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 2006, the Committee provided advice on a wide range of topics including genotoxicity of acrylamide (germ cell effects), benzimidazoles (as a common mechanism group), ethaboxam (partial review), formaldehyde (evidence for systemic mutagenicity), potassium sorbate and sodium benzoate (consideration of potential for genotoxicity to mitochondria) and terephthalic acid (used in food contact materials).

The COM also undertook a further consideration of test strategies and evaluation of chemical mutagens which included a presentation of the proposed GADD45a gene screen for genotoxins and a review of the progress towards the development of an OECD test guideline for the *in-vitro* micronucleus assay.

The COM has initiated an ongoing full review of acrylamide and use of toxicogenomics in mutagenicity evaluation. A review of the mutagenicity of mixtures of chemicals has been completed and a COM statement is in preparation.

The COM agreed to initiate a full review of its guidance document which had been published in December 2000.

Professor P B Farmer Chair
MA DPhil CChem FRSC FBTS

Acrylamide

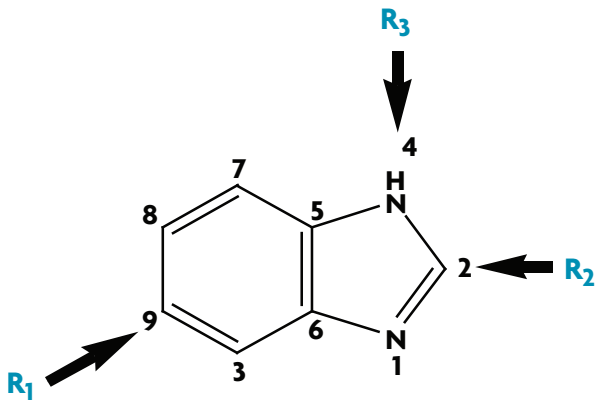
2.1 HSE had asked for an opinion on the evidence regarding germ cell mutagenicity of acrylamide and the evidence regarding a threshold for germ cell mutagenicity with this chemical. In view of the need to provide advice to HSE rapidly on the submitted data, the COM undertook a review of evidence provided by HSE using a postal consultation process. The Chair wrote to HSE in February 2008 and concluded that there were many uncertainties with regard to extrapolation of the available mutagenicity data on acrylamide for risk assessment, particularly as acrylamide (and glycidamide) seem to be clastogenic in both somatic and germ cells. Any attempt should take into account the genotoxic effect on the most sensitive cell, i.e. early spermatozoa. The default recommendation from COM is to assume no threshold.

2.2 A copy of the letter (COM statement 07/02) is appended at the end of this annual report. The COM agreed to conduct a full review of acrylamide which is ongoing (see paragraph 2.34 of this annual report).

Benzimidazoles: Consideration of a common mechanism group

2.3 The COT published its report on Risk Assessment of Mixtures of Pesticides and Similar Substances in September 2002. One of the recommendations of the report was that a scientific and systematic framework should be established to decide when it is appropriate to carry out combined risk assessments of exposures to more than one pesticide and/or veterinary medicine. One group of substances highlighted in the 2002 COT report as requiring further consideration as a possible common mechanism group was the benzimidazoles. These are substances used as pesticides (fungicides) and/or as veterinary medicines (mostly anthelmintics), which contain the benzimidazole ring (Figure 1). Additionally some pesticides and veterinary medicines are pro-benzimidazoles, i.e. they do not contain the benzimidazole ring but are metabolised *in vivo* to benzimidazoles. These compounds are considered here together with the benzimidazoles

Figure 1: Molecular structure of the benzimidazole ring



2.4 For the purposes of this review, the COM considered that aneugenicity was the toxic effect that the substances may have in common, as a result of inhibition of tubulin polymerisation.

2.5 The Committee considered a review of findings in genotoxicity studies and other data relevant to assessments of aneugenicity. On the basis of the reviewed data, there were good correlations for these compounds between inhibition of mammalian tubulin polymerisation and evidence for *in vitro* aneugenicity in mammalian cells, and between *in vitro* aneugenicity in mammalian cells and *in vivo* aneugenicity for these compounds. The Committee considered that evidence for a common functional effect of benzimidazoles on tubulin would be important for an assumption of dose additivity. The Committee considered that a decision tree would be useful to aid assessing inclusion of a benzimidazole in a common mechanism group. A flow diagram is shown in the COM statement appended at the end of this report. It was considered that genotoxicity data for benzimidazoles must be sufficient to demonstrate that mutagenic effects observed are solely due to aneugenicity.

2.6 In order to test the assumption additivity and exclude the possibility of other, less-predictable, combined effects the COM recommended that several example pairs of benzimidazole compounds which pass through the decision tree be tested alone and in combination in the *in vitro* micronucleus assay. The COM also recommended that possible combined effects of benzimidazoles with other aneugens which interact with tubulin should be considered in any research and testing strategy.

2.7 The COM statement is appended to this report.

Ethaboxam- Partial Review

Evaluation October 2006

2.8 The COM were asked for advice by PSD on a new pesticide active ingredient which is undergoing evaluation through the independent Advisory Committee on Pesticides (ACP). The referral statement is as follows; "PSD have asked for advice from the COM on the most appropriate strategy to be taken with regard to the testing for aneugenicity of ethaboxam *in vivo* and the potential for risk of site of contact *in-vivo* aneugenicity effects and their likely significance for risk assessment. This is not a full referral of the mutagenicity data to the COM." Ethaboxam is a new fungicide and is formulated as a 100 g/L suspension concentrate for control of grapevine downy mildew caused by Plasmopara viticola.

2.9 The data holder (LG Life Sciences) and representative attended the COM meeting of the 12 October 2006 to make a short presentation and to answer COM queries regarding the evaluation of ethaboxam for aneugenicity. The data holder and representative submitted reasoned arguments concerning the COM conclusions and additional investigations for polyploidy from the *in vitro* cytogenetics assay in human peripheral blood lymphocytes on 1 November 2006.

COM consideration and conclusions: October 2006

2.10 The COM considered that an appropriate investigation of polyploidy as an indicator for potential aneugenicity had been undertaken in the *in vitro* chromosomal aberration study in peripheral blood

lymphocytes given the predominant effects of ethaboxam on cell division. The COM agreed that more evaluation of the mouse lymphoma data to predict potential for aneugenicity might have been possible.

2.11 The COM agreed that a further evaluation for aneugenicity and clastogenicity should be undertaken *in vitro* to describe appropriate dose-response data and to determine the NOELs. The COM noted that the potential NOEL for non disjunction might be lower than for chromosome loss or gain and this end point should also be investigated.

2.12 The COM agreed the NOEL for MN induction in PBLs *in vitro* was likely to be lower than the value of 5 µg/ml reported in the existing study in PBLs but probably above the tested concentration of 1.0 µg/ml.

2.13 The COM considered that it would be difficult to undertake an evaluation of site of contact aneugenicity *in vivo* for ethaboxam given the current knowledge of potential approaches. A pragmatic *in vivo* testing strategy should include a re-test using intraperitoneal dosing in mice with evaluation of MN in the bone marrow to include dose response, multiple sampling times and an evaluation of whole chromosomes. Members agreed that consideration should also be given to investigating MN formation in other tissues in this study such as the liver, germ cells and splenocytes. Members considered it was important to measure plasma and tissue concentrations of ethaboxam. The COM considered that the results of the repeat *in vivo* BM MN assay in mice would be important for regulatory decision making with regard to ethaboxam.

Update Evaluation May 2007

2.14 The data holder's representative submitted an interim report of the additional studies requested by COM on 16 April 2007. The COM discussed these data at its meeting of the 15 May 2007. The data holder's representative made a short presentation of the results of the additional studies during this meeting. Additional data on the results of studies for non disjunction were submitted during the COM meeting of the 15 May 2007.

2.15 The COM considered a number of questions in deriving conclusions:

i **The interpretation of new *in vitro* MN data in PBLs**: The COM agreed that NOELS were slightly lower than the submitted test data reported; approximately 4 µg/ml (24 hour PHA stimulation) and 2 µg/ml (48 hour PHA stimulation).

ii **The need for additional *in vitro* data on aneuploidy and chromosome disjunction**: Members noted no evidence for non disjunction had been reported. The COM noted that ethaboxam treatment resulted in a greater effect in mononucleate compared to binucleate cells. The mechanism of ethaboxam induced effects *in vitro* was unclear and needed to be resolved.

iii **The interpretation of the new *in vivo* MN data in mice given ethaboxam via intraperitoneal dosing**: The small statistically significant increase in MNPCEs seen in the repeat study at high doses (2 x 300 mg/kg bw in 1% methylcellulose/0.1%Tween 80) resulting in severe toxicity and increased mortality at both the 24 hour and 48 hour post dose bone marrow sampling times is a similar

result to the first intraperitoneal study where small statistically significant increase in MN in the bone marrow was reported in mice (following intraperitoneal dosing of 2 x 486 mg/kg bw (in aqueous 1% methylcellulose) with sampling at 48 hours post dose). Severe toxicity and increased mortality were also reported in the first intraperitoneal bone marrow mouse MN assay with ethaboxam. The COM noted the evidence for aneugenicity of ethaboxam *in vitro* and considered that there was no adequate explanation for the small increases in bone marrow micronuclei seen in the intraperitoneal *in vivo* tests with ethaboxam. The COM could not exclude a direct aneugenic effect of ethaboxam in these studies. The COM did not agree that the increase in MNPCEs seen in these studies could definitely be related to a toxic-stress response. Thus overall the top dose level in the repeat intraperitoneal bone marrow MN assay of 300 mg/kg bw should be regarded as a LOAEL. The COM agreed that further evaluation of the slides from the repeat bone marrow MN assay at the top dose level with additional procedures to identify whole chromosomes should be undertaken if sufficient micronuclei could be identified.

iv **The need for additional *in vivo* MN data in other tissues (eg spleen)**: The COM agreed that further information could be provided for other tissues but this would require additional *in vivo* studies in mice using intraperitoneal dose levels below 2 x 300 mg/kg bw. There would need to be additional toxicokinetic evaluation undertaken. The COM considered that such a request would need to come from the Advisory Committee on Pesticides (ACP).

v **The use of tissue concentration data for risk assessment**: The COM did not agree from the data submitted, that ethaboxam concentrations in testes could be used as a surrogate for bone marrow. The COM noted the absence of bone marrow tissue levels from the repeat bone marrow MN assay in mice. The COM suggested that in this instance an initial risk assessment could be undertaken using the peak plasma levels of ethaboxam at the LOAEL for MN induction in bone marrow reported in the repeat intraperitoneal bone marrow MN assay, although further evaluation of the suitability of this approach for risk assessment of site of contact aneugenicity would need to be considered by the ACP.

2.16 The COM agreed two statements on ethaboxam (COM/07/S1 and COM/07/04) which were forwarded to the ACP and are reproduced at the end of this Annual report.

Formaldehyde: Evidence for systemic mutagenicity

2.17 Formaldehyde is produced worldwide on a large scale and is used in the production of phenolic, urea, melamine and polyacetal resins. Formaldehyde is also used as an intermediate in the manufacture of industrial chemicals and as an aqueous solution (formalin) as a disinfectant and preservative in many situations. The COM was asked to consider the evidence for systemic mutagenicity from animal experiments and biomonitoring studies of workers exposed to formaldehyde. The objective was to consider the potential for systemic mutagenicity following inhalation exposure, the predominant route of exposure in the occupational groups for which evidence of leukaemia had been reported by IARC.

