they agreed that such effects would not be expected to occur at low dose levels.

Additional In-vivo Mutagenicity Data

13. Members considered data from a study to investigate micronucleus induction cell proliferation and hyperdiploidy in bladder epithelium cells of rats treated with 2-phenylphenol at 2% in the diet¹¹. An increase in micronuclei, but no effect on chromosome number, was reported. However, in view of the limitations of this study, particularly the failure to be able to distinguish micronuclei from cell debris, it was felt that no conclusions could be drawn from the reported micronucleus induction.

14. The Committee also considered the results obtained in a study in rats to investigate DNA damage in stomach, liver, kidney, bladder, brain and bone marrow using a modified version of the COMET assay based on isolated nuclei¹². The results were suggestive of high dose activity in the stomach, liver, kidney and lung but not the bladder. There were concerns however as to whether the method used at that time would have adequately distinguished between genotoxicity and cytotoxicity, and it was felt that no definite conclusions could be drawn.

Conclusions

15. The Committee agreed the following overall conclusions regarding the mutagenicity of 2-phenylphenol and its sodium salt:
 - i. Data from several assays to investigate the ability of 2-phenylphenol or its sodium salt to produce gene mutation in *Salmonella typhimurium* were consistently negative.
 - ii. Positive results were obtained in *in-vitro* metaphase analysis studies in CHO cells and also in mouse lymphoma assays in both cases in the presence of an exogenous metabolic activation system. The induction of small colonies in the latter assay is consistent with the compound having clastogenic potential.
 - iii. Phenylhydroquinone (PHQ) and phenylbenzoquinone (PBQ), which are metabolites of 2-phenylphenol, PHQ usually present as conjugates, have been shown to produce oxidative DNA damage and single strand DNA breaks *in-vitro* using HL60 and V-79 cells.
 - iv. Negative results were obtained in bone marrow assays for clastogenicity *in-vivo* and also in germ cells (dominant lethal assay), suggesting that any possible clastogenic potential was not expressed in the whole mammal.
 - v. The weight of evidence from *in-vivo* studies to investigate 2-phenylphenol binding to DNA in the male rat bladder was negative: a recent study using a highly sensitive AMS techniques was particularly important in this regard. However the possibility of prolonged high level exposure producing DNA adducts cannot be entirely discounted.
 - vi. Although a contributory role of oxidative DNA damage cannot be excluded when considering the mechanisms of bladder tumour induction in the male rat, this would not be expected to occur at low dose levels.
 - vii. The committee concluded that it would be reasonable to adopt a threshold based risk assessment for 2-phenylphenol and its sodium salt.

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Statement on guidance on considering high dose positive *in-vivo* mutagenicity data in the bone marrow assays that may not be biologically significant with regard to considering a chemical to be an *in-vivo* mutagen

Introduction

1. The advice of the COM previously has been that compounds that are clearly positive in any *in-vivo* mutagenicity assay should be regarded as mammalian mutagens (and hence potential genotoxic carcinogens)¹. (<http://www.doh.gov.uk/com/guidance.pdf>). Furthermore the COM consider that for such compounds it is prudent to assume that there is no threshold for their mutagenic activity, unless appropriate mechanistic data can be provided to identify a threshold related mechanism, e.g. induction of aneuploidy, where the site of initial action is not DNA but the spindle apparatus². (<http://www.doh.gov.uk/comivm.htm>). Such mechanistic data are rarely available.
2. This approach has major implications with regard to risk assessment of such chemicals, with the assumption that there is no 'safe' level and that any exposure results in some increased health detriment, albeit this may be very small.
3. In practice this means that much weight is placed on the *in-vivo* genotoxicity assays recommended by the COM in their strategy of testing, in particular on one of the bone marrow assays for clastogenicity (usually the micronucleus test) which is usually the initial assay investigated. A positive in one of these assays results in the compound being considered as an *in-vivo* mutagen. It is thus especially important that such decisions are based on sound data, and that the positive results truly reflect *in-vivo* mutagenic activity. In some cases such positive results in the bone marrow assays have only been seen at high dose levels associated with severe toxicity including lethality, giving rise to concern at the relevance of the results. This is particularly true if the overall weight of evidence from the *in-vivo* data, including the results of carcinogenicity bioassays, is not supportive of the compound being an *in-vivo* genotoxin.
4. Furthermore it is now recognised that in the case of the *in-vivo* micronucleus test, micronuclei can be induced by mechanisms such as hypothermia, hyperthermia, stimulation of erythropoiesis and possibly indirectly due to severe cytotoxicity in the bone marrow. In such cases the positive results, if not supported by other *in-vivo* data, may be regarded as artefacts, which do not reflect mutagenicity of the compound itself in animals.
5. Draft COM guidance on the interpretation of *in-vivo* bone marrow mutagenicity data that may give rise to concern with regard to being a 'false positive' is provided in this document.