2.18 The COM were aware of a number of recent internationally recognised reviews which had reported on both carcinogenicity and mutagenicity and agreed there was no need to specifically review all of the

mutagenicity data on formaldehyde. This included a recently published evaluation of the mode of action (MOA) for nasopharyngeal cancer. The COM acknowledged that formaldehyde is a direct acting *in vitro* mutagen in bacterial and mammalian cells (including rodent and human cell lines). Mutagenic effects reported included point mutations, chromosome aberrations, sister chromatid exchanges, DNA strand breaks and UDS in rat nasal turbinate cells. With regard to the MOA for rat nasopharyngeal tumours, most reviewers had considered the formation of formaldehyde DNA-protein cross links (DPX) with a similar dose-response to the formation of nasal tumours in rats, with consequent marked local effects on cytotoxicity, cell proliferation and local site of contact mutagenic effects as key elements in the proposed MOA. The magnitude of the formaldehyde induced local site of contact cell proliferation had been emphasised in the available reviews. The Committee agreed to focus its initial discussion on the available peer reviewed scientific literature regarding toxicokinetics of absorbed formaldehyde and the evidence in the published literature for systemic *in vivo* mutagenicity.

2.19 The COM statement is appended to this report.

COM conclusions

i The COM concluded that the amount of formaldehyde systemically available following inhalation exposure at the occupational exposure standard would be negligible.

ii The COM was aware that formaldehyde was a direct acting *in vitro* mutagen. The COM concluded that there was no convincing evidence from *in vivo* mutagenicity studies in experimental animals and from biomonitoring studies of genotoxicity in workers exposed to formaldehyde for a direct *in vivo* systemic mutagenic effect of inhaled formaldehyde. A secondary mechanism might be involved in the genotoxic effects documented in peripheral blood lymphocytes in the biomonitoring studies reviewed.

iii The COM concluded that there was no reason to consider that direct systemic mutagenicity would be involved in the mechanism of formaldehyde induced systemic tumourigenicity.

iv The Committee concluded that for occupational and environmental exposure to formaldehyde, the pattern of metabolism and distribution of formaldehyde indicate that a threshold for *in vivo* systemic mutagenicity is likely.

Sodium benzoate and potassium sorbate

2.20 Sodium benzoate (E211) and potassium sorbate (E202) are two examples of organic acid food preservatives based on benzoic and sorbic acids. Benzoic acid and its sodium, potassium and calcium salts and sorbic acid and its potassium and calcium salts are permitted for use in a wide range of foods in the EU. These preservatives have been subject to a risk assessment by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).[1,2]

[1] Joint WHO/FAO Expert Committee on Food Additives (1996) Benzyl acetate, benzyl alcohol, benzaldehyde and benzoic acid and its salts. Available at: http://www.inchem.org/documents/jecfa/jecmono/v37je05.htm

[2] Joint WHO/FAO Expert Committee on Food Additives (1974) Toxicological evaluation of some food additives, including anti-caking agents, antimicrobials, antioxidants, emulsifiers and thickening agents. Available at: http://www.inchem.org/documents/jecfa/jecmono/v05je18.htm

2.21 In 1999, a study was published in Free Radical Biology and Medicine by Professor Peter Piper, then from University College London, which raised the possibility that these preservatives may be mutagenic to the yeast mitochondrial genome.[3]

2.22 This study used genetically modified yeast cells in an *in vitro* system to demonstrate the effects of potassium sorbate and sodium benzoate on the respiratory capabilities of the cells. Yeast superoxide dismutase (SOD) mutant S. cerevisiae cells were incubated with the two preservatives and the effects observed using a halo assay. The author concluded that the test substances produced an increased number of respiratory-deficient yeast cells under aerobic conditions which indicates that damage was occurring to the mitochondrial DNA in the yeast cells.

2.23 Using a postal consultation, COM members were asked by the Food Standards Agency to comment on the paper by Professor Piper whilst taking into account the large package of other toxicological data available on these preservatives.

2.24 Members were interested in the hypothesis presented by Professor Piper but were of the opinion that direct extrapolation of these results from SOD mutant yeast cells to mammalian cells *in vivo* was not possible. Members considered that mammalian mitochondria *in vivo* have sufficient anti-oxidant and DNA repair mechanisms to deal with any oxidative stress that may be attributed to the action of these preservatives in addition to that normally seen through the normal respiratory activities of the cell. The SOD mutant cells used in the study by Professor Piper have a significantly attenuated anti-oxidant and DNA repair response and therefore had a greater susceptibility to oxidative DNA damage.

2.25 In conclusion, COM members noted the evaluation of sorbates and benzoates by JECFA and were aware of the large package of toxicology data, including rodent carcinogenicity studies. COM members concluded that the study by Professor Piper did not suggest a need for a full re-evaluation of the mutagenicity data on benzoates and sorbates. On the basis of this conclusion, no further *in vivo* mutagenicity testing of these two preservatives was considered necessary at this time.

Terephthalic acid

2.26 Terephthalic acid is used as a starting material in the manufacture of polyethylene terephthalate (PET). PET may be used to coat the internal surface and welded joints (side stripes) of food cans. PET can also be used to manufacture beverage bottles.

2.27 In 2001, the Committee considered that the limited *in vitro* mutagenicity data package and absence of toxicokinetic data in the *in vivo* micronucleus assay were insufficient to determine the mutagenic potential of terephthalic acid. Therefore, the Committee recommended that an adequately conducted *in vitro* cytogenetics test in mammalian cells was needed before any definite conclusions could be reached which would indicate that the bladder tumours in the rat carcinogenicity bioassay arose from a non-genotoxic mechanism.

[3] Piper P.W. (1999) Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radical Biology and Medicine. 27 (11/12) 1219-1227

2.28 In May 2006, the COM was presented with a submission of data that BP Chemicals Ltd had commissioned, following the 2001 meeting. A mouse metabolism study was submitted to address concerns regarding bone marrow exposure at the doses selected for the mouse micronucleus study, which had been reviewed at the 2001 meeting. The Committee considered that this metabolism study was not helpful in demonstrating target tissue exposure had been achieved in the micronucleus test. A second *in vivo* study was submitted to supplement the micronucleus study. A single oral dose of terephthalic acid (2000 mg/kg bw, >99.9% purity w/w) was assessed for its ability to induce UDS in the liver of male Alpk:APfSD rats. Members agreed that this unscheduled DNA synthesis (UDS) study had been adequately conducted and was negative. As per the original COM data request, *in vitro* cytogenetics data using human lymphocytes to assess the clastogenicity of terephthalic acid were provided. This assay was limited to 500 µg/ml at which the pH of the culture medium was reduced from 7.10 to 6.74. under the conditions of this initial study, terephthalic acid was found to be clastogenic. A second study was submitted using the sodium salt of terephthalic acid, sodium terephthalate (99% purity w/w). In this study, no reduction in pH was observed when tested up to 2100 µg/ml, the maximum concentration stipulated by the protocol for this assay (10 mM). The author of the study report concluded that sodium terephthalate was not clastogenic under the conditions of this study.

2.29 The Committee agreed that the two *in vivo* studies were adequate and negative, indicating that terephthalic acid is not an *in vivo* mutagen. The available evidence supported the previous COM conclusion of a non-genotoxic mechanism for the bladder tumours seen in the rat carcinogenicity study.

2.30 A full statement is appended at the end of this Annual report.

Horizon Scanning

2.31 The annual horizon scanning exercise was intended to provide an opportunity for members and advisers from Government Departments/Agencies to discuss and suggest topics for further work. Considerable progress on the items identified in the 2006 horizon scanning exercise had been made, although it was noted that the review on mutational spectra had not been initiated and this would be carried over to next year's work programme. The 2007 literature search used PUBMED and initially indicated several thousand publications in 2006/7, which could be potentially relevant. The search strategy subsequently focused on a number of areas such as, genotoxicity test strategy, novel mutagens, mutagens in the environment, genotoxicity biomonitoring, high potency mutagens, potency of genotoxins, mutagen spectra/spectrum and chemical mutagens. Members were asked for their views on the identified areas and for additional suggestions for further work.

2.32 With regard to specific chemicals, phenol was another chemical that had been identified for further work. The COM had reviewed unpublished data in 2003, which suggested a plausible mechanism for secondary indirect effect for positive mutagenic results seen in the bone marrow i.e. hypothermia occurring at dose levels associated with positive micronucleus assays. Members had agreed that before definite conclusions could be drawn on the significance of such data they would need to see a peer reviewed published report of the study. A publication on these data was now available. The committee agreed that it could review this hypothesis i.e. the potential for false positive effects due to hypothermia or hyperthermia using phenol as one example compound.

2.33 The committee agreed that the review of the approaches to the genotoxicity testing of mixtures should be completed. Members agreed to initiate an update of the current COM guidance on mutagenicity testing, but noted that this would be a long process. Members' suggestions for other areas for future work included the significance of aneuploidy, its causes and possible approaches to risk assessment, also mitochondrial mutation and its potential involvement in various diseases, and epigenetics. Members agreed a focused review of the utility of the Ames test for evaluation of low levels of mutagenic impurities in test materials would be valuable.

Test Strategies and Evaluation

2.34 The COM has an ongoing remit to review and provide advice on mutagenicity testing strategies. During this year, the COM heard a presentation from Dr R Walmsley (University of Manchester) on the TK6 GADD45a GFP genotoxicity assay which had been proposed as an early screening asay to be undertaken prior to *in-vitro* mutagenicity testing. The COM considered this to be an interesting presentation and that initial results had suggested both good specificity and sensitivity. Members were interested to see the results when the dataset had been expanded, including further exogenous activation results. Members asked for more information on the validation of the exogenous metabolising fraction to be used in the GADD 45a GFP assay. This would involve studies on source of exogenous metabolising fraction (e.g. rat or hamster liver) and the level of incorporation in the assay and effect of exogenous metabolising fraction on the growth of TK cells in the assay and influence on the flow cytometry method. The Committee looked forward to reviewing more information on this developing approach to genotoxicity screening.

2.35 The COM discussed the ongoing development of an OECD guideline for the *in vitro* micronucleus test at the May 2007 meeting which was attended by representatives of the Industrial Genotoxicity group (IGG). The COM advice was passed to U.K. representatives to take forward at an OECD working group to be convened in October 2007.

Ongoing Reviews

2.36 *Acrylamide*; The HSE have requested a further evaluation from the COM regarding the information cited by the PPG in its letter to the chair of COM (dated 8 May 2007) (COM statement 07/02). The Food Standards Agency have also requested that a consideration be given to all available genotoxicity data on acrylamide by COM. The COM has agreed that the ESR review completed by HSE (EU Risk Assessment report 2002) could be used as a basis for the review. A submission of data from the Polyelectrolyte Producers Group (PPG) was reviewed at the October 2007 meeting. Further consideration of the genotoxicity data on acrylamide will be undertaken by the COM during 2008.

2.37 *Toxicogenomics*; The COT/COC/COM held a joint symposium on the issue of genomics and proteomics in October 2001 and published a joint statement in December 2004 on the use of Toxicogenomics in toxicology. This was based on literature review of 50 studies and included information from the International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) collaborative programme of research. This topic was identified during the 2006 horizon

scanning exercise for an updated review. The DH Toxicology unit drafted a short overview of a number of new relevant *in vitro* studies, which included data on gene expression changes in studies on DNA adducts and mutagenicity for the October 2007 meeting. A large number of papers had been retrieved, but those selected for review were specifically chosen with the aim of identifying any advancement in the field, which may affect the conclusions drawn in the last statement. The COM noted that further information was available which would be considered during 2008.

2.38 *Chemical Mixtures*; The COM expressed an interest in the evaluation of the mutagenicity of chemical mixtures during the 2005 and 2006 horizon scanning exercises. One important recommendation was to consider the possibility occurrence of synergistic interactions regarding mutagenic effects of chemical mixtures, the possible mechanisms for any synergistic effects and the implications of such a finding for risk assessment. The COM considered two discussion papers during 2007 and a draft working paper is in preparation for the February 2008 meeting.

Statements of the COM

Statement on partial review of Ethaboxam

COM/07/S1 - February 2007

Introduction

1 The COM have been asked for advice by PSD on a new pesticide active ingredient which is undergoing evaluation through the independent Advisory Committee on Pesticides (ACP). The referral statement is as follows; "PSD have asked for advice from the COM on the most appropriate strategy to be taken with regard to the testing for aneugenicity of ethaboxam *in-vivo* and the potential for risk of site of contact *in-vivo* aneugenicity effects and their likely significance for risk assessment. This is not a full referral of the mutagenicity data to the COM."

2 Ethaboxam is a new fungicide and is formulated as a 100 g/L suspension concentrate for control of grapevine downy mildew caused by Plasmopara viticola. The proposed pattern of use is for up to 5 applications per season, with an interval of 7 to 10 days depending on weather conditions and disease pressure. Thus the key sites of contact for evaluation are the upper respiratory tract (in particular the nasal passages) and possible skin contact to concentrate and in-use dilution.

3 The data holder (LG Life Sciences) and representative (Huntingdon Life Sciences) attended the COM meeting of the 12 October 2006 to make a short presentation and to answer COM queries regarding the evaluation of ethaboxam for aneugenicity.

COM consideration of areas for discussion

4 The Committee considered the submitted data which included the draft PSD evaluation which presented information on structure, use, ADME studies, general toxicology, mutagenicity, carcinogenicity and reproduction,[1,2] and copies of the mutagenicity studies relevant to the evaluation of aneugenicity which had [1,2] been submitted to PSD.[3-8] (Some information on a possible approach to the assessment of aneugenicity *in-vivo* in the gastrointestinal tract and the evaluation of aneugenicity data was also presented to members.[9,10]) Members derived an evaluation of these studies and considered the areas of mutagenicity evaluation to raise with the data holder and their representative.

5 The areas of discussion related to:

i the evaluation of the data from the available *in-vitro* chromosomal aberration assay in human peripheral blood lymphocytes (PBLs) and the mouse lymphoma assay (MLA) with regard to identifying biological alerts for potential aneugenicity *in-vitro*.[3,4]

ii the evaluation of the data from initial *in-vitro* study of effects of ethaboxam on cell division in cytochalasin B blocked human PBLs[5] and the subsequent *in-vitro* micronucleus (MN) assay in human PBLs and evaluation of a NOEL for MN formation.[6]

iii the evaluation of data from the *in-vivo* oral bone marrow MN assay in rats[7] and the intraperitoneal bone marrow MN assay in mice.[8]

6 The primary objective of the COM was to consider an appropriate testing strategy for aneugenicity which could be recommended to the ACP.

Data holder presentation

7 The data holder (LG Life Sciences) and representative (Huntingdon Life Sciences; HLS) were asked to make a short presentation to the COM and to answer members queries. All comments were made by the data holders representative.

8 HLS noted that some of the fungicidal activity of ethaboxam was due to interference with the fungal cytoskeleton possibly by inhibition of tubulin subunits. The negative findings in the Ames and MLA tests indicated no potential for gene mutagenicity. The *in-vitro* cytogenetics and MN tests in PBLs were considered to be positive in the absence of exogenous metabolic activation. However two negative bone marrow MN assays were available in the rat (oral dosing) and in the mouse (intraperitoneal dosing). In addition following a request from the ACP, a re-analysis of sections from the gastrointestinal tract from the rat 2 year chronic toxicity/carcinogenicity assay had not indicated any effects on mitosis. HLS also noted that no adverse toxicity had been reported in a 28 day dermal toxicity study in the rat. It was also considered that potential site of contact concentrations (skin and via oral ingestion) would be low.

COM questions for data holder and representative

9 A summary of the response given by the data holder's representative on the areas for discussion (see paragraphs 5, 7 and 8 above) is given below.

10 HLS noted that the initial strategy had placed a weight of evidence on the mouse lymphoma assay and the available *in-vivo* MN assays and thus the initial *in-vitro* study in PBLs had not specifically incorporated an investigation of MN formation. The data holder's representative noted the comments made by the COM and evaluation of the *in-vitro* chromosomal aberration assay and the mouse lymphoma assay. HLS noted the evaluation of the COM with regard to the *in-vitro* MN assay in PBLs regarding the assessment of dose response for aneugenicity and clastogenicity and the possible effects of adding exogenous metabolic activation systems. HLS considered that the use of 0.5% carboxymethyl cellulose was a relatively normal approach to oral dosing in *in-vivo* bone marrow MN assays. The COM considered that oral absorption would have been limited in comparison to that seen in the oral rat kinetics study (up to 60%) using radiolabelled ethaboxam which had used 1% CMC and Tween 80 as a surfactant to aid solubility in the dosing vehicle. HLS noted the interpretation of the *in-vivo* bone marrow MN assays in rats and mice reached by the COM.

11 The data holder and HLS withdrew from the meeting so that the COM could derive its conclusions.

Additional data submitted by industry: LG Life Sciences (1 November 2006)

12 The data holder and representative submitted reasoned arguments concerning the COM conclusions and additional investigations for polyploidy from the *in-vitro* cytogenetics assay in human peripheral blood lymphocytes.[4]

13 The COM noted the new data but agreed that it would still be appropriate to investigate the aneugenicity and clastogenicity *in-vitro* to describe appropriate dose-response data and to determine the NOELs. The COM noted the data holder had agreed to undertake a further *in-vivo* BM MN assay in mice using intraperitoneal administration. Members agreed that this study should include dose-response, multiple sampling times, and an evaluation of whole chromosomes. The COM considered that the results of the repeat *in-vivo* BM MN assay in mice would be important for regulatory decision making with regard to ethaboxam.

COM consideration and conclusions

14 The COM considered that an appropriate investigation of polyploidy as an indicator for potential aneugenicity had been undertaken in the *in-vitro* chromosomal aberration study in peripheral blood lymphocytes given the predominant effects of ethaboxam on cell division. The COM agreed that more evaluation of the mouse lymphoma data to predict potential for aneugenicity might have been possible.

15 The COM agreed that a further evaluation for aneugenicity and clastogenicity should be undertaken *in-vitro* to describe appropriate dose-response data and to determine the NOELs. The COM noted that the potential NOEL for non disjunction might be lower than for chromosome loss or gain and this end point should also be investigated.

16 The COM agreed the NOEL for MN induction in PBLs *in-vitro* was likely to be lower than the value of 5 µg/ml reported in the existing study in PBLs but probably above the tested concentration of 1.0 µg/ml.

17 The COM considered that it would be difficult to undertake an evaluation of site of contact aneugenicity *in-vivo* for ethaboxam given the current knowledge of potential approaches. A pragmatic *in-vivo* testing strategy should include a re-test using intraperitoneal dosing in mice with evaluation of MN in the bone marrow to include dose response, multiple sampling times and an evaluation of whole chromosomes. Members agreed that consideration should also be given to investigating MN formation in other tissues in this study such as the liver, germ cells and splenocytes. Members considered it was important to measure plasma and tissue concentrations of ethaboxam. The COM considered that the results of the repeat *in-vivo* BM MN assay in mice would be important for regulatory decision making with regard to ethaboxam.

February 2007
COM/07/S1

References

1 General information on ethaboxam volume 1 submission 91/414/EEC (draft in-confidence PSD evaluation) 2006.

2 Detailed evaluation volume 3 91/414/EEC (draft in confidence PSD) 2006.

3 Mouse Lymphoma Assay. In confidence Huntingdon Life Sciences project LKF 038, 21 December 2001. (in confidence report).

4 *in-vitro* Chromosome aberration assay in human peripheral blood lymphocytes. Huntingdon life Sciences project LKF/039, 24 October 2001. (in confidence report).

5 In vitro investigation of toxicity using Cytochalasin B in human peripheral blood lymphocytes. Huntingdon Life Sciences project LKF/087, 12 February 2003.

6 *in-vitro* MN assay in human PBLs. Huntingdon Life Sciences report on LGC-30473. Draft in confidence report July 2006.

7 Rat bone marrow MN assay. Huntingdon Life Sciences project LKF/046, 15 November 2001. (in confidence report).

8 *in-vivo* mouse micronucleus test. Huntingdon Life Sciences project LKY24, 13 November 1996. (in confidence report).

9 Rosefort C *et al* (Mutagenesis, 19, 277-284, 2004).

10 Vanhauwaert A *et al* (Mutagenesis, 16, 39-50, 2001).

Statement on Acrylamide

COM/07/S2 - February 2007

Request for advice on Germ Cell Mutagenicity of Acrylamide

Text of letter from COM Chair to Health & Safety Executive (HSE)

Thank you for your enquiry which was received by the secretariat on the 5 January 2007. HSE has asked for an opinion on the evidence regarding germ cell mutagenicity of acrylamide and the evidence regarding a threshold for germ cell mutagenicity with this chemical. I have consulted with a number of members by post and have also asked for a view from Professor David Phillips (chair of COC) who has agreed the contents of this letter. The need to reply to you before the 1 February 2007 COM meeting has meant that it is not possible to consider this at a formal meeting of COM.

You provided a number of documents for members to consider. These included a copy of the EU Risk Assessment Document. A Submission from Polyelectrolyte Producers Group May 2006: Acrylamide: Recent Scientific Data relating to carcinogenicity, mutagenicity and human exposure. You also submitted 7 published references selected by HSE. (References 1-7, see end of this letter). The secretariat drafted a briefing note for COM members, who also had access to the evaluation from the Polyelectrolyte producers and references 1-7.

I give below agreed answers to the questions you have raised:

i) Does the new evidence confirm that acrylamide is a germ cell mutagen?

There is overwhelming evidence in the EU RAR that acrylamide is a male germ cell mutagen. The additional references submitted support this conclusion.[1,2,7] The possibility exists that acrylamide is also a female germ cell mutagen.

ii) Can a threshold be identified for the end point?