Guidance on 'false positive' *in-vivo* mutagenicity data

6. The COM guidance on a strategy for testing chemicals for mutagenicity is based on the identification of compounds with mutagenic potential on the basis of a small number of well conducted *in-vitro* tests. This is followed by establishing whether activity seen *in-vitro* can be expressed *in-vivo* in somatic cells, to establish whether the compound can be regarded as an *in-vivo* somatic cell mutagen and hence a potential carcinogen. In the initial *in-vitro* testing it is recognised in the guidelines that artifactual positives may be obtained in the mammalian cell assays which do not reflect intrinsic mutagenic activity, and that this uncertainty may be resolved by *in-vivo* assays, and specifically the bone marrow assays for clastogenicity. Until recently there has been little consideration as to whether artifactual positive results can also be obtained in the *in-vivo* bone marrow assays.
7. However it is now known that effects such as hyperthermia^{3,4} or hypothermia^{5,6}, which can be produced as a secondary toxic effect at high dose levels with certain compounds, can produce micronuclei in bone marrow cells *in-vivo*. Another possible mechanism is the induction of erythropoiesis. This was demonstrated following unexpected positive results in the bone marrow micronucleus test with a series of recombinant human erythropoietin products; similar effects were later shown with native erythropoietin^{7,8,9}. The induction of micronuclei by such non-DNA reactive compounds has been shown to be due to the accelerated proliferation and differentiation of erythrocytes and promotion of early release of PCEs resulting in the micronucleated PCEs.
8. Another possible mechanism is excessive toxicity at high dose levels, associated with severe toxicity including lethality, i.e. higher levels than recommended in the current OECD guidelines. Such 'high dose' studies may possibly give positive results due to the severe toxicity seen in the bone marrow. Thus there are a number of reasons why the *in-vivo* data may not indicate that the compound is an *in-vivo* mutagen but further information would be needed to draw any definite conclusions.
9. In considering whether positive results in *in-vivo* bone marrow mutagenicity data may be caused by factors other than mutagenicity of the compound itself, or its metabolites, it is important to consider all the available data regarding the mutagenic profile of the chemical and whether the hypothesis that it is not an *in-vivo* mutagen is biologically plausible. This will include information on structure-activity relationships, data from *in-vitro* mutagenicity assays, other *in-vivo* mutagenicity data, information on toxicokinetics of the substances and any available data from carcinogenicity bioassays. Each compound needs to be considered on a case-by-case basis.
10. An important consideration with regard to data from bone marrow assays for clastogenicity (metaphase analysis or micronuclei) is the highest dose level used. The guidance in the current OECD guidelines is that the top dose should produce signs of toxicity such that higher dose levels, based on the same dosing regime, would be expected to produce lethality (or for non-toxic compounds it should be set at a limit dose of 2 grams/kg for single doses and 1 gram/kg for multiple dosing regimes)¹⁰. In the past considerably higher dose levels have been used, and on occasions the relevance of such data needs to be assessed. In the most extreme case, when positive results have been

obtained only at highly toxic or lethal dose levels, the Committee believe that the results obtained are confounded by toxicity and cannot be interpreted with any certainty in these circumstances; it is thus necessary to repeat the test using lower, more appropriate dose levels. It may, however, be possible to utilise the available data from the lower dose levels, together with other relevant information, to enable the isolated high dose *in-vivo* positive data to be discounted.