No. The mechanism for acrylamide induced germ cell mutagenicity is not fully understood. There is evidence to show that metabolism of acrylamide to glycidamide, a DNA reactive epoxide, is a key step in the *in-vivo* mutagenic responses to acrylamide. There is no evidence to support a threshold mechanism for acrylamide or glycidamide induced mutagenicity available in the submitted papers. The default approach recommended by COM is to assume no threshold for what appears to be a clastogenic mode of action. (http://www.advisorybodies.doh.gov.uk/com/comivm.htm). The submitted references which evaluated dosimetry for mutagenic response of acrylamide can neither prove nor disprove a threshold.[1,3] It is noted that the submitted data is consistent with a linear dose-response for mutagenicity.[3]

iii) Is there sufficient evidence to show that both somatic and germ cell mutagenicity are dependent upon metabolism of acrylamide to its epoxide glycidamide?

There is evidence from the EU RAR for DNA binding in germ cells following dosing of rodents with acrylamide. An abolition of acrylamide induced MN formation DNA damaging effects was noted in CYP2E1 mice which lack the ability to metabolise acrylamide to glycidamide.[5] These data taken with a review of existing germ cell mutagenicity data[7] suggest that glycidamide is a critical metabolite in the *in-vivo* mutagenicity of acrylamide including the germ cell mutagenicity.

iv) Is there sufficient evidence to show that humans metabolise acrylamide to glycidamide to a lesser extent than rodents (with humans < rats < mice)? In view of iii) and iv) what can be said about risks to human health?

Fennell showed that overall AUC values for glycidamide formation in humans and rats from absorbed acrylamide were similar.[6] The rate constants for acrylamide and glycidamide reaction with haemoglobin reported in this paper were slightly higher in humans compared to rats. Paulsson *et al* reported a higher level of acrylamide metabolism and protein adduction in mice compared to rats.[4] Overall mice metabolise acrylamide to a greater extent than humans, but rats appear to be relatively similar to humans regarding extent of metabolism. Its not possible to extrapolate these data to predict mutagenic risk to humans. The default COM approach in such situations where there is insufficient information to allow a conclusion regarding a threshold approach to risk assessment is to assume a no threshold approach to risk assessment.

v) Assuming that a threshold for germ cell mutagenicity is not supported, what are the views of the committee concerning the use of a toxicological reference point or dose descriptor on the dose-response relationship as a basis for assessment of risks (calculation of Margins of Exposure) taking into account interspecies differences in metabolism? In this regard, it is noted that the Allen paper derives a toxicological reference point (claimed to be a threshold for mutagenicity) from combining the dose-response relationship for somatic cell mutagenicity and that for germ cell mutagenicity. As the data appear to indicate that acrylamide is a more "potent" germ cell mutagen than a somatic cell mutagen, how valid is this approach? Are there any robust dose-response data for germ cell mutagenicity of acrylamide (in the totality of the available database) from which to identify a dose descriptor to use for risk assessment purposes? What are the uncertainties involved in such an approach?

COM members cautioned the dose-response modelling undertaken by Allen *et al*, and considered that the interpretation of the results of the modelling was problematic. COM members agreed it was too premature to derive any conclusions regarding genotoxic potency on the basis of this paper.

I would therefore consider there are too many uncertainties with regard to extrapolation of the available mutagenicity data on acrylamide for risk assessment, particularly as acrylamide (and glycidamide) seem to be clastogenic in both somatic and germ cells. Any attempt should take into account the genotoxic effect on the most sensitive cell, ie early spermatozoa. The default recommendation from COM is to assume no threshold.

I hope the answers we have provided are useful to HSE in its consideration of acrylamide. Please do not hesitate to contact me or the secretariat if you have any further questions.

February 2007
COM/07/S2

References

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4 Paulsson B *et al* Mutation Research, 516, 101-111, 2002.

5 Ghanayem B *et al* Mutation Research, 578, 284-297, 2005.

6 Fennell TR *et al* Toxicological Science, 84, 1-13, 2005.

7 Favor J and Shlelby MD. Mutation Research, 580, 21-30, 2005.

Statement: Benzimidazoles: An approach to defining a common Aneugenic Grouping

COM/07/S3

Introduction

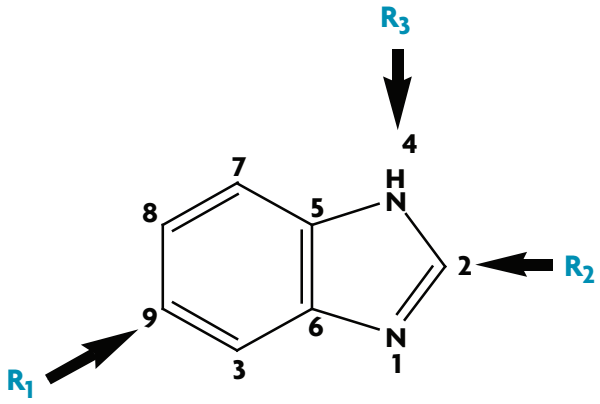
1 The COT published its report on Risk Assessment of Mixtures of Pesticides and Similar Substances in September 20021. One of the recommendations of the report was that a scientific and systematic framework should be established to decide when it is appropriate to carry out combined risk assessments of exposures to more than one pesticide and/or veterinary medicine. The COT recommended that the default assumptions in risk assessments of combined exposure should be that chemicals with different toxic action will act independently, and that those with the same toxic action will act additively[1]. The COT has observed that where groups of chemicals with similar modes of action have been studied, additivity (or less than additivity) has been demonstrated rather than synergy. Examples include endocrine disrupters that act by agonism of oestrogen receptors, dioxin-like compounds and organophosphates[1,2].

2 Under dose additivity (also called simple similar action or simple joint action) the effect of a mixture is obtained by summing the doses of the individual compounds after adjustment for differences in their potencies[1]. Because it occurs across the full dose-response curve, it is relevant to low doses. In contrast, if compounds have independent toxic actions, combined effects may be seen at high doses (e.g. due to effect addition or pharmacokinetic interactions), but would not be expected at doses below thresholds for the individual chemicals1.

3 A new draft regulation proposed by the European Commission concerning the placing of plant protection products on the market (to replace Directive 91/414/EEC) requires additive and synergistic effects of pesticides to be taken into account in the approvals process when the methods to assess such effects are available[3]. The US EPA is already required by the 1996 Food Quality Protection Act in the USA to assess risks from exposures to combinations of pesticides.

4 One group of substances highlighted in the 2002 COT report as requiring further consideration as a possible common mechanism group was the benzimidazoles. These are substances used as pesticides (fungicides) and/or as veterinary medicines (mostly anthelmintics), which contain the benzimidazole ring (Figure 1). Additionally some pesticides and veterinary medicines are pro-benzimidazoles, i.e. they do not contain the benzimidazole ring but are metabolised *in vivo* to benzimidazoles. These compounds are considered here together with the benzimidazoles.

Figure 1: Molecular structure of the benzimidazole ring



5 The mechanism of action of benzimidazole compounds as fungicides is widely considered to be binding to free tubulin, particularly β-tubulin at the colchicine binding site, disrupting microtubule formation and thereby inhibiting mitosis[4,5,6,7]. The primary mechanism of action of benzimidazoles as anthelmintics is also considered to be by binding to free β-tubulin and inhibiting its polymerisation[8,9], as a result affecting microtubule-dependent glucose uptake[10,11]. A number of benzimidazoles have been shown to also inhibit mammalian tubulin polymerisation and to be aneugenic *in vivo*.

Definition of "common mechanism" of toxicity

6 The COT report did not specifically define "common mechanism" in its 2002 report. However, it variously referred to chemicals which would be in a common mechanism group as "similarly acting", having "the same" toxic action, or having a "common mode of action"[1]. The US Environmental Protection Agency (EPA), which is required by the 1996 Food Quality Protection Act in the USA to assess risks from exposure to combinations of pesticides, has defined a common mechanism group as consisting of "Two or more chemicals or other substances that cause a common toxic effect(s) by the same, or essentially the same, sequence of major biochemical events (i.e., interpreted as mode of action)." (http://www.epa.gov/pesticides/glossary/index.html#common_mechanism, accessed 27 November 2006).

7 For the purposes of this review, aneugenicity was considered to be the toxic effect that the substances may have in common, as a result of inhibition of tubulin polymerisation.

Risk assessment of individual benzimidazoles

8 The COM has previously advised that it is reasonable to assume that aneuploidy-inducing chemicals (particularly those that function by damaging the cell division apparatus) have a threshold of action[12]. In 1993 the COM provided advice on methodology for identifying thresholds for aneugens acting by spindle inhibition[12]. In 1996, the Committee considered the results of experiments undertaken with the benzimidazoles benomyl and carbendazim and concluded that the studies had been satisfactorily

conducted and the data indicated No Observed Effect Levels (NOELs) for these two chemicals[13]. The Committee also saw similar data for thiophanate-methyl[14]. The Committee advised in 2000 that there is a sound scientific basis to assume that these chemicals have a threshold of action in both somatic and germ cells[15]. Other committees have drawn similar conclusions for other benzimidazoles where genotoxicity data have provided evidence for aneugenicity.

Review of the data on benzimidazoles

9 The Committee considered a review of findings in genotoxicity studies and other data relevant to assessments of aneugenicity, both from the regulatory assessments of authorised benzimidazoles (and pro-benzimidazoles) and from additional relevant papers identified in the published peer-reviewed literature. Since this was a consideration of a general approach to grouping these compounds, the Committee was not asked to evaluate the individual compounds. The findings are summarised in Table 1.

10 The data indicate that helminth tubulin binding is a relatively poor predictor of *in vivo* aneugenicity, and the Committee considered that aneuploidy in fungi could also not be considered to be a reliable predictor of *in vivo* aneugenicity in mammals. However, there were good correlations for these compounds between inhibition of mammalian tubulin polymerisation and evidence for *in vitro* aneugenicity in mammalian cells, and between *in vitro* aneugenicity in mammalian cells and *in vivo* aneugenicity for these compounds.

11 Results in the mouse lymphoma assay did not appear to be a good predictor of results in the *in vivo* micronucleus assay (data not shown in Table 1). For 4 compounds which were positive in an *in vivo* micronucleus assay and were studied in the mouse lymphoma assay, two were negative in the mouse lymphoma assay, one was clearly positive, and one was equivocal as there was not a clear dose-response relationship.

Additional relevant data

12 Two non-benzimidazole substances which both reduce tubulin polymerisation but by binding to different sites of tubulin (dilanatin and vinblastine) were reported to have dose-additive inhibitory effects on mammalian microtubule assembly when tested in combination[16].

In contrast possible synergy in the antiproliferative effects on mammalian cells was reported for two compounds which have different effects on tubulin (paclitaxel and vinorelbine), one reducing polymerisation and the other stabilising polymerised tubulin, preventing microtubule disassembly which is necessary for completion of cell division[17]. The Committee considered that evidence for a common functional effect of benzimidazoles on tubulin would be important for an assumption of dose additivity.

Table 3: : Summary of positive and negative results indicating action on tubulin and *in vitro* and *in vivo* aneugenicity. Note that some of the results are from studies reported in the peer-reviewed literature, which have not been critically assessed by this Committee.

Chemical	Binding to helminth tubulin	Inhibition of mammalian tubulin polymerisation *in vitro*	Aneugenicity in non-mammalian cells†	In vitro aneugenicity in mammalian cells‡	In vivo aneugenicity (e.g. bone marrow micronucleus assay)
Albendazolea	Positive	Positive	ND	Positive in micronucleus assay. No kinetochore staining	Positive in bone marrow micronucleus assay. No kinetochore staining
Albendazole oxidea	Positive	ND	Positive	Positive in micronucleus assay. No kinetochore staining	Positive in bone marrow micronucleus assay. No kinetochore staining
Benomylb	ND	Positive	Positive	Positive	Positive
Carbendazimb	ND	Positive	Positive	Positive	Positive
Febantelc	ND	ND	ND	ND	Negative
Fenbendazolec	Positive	ND	ND	ND	Negative
Flubendazole	Positive	Positive	ND	Polyploidy and cell transformation	Negative
Fuberidazole	ND	ND	ND	Inhibition of mitosis	Negative
Mebendazole	Positive	Positive	Positive	Positive	Positive
Netobimina	ND	ND	ND	ND	Positive in bone marrow micronucleus assay. No kinetochore staining)
Omeprazole	ND	ND	ND	Positive in micronucleus assay. No kinetochore staining	Positive in micronucleus study in hepatocytes. No kinetochore staining
Oxfendazolec	Positive	Positive	ND	ND	ND
Oxibendazole	Positive	Positive	ND	Polyploidy and metaphases of abnormal morphology	Negative
Thiabendazole	Positive	Positive	Positive	Positive	Positive
Thiophanate methylb	ND	ND	ND	ND	Positive
Triclabendazole	No	ND	ND	ND	Negative

ND: No data identified

a Netobimin is metabolised to albendazole which is metabolised to albendazole oxide

b Benomyl and thiophanate-methyl are metabolised to carbendazim

c Febantel is metabolised to fenbendazole, which is metabolically interconvertible with oxfendazole

† Tests for mitotic aneuploidy in yeast and *Aspergillus nidulans*

‡ Includes studies in human lymphocytes, human/mouse hybrid cell line R3-5, human ovarian granulosa cells, Chinese hamster LUC2 and DON:Wg3h cells, Chinese hamster primary cells, rat primary heptocytes, with additional data from CHO cells and mouse embryo fibroblast C3H/10T1/2 clone 8 cells.

Decision tree for inclusion in a common mechanism group

13 The Committee considered that a decision tree would be useful to aid assessing inclusion of a benzimidazole in a common mechanism group. Key information required would include the chemical structure, results from *in vitro* studies in mammalian cells and/or *in vivo* micronucleus assays, and data to indicate that compounds act via a common functional effect on mammalian tubulin, i.e. inhibition of polymerisation (See Figure 2). It was not considered essential for a benzimidazole to have been studied *in vivo* for it to be included in the common mechanism group since the available data indicated that *in vitro* aneugenicity is a good predictor of *in vivo* aneugenicity for the benzimidazoles.

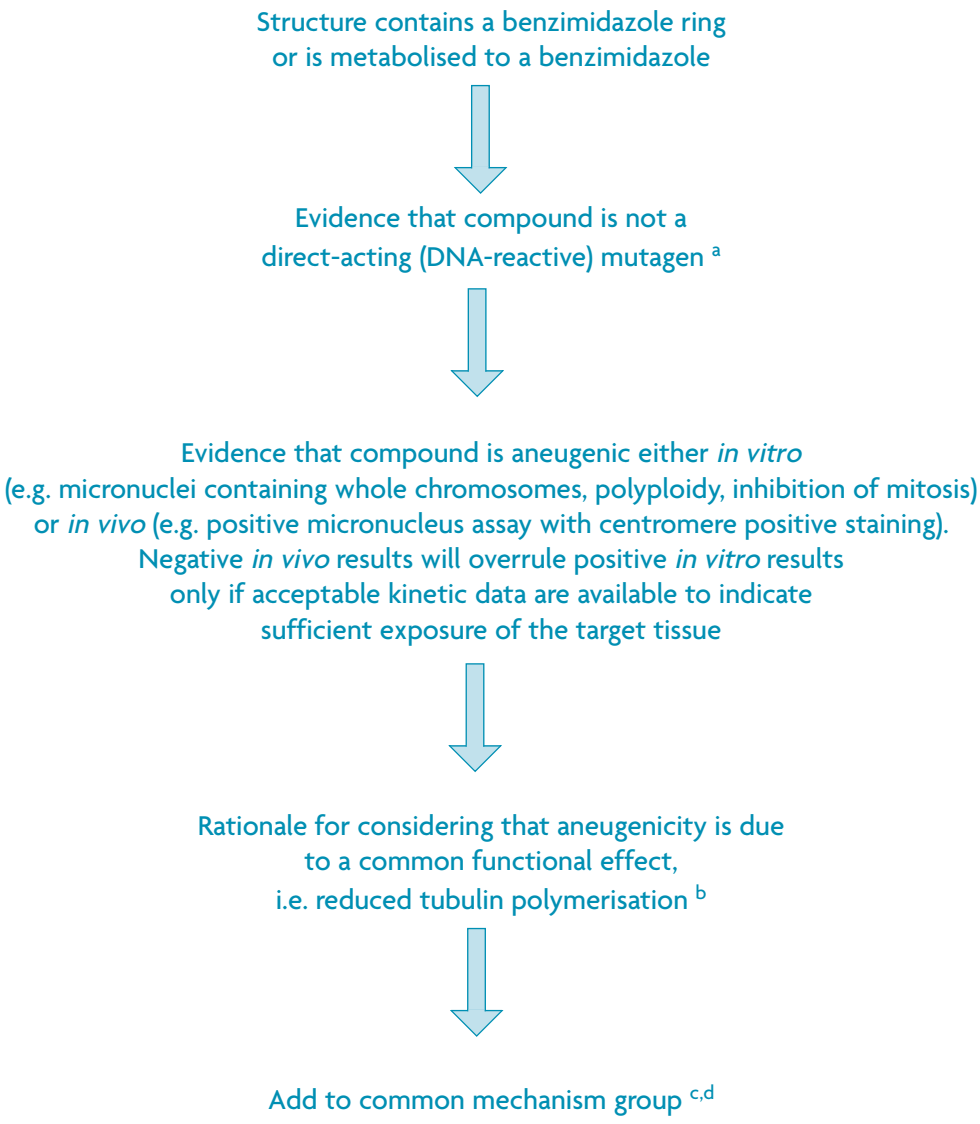
14 Since aneugens may also be clastogens, or may produce clastogenic metabolites, it was considered that genotoxicity data for benzimidazoles must be sufficient to demonstrate that mutagenic effects observed are solely due to aneugenicity, and this is reflected in the decision tree.

Research recommendations

15 The Committee recognised that an assumption of dose addition from combined exposure is pragmatic and is supported by data previously seen by the COT for compounds that act by various common modes of (non-genotoxic) action. However, in order to test the assumption and exclude the possibility of other, less-predictable, combined effects the COM recommended that several example pairs of benzimidazole compounds which pass through the decision tree be tested alone and in combination in the *in vitro* micronucleus assay.

16 We recognise that there is also a need to consider possible combined effects of benzimidazoles with other aneugens which interact with tubulin. We recommend that a suitable approach would be to test pairs of benzimidazoles and other aneugens which interact with tubulin in a similar manner as benzimidazoles (inhibition of tubulin polymerisation). In addition, any identified aneugens to which there may be co-exposure with benzimidazoles but which interact with tubulin in a different manner (e.g. by enhancing tubulin polymerisation) should also be tested in pairs with benzimidazoles.

Figure 2: Decision tree for including a compound in a benzimidazole common mechanism group for aneugenicity



Notes:

a In some instances there may additionally be a need to assess risks from combined exposure with other benzimidazoles which are aneugens even if the compound may also be a direct acting mutagen, in which case the substance may continue through the procedure.

b Any *in vitro* data showing reduced polymerisation of mammalian tubulin would be suitable

c Mixtures of selected benzimidazoles assigned to the common mechanism group should be studied *in vitro* in order to test the default assumption of dose additivity and exclude the possibility of greater than additive effects (i.e. synergy).

d Non-benzimidazoles which are considered to be aneugens may also be added to the common mechanism group if dose addition with at least one benzimidazole in the common mechanism group is demonstrated.

References

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Updated Statement: Partial review of Ethaboxam Consideration of initial data from studies requested by COM in statement COM/07/S1

COM/07/S4 - October 2007

Background

1 The COM has been asked for advice by PSD on a new pesticide active ingredient which is undergoing evaluation through the independent Advisory Committee on Pesticides (ACP). The referral statement is as follows; "PSD have asked for advice from the COM on the most appropriate strategy to be taken with regard to the testing for aneugenicity of ethaboxam *in-vivo* and the potential for risk of site of contact *in-vivo* aneugenicity effects and their likely significance for risk assessment. This is not a full referral of the mutagenicity data to the COM." Ethaboxam is a new fungicide and is formulated as a 100 g/L suspension concentrate for control of grapevine downy mildew caused by Plasmopara viticola. The proposed pattern of use is for up to 5 applications per season, with an interval of 7 to 10 days depending on weather conditions and disease pressure. Thus the key sites of contact for evaluation are the upper respiratory tract (in particular the nasal passages) and possible skin contact to concentrate and in-use dilution.

2 The Committee considered the submitted data at the October 2006 meeting which included the draft PSD evaluation which presented information on structure, use, ADME studies, general toxicology, mutagenicity, carcinogenicity and reproduction and copies of the mutagenicity studies relevant to the evaluation of aneugenicity which had been submitted to PSD. (Some information on a possible approach to the assessment of aneugenicity *in-vivo* in the gastrointestinal tract and the evaluation of aneugenicity data was also presented to members.) The COM heard a presentation from the data holder (LG Life Sciences) and representative (Huntingdon Life Sciences).

3 The COM agreed that a number of additional studies should be undertaken to further evaluate the potential for *in vitro* and *in vivo* aneugenicity as summarised from statement MUT/07/S1 below:

i. The COM agreed that a further evaluation for aneugenicity and lastogenicity should be undertaken *in-vitro* to describe appropriate dose-response data and to determine the NOELs*. The COM noted that the potential NOEL for non disjunction might be lower than for chromosome loss or gain and this end point should also be investigated.

ii. The COM considered that it would be difficult to undertake an evaluation of site of contact aneugenicity *in-vivo* for ethaboxam given the current knowledge of potential approaches. A pragmatic *in-vivo* testing strategy should include a re-test using intraperitoneal dosing in mice with evaluation of MN** in the bone marrow to include dose response, multiple sampling times and an evaluation of whole chromosomes. Members agreed that consideration should also be given to investigating MN formation in other tissues in this study such as the liver, germ cells and splenocytes. Members considered it was important to measure plasma and tissue concentrations of ethaboxam. The COM considered that the results of the repeat *in-vivo* BM*** MN assay in mice would be important for regulatory decision making with regard to ethaboxam.