Consideration of carcinogenicity data (if available)

11. The implications of mutagenicity data with regard to carcinogenicity was considered in some detail in the 1989 COM guidelines for the testing of chemicals for mutagenicity and also in the 1991 COC guidelines on the evaluation of chemicals for carcinogenicity^{11,12}. At that time both committees concluded that it was reasonable and prudent to regard compounds that were mutagens *in-vivo* in the somatic cell assays recommended by the COM to also have carcinogenic potential even though such potential had not been examined or had not been demonstrated in formal carcinogenicity tests.
12. The updated COM guidelines on a strategy for testing chemicals for mutagenicity published in December 2000 endorsed the view that *in-vivo* somatic cell mutagens should be regarded as potential carcinogens¹. These conclusions have implications with regard to the testing of chemicals for carcinogenicity. The need to use large numbers of animals in carcinogenicity bioassays on compounds that were clear *in-vivo* somatic cell mutagens is unlikely to be justified; this is recognised in the 1991 COC guidelines. The COC is currently updating its guidance on risk assessment of carcinogens.
13. These conclusions also have implications regarding how carcinogenicity bioassay data that are not supportive of a compound being a genotoxic carcinogen may be used to assist in the interpretation of *in-vivo* mutation data when there is concern about the relevance of such data.
14. On occasions in the past the COM have used negative data from carcinogenicity bioassays in this way. An example was the chemical incapacitant CS (2-chlorobenzylidene malonitrile). This compound is mutagenic *in-vitro* inducing both clastogenic and aneugenic effects in mammalian cells. Although there were negative results from *in-vivo* bone marrow micronucleus assays it was noted that no data were available to indicate whether adequate amounts of CS, or short-lived reactive metabolites, reached the target organ. Data from DNA binding studies in the liver and kidney did not help with regard to concerns in tissues of initial contact due to the direct acting mutagenic potential. However the COM took into account the negative carcinogenicity bioassay data from the NTP programme comprising inhalation studies in the rat and the mouse¹³. The advice from the COC was that these assays had been adequately conducted and were negative. The COM agreed these negative data provided reassurances that the *in-vitro* effects seen with CS do not occur *in-vivo* at the site of initial contact in animals. (Full information can be found at www.doh.gov.uk/cot/csgas/htm.)
15. Thus consideration of the results from carcinogenicity bioassays, if available, is important when assessing the overall weight of evidence as to whether the compound should be regarded as an *in-vivo* mutagen. In this regard an assessment needs to be made as to whether the carcinogenicity data are consistent with the compound being a genotoxic carcinogen. Alternatively the tumour profile may be

suggestive of a non-genotoxic mechanism of chemical carcinogenesis, or that the chemical is not carcinogenic. Consideration may need to be taken of any difference in dose levels used but it must be recognised that the difference in duration of the mutagenicity and carcinogenicity bioassays will severely limit the utility of any comparison. However, consideration of the available information on the toxicokinetics of the compound will often be useful.

16. It is not possible to give more than generic guidance in this area, as each compound will need to be considered on a case-by-case basis. Expert judgement will be needed with particular consideration of a number of key points that are summarised in the conclusions.

Conclusions

17. The following points need to be considered when deciding whether positive *in-vivo* bone marrow clastogenicity data can be discounted with regard to considering a compound as an *in-vivo* mutagen:
 - i) The totality of the relevant data relating to the specific compound in question needs to be considered, including any information on its chemical reactivity, toxicokinetics, its mutagenic profile (*in-vitro* and *in-vivo*), and also any available data from carcinogenicity bioassays to enable an assessment to be made as to whether the compound appears to be a genotoxic carcinogen.
 - ii) Positive bone marrow *in-vivo* mutagenicity data from dose levels that are associated with high levels of toxicity or lethality (i.e. above the maximum dose level recommended in the current OECD guidelines) cannot be interpreted with any certainty because of the confounding effects of toxicity. If adequate information is not available at lower, non-lethal dose levels then retesting is necessary.
 - iii) It is also important to consider whether there is any evidence for a plausible mechanism to support the contention that the observed positive results in the bone marrow assays at high dose levels may be secondary to other non-genotoxic effects rather than being a mutagenic effect of the compound (or its metabolites). Examples of such mechanisms include (but are not limited to) hypothermia, hyperthermia, and erythropoiesis.
 - iv) Data from adequately conducted carcinogenicity bioassays, if available, provide important information to help in the assessment of the significance of such high dose bone marrow mutagenicity results. Such data may indicate that the carcinogenic profile of the chemical is consistent with either a genotoxic or a non-genotoxic mechanism, or that the compound is not carcinogenic.
 - v) Only generic advice can be given in this area and it should be emphasised that each compound needs to be considered in a case-by-case basis. However consideration of the above factors, with expert judgement, may provide sufficient evidence to conclude that the positive *in-vivo* bone marrow data at high dose levels was due to a non-genotoxic effect. A threshold based risk assessment may thus be appropriate.

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Declaration of COM Members' Interests during the period of this report

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	Company	Interest	Company	Interest
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