*= No Observed Effect Level, ** MN = micronucleus, *** = Bone Marrow

Introduction to current review

4 HLS submitted an interim report of the additional studies requested by COM on 16 April 2007. The COM discussed these data at its meeting of the 15 May 2007. HLS made a short presentation of the results of the additional studies during this meeting. Additional data on the results of studies for non disjunction were submitted during the COM meeting of the 15 May 2007.[2]

COM consideration of areas for discussion

5 The COM considered the new data submitted.[1,2] There were a number of questions relating to the interpretation of the *in vitro* MN assay with regard to the derivation of NOELs in binucleate and mononucleate cells and the appropriate statistical level of significance to apply. With regard to the repeat intraperitoneal *in vivo* bone marrow MN assay in mice, members considered that the justification of the top dose needed further explanation as there was evident excessive toxicity and mortalities reported at the top dose of 300 mg/kg bw in the main study. Members also agreed that HLS should be questioned on the selection of tissue levels in the testes as a surrogate for the bone marrow and whether *in vitro* data on non disjunction were available (subsequently submitted during the COM meeting of 15 May 2007).[2]

Presentation to COM from HLS

6 HLS reported the data from the repeat *in vitro* MN assay in peripheral blood lymphocytes (PBLs) using both 24 hour and 48 hour PHA stimulation. FISH analysis using probes for chromosomes 1,8,11,17 and 18 in binucleate cells was undertaken. Overall NOELs after 24 hour and 48 hour PHA stimulation were interpreted to be 7 µg/ml and 4 µg/ml respectively. There was no evidence for non disjunction.

7 With regard to the repeat *in-vivo* bone marrow MN assay in mice (groups of 7 male mice dosed intraperitoneally twice at 50 mg/kg bw, 150 mg/kg bw or 300 mg/kg bw (24 hours apart) with bone marrow sampling 24 hours and 48 hours after the last dose), a small statistically significant ($P<0.01$) increase in the group mean incidence of micronucleated polychromatic erythrocytes (MNPCEs) was reported at the 24 hour and 48 hour sampling times after the second intraperitoneal dose of 300 mg/kg bw ethaboxam (in 1% methylcellulose/0.1% Tween 80). The trend analysis was statistically significant at the 48 hour sampling time point. A statistically significant reduction in percentage of polychromatic erythrocytes (PCEs) had been documented at both 150 mg/kg bw and 300 mg/kg bw ethaboxam ($P <0.001$ or <0.0001) with a statistically significant trend ($P<0.001$). The increase in MNPCEs at the top dose of 300 mg/kg bw reported at both the 24 hour and 48 hour sampling time points was not considered biologically relevant as it was only seen at the high dose where there was significant systemic toxicity and mortality reported, the individual and group mean data were within the historical control ranges and the low vehicle control MNPCE rate increased the statistical significance.

8 The toxicokinetic analysis for ethaboxam revealed evidence of exposure in the plasma, liver, spleen and testes. Tissue concentrations had not been measured in the bone marrow due to the small amount of tissue. HLS considered that tissue concentrations of ethaboxam were at least equivalent to testes and this was supported by evidence of bone marrow toxicity (reduced PCE frequency). Overall HLS suggested ethaboxam did not induce biologically significant increases in MNPCEs and therefore no subsequent FISH analysis for whole chromosomes had been undertaken.

9 HLS concluded that the request for additional data from COM had been adequately fulfilled. NOELs for binucleate PBLs were interpreted to be 6 µg/ml (24 hour stimulation) 3 µg/ml (48 hour stimulation). There was no evidence for induction of non disjunction. Ethaboxam was not mutagenic in a repeat intraperitoneal bone marrow MN assay in mice with additional toxicokinetic data in directly and indirectly exposed tissues. Overall HLS concluded there was no evidence for genotoxicity *in vivo*.

COM questions for HLS

10 In response to a question from the COM on the evaluation of NOELs for the *in vitro* MN assay, HLS reported that the dose spacing in the study was very small and the size of increase in MN frequency at low concentrations was small.

11 In answer to questions on the conduct of the repeat *in vivo* bone marrow mouse MN assay. HLS considered the process had been adequate and the top does level (300 mg/kg bw) satisfactory. The COM considered the level of toxicity seen at the top dose level was excessive. Members asked for an explanation of the cause of the small increase in MNPCEs seen in the study. HLS noted the response in animals was within the historical control range, and the response had been seen at highly toxic doses at the limit of the MTD and could be interpreted as a toxic-stress related response. Members asked if further investigation using procedures to identify whole chromosomes would be useful. HLS considered there were too few MNPCEs on slides at the top dose level for any meaningful analysis for the presence of whole chromosomes.

12 The COM asked for more explanation regarding the assertion that tissue concentrations in the testes could be regarded as equivalent to bone marrow. HLS considered that testes were representative of peripherally exposed tissue and hence similar to bone marrow. HLS noted that higher tissue levels in liver and spleen might in part represent surface retention of ethaboxam following intraperitoneal dosing. Members asked if tissues had been specially prepared to avoid such contamination by intraperitoneally dosed ethaboxam. HLS reported that standard operating procedures had been used. COM members considered that surface retention of ethaboxam in spleen and liver was unlikely at 24 hours post dose. Overall the time course seen for all tissues and plasma suggested a reduction in tissue concentrations at 6-12 hours post dose, a peak at between 12-24 hours post dose, with a relatively slow elimination (compared to plasma) for liver, spleen and testes. Overall COM members were not convinced that testes could be selected as a representative tissue of bone marrow.

13 The data holder, HLS and JSCI withdrew from the meeting so that COM could derive conclusions.

COM consideration and conclusions

14 The COM considered a number of questions in deriving conclusions:

i. **The interpretation of new *in vitro* MN data in PBLs**: The COM agreed that NOELS were slightly lower than the submitted test data reported; approximately 4 µg/ml (24 hour PHA stimulation) and 2 µg/ml (48 hour PHA stimulation).

ii. **The need for additional *in vitro* data on aneuploidy and chromosome disjunction**: Members noted no evidence for non disjunction had been reported. The COM noted that ethaboxam treatment resulted in a greater effect in mononucleate compared to binucleate cells. The mechanism of ethaboxam induced effects *in vitro* was unclear and needed to be resolved.

iii. **The interpretation of the new *in vivo* MN data in mice given ethaboxam via intraperitoneal dosing**: The small statistically significant increase in MNPCEs seen in the repeat study at high doses (2 x 300 mg/kg bw in 1% methylcellulose/0.1%Tween 80) resulting in severe toxicity and increased mortality at both the 24 hour and 48 hour post dose bone marrow sampling times is a similar result to the first intraperitoneal study where small statistically significant increase in MN in the bone marrow was reported in mice (following intraperitoneal dosing of 2 x 486 mg/kg bw (in aqueous 1% methylcellulose) with sampling at 48 hours post dose). Severe toxicity and increased mortality were also reported in the first intraperitoneal bone marrow mouse MN assay with ethaboxam. The COM noted the evidence for aneugenicity of ethaboxam *in vitro* and considered that there was no adequate explanation for the small increases in bone marrow micronuclei seen in the intraperitoneal *in vivo* tests with ethaboxam. The COM could not exclude a direct aneugenic effect of ethaboxam in these studies. The COM did not agree that the increase in MNPCEs seen in these studies could definitely be related to a toxic-stress response. Thus overall the top dose level in the repeat intraperitoneal bone marrow MN assay of 300 mg/kg bw should be regarded as a LOAEL. The COM agreed that further evaluation of the slides from the repeat bone marrow MN assay at the top dose level with additional procedures to identify whole chromosomes should be undertaken if sufficient micronuclei could be identified.

iv. **The need for additional *in vivo* MN data in other tissues (eg spleen)**: The COM agreed that further information could be provided for other tissues but this would require additional *in vivo* studies in mice using intraperitoneal dose levels below 2 x 300 mg/kg bw. There would need to be additional toxicokinetic evaluation undertaken. The COM considered that such a request would need to come from the Advisory Committee on Pesticides (ACP).

v. **The use of tissue concentration data for risk assessment**: The COM did not agree from the data submitted, that ethaboxam concentrations in testes could be used as a surrogate for bone marrow. The COM noted the absence of bone marrow tissue levels from the repeat bone marrow MN assay in mice. The COM suggested that in this instance an initial risk assessment could be undertaken using the peak plasma levels of ethaboxam at the LOAEL for MN induction in bone marrow reported in the repeat intraperitoneal bone marrow MN assay, although further evaluation of the suitability of this approach for risk assessment of site of contact aneugenicity would need to be considered by the ACP.

COM/07/S4
October 2007

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Formaldehyde: Evidence for Systemic Mutagenicity

COM/07/S5 - November 2007

Introduction

Background to COM consideration of Formaldehyde

Exposure

1 Formaldehyde is produced worldwide on a large scale and is used in the production of phenolic, urea, melamine and polyacetal resins. Formaldehyde is also used as an intermediate in the manufacture of industrial chemicals and as an aqueous solution (formalin) as a disinfectant and preservative in many situations. Formaldehyde also occurs as a natural product in most living systems and in the environment. There are also a number of non occupational sources of exposure including vehicle emissions, from building and household materials, from food and cooking and from tobacco smoke. Most formaldehyde released to the environment is rapidly degraded and human exposure from environmental sources is most likely to occur when there is a continuous source present.[1,2]

Toxicokinetics

2 Formaldehyde is a normal intermediary metabolite in humans. It is estimated that endogenous blood concentrations of formaldehyde are approximately 0.1 mM. Absorbed formaldehyde can be metabolised to formate and enter the one carbon pool for incorporation in DNA, RNA and proteins. Other pathways of metabolism include oxidation to carbon dioxide. Absorbed formaldehyde is rapidly excreted. A small proportion of material may be metabolised by a number of non saturable pathways at the site of contact to form cross links with proteins and a small amount to DNA. The Committee agreed that a consideration of the toxicokinetics of formaldehyde would be important with regard to consideration of potential for systemic mutagenicity.

Mutagenicity and Carcinogenicity of Formaldehyde

3 Formaldehyde has recently been considered by IARC and placed into group 1 (carcinogenic to humans).[1] There is sufficient evidence that formaldehyde causes nasopharyngeal cancer in humans. There was limited evidence that formaldehyde causes sinonasal cancer in humans. The working group concluded that '.. there is strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to formaldehyde. Increased risk for leukaemia has consistently been observed in studies of professional workers and in two of three of the most relevant studies of industrial workers. These findings fall slightly short of being fully persuasive because of some limitations in the findings from cohort and garment workers in the USA and because they conflict with the non-positive findings from the British cohort of workers'. This conclusion stimulated some discussion in the published literature regarding the possible mechanism of formaldehyde induced increased risk of leukaemia.[3] The IARC working group commented on the possible mechanism and '..noted evidence for clastogenic damage to circulatory cells, but overall since there was no good

animal models for acute myeloid leukaemia, did not reach a conclusion with regard to the mechanism of acute myeloid leukaemia reported in epidemiological studies'.[1]

Introduction to COM discussion

4 The COM was asked to consider the evidence for systemic mutagenicity from animal experiments and biomonitoring studies of workers exposed to formaldehyde. The objective was to consider the potential for systemic mutagenicity following inhalation exposure, the predominant route of exposure in the occupational groups for which evidence of leukaemia had been reported.

5 The COM were aware of a number of recent internationally recognised reviews which had reported on both carcinogenicity and mutagenicity and agreed there was no need to specifically review all of the mutagenicity data on formaldehyde.[1,2,4] This included a recently published evaluation of the mode of action (MOA) for nasopharyngeal cancer.[4] The COM acknowledged that formaldehyde is a direct acting *in vitro* mutagen in bacterial and mammalian cells (including rodent and human cell lines). Mutagenic effects reported included point mutations, chromosome aberrations, sister chromatid exchanges, DNA strand breaks and UDS in rat nasal turbinate cells.[1,24] With regard to the MOA for rat nasopharyngeal tumours, most reviewers had considered the formation of formaldehyde DNA-protein cross links (DPX) with a similar dose-response to the formation of nasal tumours in rats, with consequent marked local effects on cytotoxicity, cell proliferation and local site of contact mutagenic effects as key elements in the proposed MOA. The magnitude of the formaldehyde induced local site of contact cell proliferation had been emphasised in the available reviews.[2,4]

6 The Committee agreed to focus its initial discussion on the available peer reviewed scientific literature regarding toxicokinetics of absorbed formaldehyde and the evidence in the published literature for systemic *in-vivo* mutagenicity.[1-30]

The kinetics of absorbed formaldehyde

Studies in experimental animals[3,14-16]

7 The COM considered information from published inhalation studies in rats and Rhesus monkeys (using either single exposure to 6 ppm for 6 h or repeated exposure to the same concentrations 5 days/week for 4 weeks with sampling after the last exposure) and agreed the majority of absorbed formaldehyde was incorporated into intermediary metabolism (≥91%) with small amounts bound as DPX (ca ≤9%).[3,14,15] Importantly there was no evidence for an increase in blood formaldehyde concentrations in these studies. In some relatively old published experiments the metabolic incorporation and covalent binding of formaldehyde was investigated in nasal tissue and bone marrow following inhalation exposure of rats to formaldehyde (dual labelled ^{3}H- and ^{14}C-) at a number of concentrations up to 15 ppm for 6 h. There was evidence for covalent binding of formaldehyde to rat nasal DNA but no evidence for binding to bone marrow.[15] In a further study using a similar inhalation exposure rats were pretreated with an intraperitoneal injection of phorone designed to deplete non protein sulphydryl levels and hence increase the potential for DNA binding of absorbed formaldehyde. Metabolic incorporation into rat nasal tissue and bone marrow were significantly decreased, but there was no evidence for covalent binding to bone marrow DNA.[16]

Modelling of toxicokinetics in humans

8 The committee noted that a number of research groups had attempted to model the systemic uptake and distribution of formaldehyde in humans exposed to formaldehyde at exposure levels close to or at the UK occupational exposure standard of 2 ppm for 8 hours.[2,3,5,29] These studies had concluded that the systemic blood levels of formaldehyde resulting for such exposures would be ≤0.1% of the endogenously formed blood concentrations of formaldehyde (ie approximately 0.0001mM compared to an endogenous level of approximately 0.1 mM).

9 The Committee concluded that systemic exposure to formaldehyde at potential cancer target organs resulting from inhalation exposure would be a negligible amount compared to endogenously formed formaldehyde. Members acknowledged that there was limited information on the proportion of free and bound formaldehyde and the potential for release of adsorbed formaldehyde bound to macromolecules but overall considered that the potential for redistribution of formaldehyde was very small compared to endogenously formed formaldehyde.

Potential for *in vivo* systemic mutagenicity

Studies in experimental animals[6-13]

10 The COM noted that a number of test materials had been used in *in vivo* studies in experimental animals which included aqueous solutions (30-50%) stabilised with 10% methanol. Alternatively paraformaldehdye hydrolysed using sodium hydroxide might be used as a source of formaldehyde generation. Formaldehyde is intrinsically reactive and thus unstabilised solutions may be oxidised to formic acid and at low temperatures a precipitate of trioxymethylene may be formed.[2] The precise composition of the test material used was not clear in many of the studies reviewed but was presumed to be predominantly formaldehyde. Members agreed the presence of methanol would complicate the evaluation of *in vivo* genotoxicity studies.

11 The COM agreed that the available *in vivo* tests for micronucleus induction and chromosome aberrations in bone marrow in rodents using inhalation exposure or intraperitoneal administration were predominantly negative.[7-11] A slight and apparently dose related increase in micronucleated cells/1000 PCEs was noted in mice given two intraperitoeneal doses of formaldehyde (derived from paraformaldehdye at 6.25, 12.5 or 25 mg/kg bw) separated by 24 hours at two samplings (16 h and 40 h post final dose).[8] It was noted that the study authors had not considered that a statistically significant increase had been documented in this study. The Committee considered a recently published *in vivo* comet assay in rats exposed by inhalation to up to 10 ppm 6 h/day for 5 days/week. An apparent dose-related increase in comet tail moment had been reported following examination of 50-100 cells/animal in peripheral blood lymphocytes and in the liver. It was noted that in principle a cross linking agent such as formaldehyde might be expected to reduce comet tail moment and it was possible that the effects might have been due to oxidative damage and possibly apoptosis. The COM noted that formaldehyde induced disturbances of protein and lipid oxidation in this study.[13]

12 The Committee considered the evidence for a dominant lethal effects and noted two positive results had been reported. Thus a single intraperitoneal dose of formaldehyde (50 mg/kg bw) administered to Q strain mice with separate matings each week for seven weeks resulted in increased embryonic death

in the 1st and 3rd weeks.[6] There was no evidence for a clastogenic effect on spermatocytes in this study. The COM concluded it was unlikely that the effects reported resulted from a systemic mutagenic effect of formaldehyde.

13 In a separate investigation an increase in the number of abnormal spermatozoa and evidence for a dominant lethal effect was reported in a study using isogenic University of Lagos rats given intraperitoneal doses of 0.125-0.5 mg/kg bw of formaldehyde.[12] These doses were reported to be between 1/16 and ¼ of the intraperitoeneal LD50 in this strain of rat and are very much lower than the dose levels used in other *in-vivo* mutagenicity studies with formaldehyde. The dominant lethal study examined matings 1-7 days, 8-14 days and 15-21 days post dosing. A significant increase in the number of dead implants was reported in the period 1-7 days post dose which was accompanied by a reduction in sperm counts and abnormal spermatozoa.[12] Members noted that the use of methanol to stabilise formaldehyde could have contributed to the observed effects on spermatozoa morphology reported in rats.

14 The COM concluded that the mechanism by which formaldehyde could have induced the observed effects in the dominant lethal studies was unclear but did not involve a direct systemic mutagenic response.

Other site of contact *in vivo* mutagenicity studies

15 The Committee noted clear evidence for an increase in micronucleated cells of the basal epithelium of the stomach in a study in rats where a single oral dose of 200 mg/kg formaldehyde was administered.[11] In a separate study using repeated inhalation exposure of rats to formaldehyde (up to 15 ppm 6 h/day for 1 or 8 weeks), lung lavage samples in addition to bone marrow samples were examined for chromosome aberrations. There was no evidence for a clastogenic effect in bone marrow samples but a statistically significant increase in chromosome aberrations was noted in lung lavage samples.[10]

16 The COM agreed that formaldehyde was a site of contact *in-vivo* mutagen. Members noted that the site of contact effects in the gastrointestinal tract might not all be due to formaldehyde reacting directly within the target cells as the effects extended down the gastrointestinal tract further than would be expected for a highly reactive chemical.

Biomonitoring studies of formaldehyde exposure[17-28]

17 Biomonitoring studies of genotoxicity in workers exposed during a variety of activities including manufacture of formaldehyde and use of formaldehyde in mortuary and anatomy departments and in paper impregnation were evaluated. A further group used in biomonitoring studies were dialysis patients where the dialysis equipment was sterilised with formaldehyde. A recent study of volunteers exposed to formaldehyde at levels below the occupational exposure standard was also retrieved.[31] A number of these studies had reported evidence for an increase in MN or DPXs in PBLs.[17-28]

18 The COM considered these data with regard to the evaluation of data from toxicokinetic studies and *in-vivo* genotoxicity studies in experimental animals which suggested there would be no biological rationale for a direct systemic mutagenic effect of formaldehyde in biomonitoring studies. None of the studies collected the appropriate information previously identified by COM to assess background variation in results. Thus members noted that the quality of the biomonitoring studies was poor with limited account for confounding factors, including age, and also considered that the method for determination of DNA-protein cross links (SDS separation of protein-linked DNA) in PBLs had not been adequately validated. The COM suggested that a secondary mechanism could be responsible for the positive findings in peripheral blood lymphocytes.

19 The COM considered that no definite conclusions could be reached with regard to the small increases in DNA-protein cross links reported in the studies published by Saham *et al.*[22,26]

20 The Committee considered if any of the available studies was of sufficient quality to draw conclusions with regard to systemic mutagenicity of formaldehyde. Members noted the study by Ye and colleagues[27] clearly showed an increase in micronuclei in nasal mucosal cells in workers exposed to formaldehyde during manufacture whilst no concurrent increase in micronuclei in peripheral blood lymphocytes was noted.[27] The increase in SCE formation in peripheral blood lymphocytes in this study may have resulted from a secondary mechanism following oxidative DNA damage.

21 Members commented on the publication by Orsiere T *et al* (2006).[28] The apparent increase in micronuclei with centromeres was not consistent with the proposed mechanism of formaldehyde effects cross linking DNA and proteins. It was noted the protocol was not optimal for identification of aneuploidy, and that individual data were not available. Members considered that no definite conclusions could be reached on the data presented in this publication.

22 There was no evidence for an increase in micronuclei in buccal smears from volunteers exposed for up to 4 h/day for 10 working days to levels of formaldehyde below the UK occupational exposure limit (ie < 2 ppm).[31]

23 The Committee concluded that there was no convincing evidence regarding direct systemic mutagenic effects of formaldehyde from the available biomonitoring studies. The COM agreed a secondary mechanism might be involved with regard to the genotoxic effects documented in peripheral blood lymphocytes in the biomonitoring studies reviewed.

Additional *in vitro* study of formaldehyde induced DPX

24 The Committee noted that recent *in vitro* studies had demonstrated that DPXs formed from formaldehyde were effectively removed at concentrations up to 100 μM.[30]

COM conclusions

25 The COM concluded that the amount of formaldehyde systemically available following inhalation exposure at the occupational exposure standard would be negligible.

26 The COM was aware that formaldehyde was a direct acting *in vitro* mutagen. The COM concluded that there was no convincing evidence from *in vivo* mutagenicity studies in experimental animals and from biomonitoring studies of genotoxicity in workers exposed to formaldehyde for a direct *in vivo* systemic mutagenic effect of inhaled formaldehyde. A secondary mechanism might be involved in the genotoxic effects documented in peripheral blood lymphocytes in the biomonitoring studies reviewed.

27 The COM concluded that there was no reason to consider that direct systemic mutagenicity would be involved in the mechanism of formaldehyde induced systemic tumourigenicity.

28 The Committee concluded that for occupational and environmental exposure to formaldehyde, the pattern of metabolism and distribution of formaldehyde indicate that a threshold for *in vivo* systemic mutagenicity is likely.

COM/07/S5
November 2007

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Statement on the Mutagenicity of Terephthalic Acid

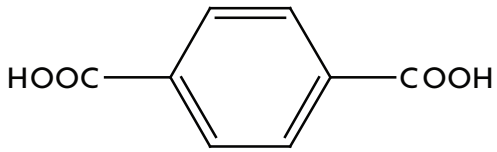
COM/07/S6 – December 2007

Background

1 Terephthalic acid (Figure 1) is used as a starting material in the manufacture of polyethylene terephthalate (PET). PET may be used to coat the internal surface and welded joints (side stripes) of food cans. PET can also be used to manufacture beverage bottles.

2 Terephthalic acid has been found to migrate from food contact materials at concentrations below 0.7 mg/kg food[1,2]. Migration from food contact materials is specifically controlled by European Regulation, which stipulates a specific migration limit (SML) for terephthalic acid of 7.5 mg/kg food.

Figure 1: Terephthalic acid



Previous Committee Evaluations

3. In October 2000, the Committee on Toxicity (COT) considered the health effects of terephthalic acid in the context of a survey on the migration of this compound from can coatings into food [1]. The COT concluded that the concentrations of terephthalic acid that had been determined in foods analysed in the survey were not of concern for public health on the basis of the then available information. However, the COT requested that, in the light of the bladder tumours occurring in rats fed the highest dietary concentration of terephthalic acid (5% in the diet, equivalent to 2.5 g/kg bw/day) in long-term studies, the view of the COM be sought on the potential *in vivo* genotoxicity of this compound [3].

4. In November 2001, the COM considered the mutagenicity of terephthalic acid based on a limited data set. *In vitro* assays included several bacterial mutagenicity assays that, although finding terephthalic acid to be negative, were either poorly reported or had inadequate protocols [4,5,6]. Overall, the Committee accepted that the evidence from the bacterial studies suggested that terephthalic acid is not mutagenic in a limited number of Salmonella typhimurium strains. An *in vitro* cytogenetics test in lung fibroblasts was also considered by the Committee [7]. Although terephthalic acid was found to be negative when tested at a concentration of 2 mg/ml using an exposure period of 48 hours, the study did not address the influence of an exogenous metabolic activation system. In addition, the effect of shorter exposure periods were not investigated. Finally, members reviewed a negative *in vivo* micronucleus assay conducted with terephthalic acid in ICR mice [8]. This was conducted to current standards but lacked toxicokinetic data, so gave no direct measurement of bone marrow exposure.

However, signs of toxicity were reported which suggested that the test material had been absorbed into the systemic circulation and thus dose selection had been adequate.

5 The Committee considered that the limited *in vitro* mutagenicity data package and absence of toxicokinetic data in the *in vivo* micronucleus assay were insufficient to determine the mutagenic potential of terephthalic acid. Therefore, the Committee recommended that an adequately conducted *in vitro* cytogenetics test in mammalian cells was needed before any definite conclusions could be reached which would indicate that the bladder tumours in the rat carcinogenicity bioassay arose from a non-genotoxic mechanism[9] .

6 Subsequently the COT has evaluated a multi-generation reproductive toxicity study. Whilst dietary administration of up to 20 g/kg diet terephthalic acid for two successive generations did not result in any alterations in reproductive performance, histopathological changes in the urinary bladder and the kidney were reported at this dose[10]. Further histopathological examination was conducted at the request of the COT. These changes comprised transitional epithelial hyperplasia, cystitis, inflammatory or mononuclear cell infiltration and haemorrhage. This expert report concluded that these treatment related changes indicated an irritant effect of the compound on the bladder mucosa at this dose level at 20 g/kg diet; however, no changes were observed in the bladder of animals receiving lower doses[11,12]. The COT were satisfied with this analysis, determining a NOAEL of 425 mg/kg bw/day for this study, equivalent to the 5 g/kg diet dose group. However, the COT decided that a final statement should not be issued until the additional mutagenicity data on terephthalic acid had been evaluated by the COM[13].

Discussion of Submitted Data

7 In May 2006, the COM was presented with a submission of data that BP Chemicals Ltd had commissioned, following the 2001 meeting.

Mouse Metabolism Study

8 A mouse metabolism study was submitted to address concerns regarding bone marrow exposure at the doses selected for the mouse micronucleus study, which had been reviewed at the 2001 meeting. The test material for this study was prepared from unlabelled terephthalic acid (756.2 mg, 99.9% purity w/w) mixed with [^{14}C]-terephthalic acid (4.51 MBq = 0.25mg, 99.2% purity w/w) in aqueous carboxymethylcellulose (8.69g, 0.5% w/w). Seven groups of three male Crl:CD-1TM(ICR) BR mice were administered this test material (800 mg terephthalic acid/kg bw) via the intraperitoneal route. This dose, strain and administration route were selected to be consistent with those used in the micronucleus study. Groups of mice were sacrificed at 2, 4, 6, 12, 24 and 48 h post treatment and the excreta of the 48 h dose group were collected for the duration of treatment.

9 The administered dose was extensively absorbed into the systemic circulation, widely distributed and rapidly excreted. Greater than 70% of the administered dose was excreted in the urine by 24 h. The highest mean tissue concentration was found in the kidney 2 h post administration (563 µg equiv/g, representing 1% of the administered dose) followed by bone (74 µg equiv/g). Difficulties in isolating murine femur bone marrow were experienced and bone marrow could be extracted from two of the three mice in the high dose group, with one below the limit of detection and the other 92 µg equiv/g, compared to blood (167 µg equiv/g) and plasma (221 µg equiv/g). Radioactivity declined rapidly in all

tissues, with levels in most tissues below the limit of detection by 48 h. Analysis of the urine found a single peak, which was reported to comprise the parent compound and a sulphate conjugate of terephthalic acid (based on mass spectrometry data).

10 It was noted that there was considerable variation in tissue levels of terephthalic acid between the three animals used in each dose group, which was most apparent at 2 hours. Members also noted that the vehicle was different to the one that had been used in the micronucleus study, further complicating interpretation of the data.

11 The Committee considered that this metabolism study was not helpful in demonstrating target tissue exposure had been achieved in the micronucleus test. However, members noted there was evidence of systemic toxicity in the preliminary toxicity study and in the micronucleus study. Also, that there had been a reduction in the Polychromatic/Normochromatic erythrocyte ratio (P/N ratio) in the micronucleus test, which suggested that target tissue exposure to terephthalic acid, had occurred.

Unscheduled DNA Synthesis Study (UDS)

12 A second *in vivo* study was submitted to supplement the micronucleus study. A single oral dose of terephthalic acid (2000 mg/kg bw, >99.9% purity w/w) was assessed for its ability to induce UDS in the liver of male Alpk:APfSD rats. Groups of three rats were sampled at 2 and 16 h post administration. Assessment of the mean net nuclear grain counts and percentage of cells in repair indicate terephthalic acid did not induce UDS at either time point. This study was performed to GLP, adhering to OECD guideline 486 (1997). Negative (vehicle) and positive (N-nitrosodimethylamine, 10 mg/kg bw) controls behaved as expected in this assay.

13 Members agreed that this unscheduled DNA synthesis (UDS) study had been adequately conducted and was negative.

In Vitro Cytogenetics Studies

14 As per the original COM data request, *in vitro* cytogenetics data using human lymphocytes to assess the clastogenicity of terephthalic acid were provided. In the first study, concentrations of 50, 250 and 500 µg/ml terephthalic acid (99.9% purity) were applied in the presence and absence of S9 metabolic activation. This assay was limited to 500 µg/ml at which the pH of the culture medium was reduced from 7.10 to 6.74. At 1000 µg/ml the pH of the medium was reduced to pH 6.00.

15 Following the standard protocol, two independent experiments were performed; Experiment 1 assessed the clastogenicity of terephthalic acid following 3 h incubation in the presence and absence of S9 metabolic activation and Experiment 2 assessed terephthalic acid following 3 h incubation in the presence of S9 and 20h in the absence of S9. All cultures were harvested 20 h after dosing (68 h after culture initiation).

16 A dose related reduction in mitotic index was observed in both experiments of this study. Statistically significant increases in the percentage of aberrant cells were observed following 20 h incubation in the absence of S9 metabolic activation ($p<0.01$ at 250 and 500 µg/ml). In addition, there were small increases in the percentage of aberrant cells following 3 h incubation in the presence and absence of S9 metabolic activation. Therefore, under the conditions of this initial study, terephthalic acid was found to be clastogenic.

17. A second study was submitted using the sodium salt of terephthalic acid, sodium terephthalate (99% purity w/w). In this study, no reduction in pH was observed when tested up to 2100 µg/ml, the maximum concentration stipulated by the protocol for this assay (10 mM). Therefore, concentrations of 1000, 1500 and 2100 µg/ml were examined. As before, independent experiments assessed the clastogenicity of sodium terephthalate following 3 h incubation in the presence and absence of S9 metabolic activation and 3 h in the presence of S9 and 20 h in the absence of S9. All cultures were harvested 20 h after dosing (68 h after culture initiation).

18 Small but statistically significant increases in the percentage of aberrant cells were observed, when compared to the vehicle control, following 3 h incubation in the presence and absence of S9 metabolic activation. These were not concentration related and were within the range of the historical control. Treatment did not reduce the mitotic index. No statistically significant increases in aberrant cells were observed in cultures incubated for 20 h in the absence of S9 metabolic activation. The author of the study report concluded that sodium terephthalate was not clastogenic under the conditions of this study. It was also argued that the positive finding for terephthalic acid in this study should be taken in the context of the negative result for sodium terephthalate in the second study. The author suggested that the clastogenicity observed in the initial study was not associated with the terephthalate anion itself.

19 Both studies were performed to GLP, adhering to OECD guideline 473 (1997). Negative (vehicle) and positive (mitomycin C, 0.5 µg/ml; cyclophosphamide, 50 µg/ml) behaved as expected in both studies.

20 Members were concerned that a relatively small reduction of 1 pH unit could not fully account for the clastogenicity observed in the first study. Whilst the criteria for a positive response had not been fulfilled in the second study, the low incidence of aberrations in the control cultures meant it was not possible to determine that terephthalic acid produced no effect. It was noted that 100 metaphases had been scored in the controls and at each dose group level. Members agreed that this should be increased to 200 to aid interpretation of the data.

21 These additional counts were provided for the May 2007 meeting. Members considered that the additional metaphase counts had reaffirmed the previous result, which suggested a weak clastogenic effect *in vitro*, and that the mechanism for this effect was unclear. Interpretation of this response was complicated by the incidence of aberrations in the control cells, which was much lower than the historical control. However, this study did not meet the criteria for a positive effect.

Conclusions

22 The Committee agreed that the two *in vivo* studies were adequate and negative, indicating that terephthalic acid is not an *in vivo* mutagen. The available evidence supported the previous COM conclusion of a non-genotoxic mechanism for the bladder tumours seen in the rat carcinogenicity study.

COM/07/S6
December 2007

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2007 Membership of the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment

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Professor Peter B Farmer MA DPhil CChem FRSC
Professor of Biochemistry, Cancer Studies and Molecular Medicine, Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester

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Non-specialist Member

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Professor David H Phillips BA PhD DSc FRCPath
Professor of Environmental Carcinogenesis, Institute of Cancer Research

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Dr D Benford BSc PhD	*Joint Scientific Secretary – Food Standards Agency*
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Dr L Hetherington BSc PhD	*Scientific – Health Protection Agency*
Ms F Pollitt MA DipRCPath	*Scientific– Health Protection Agency*
Mr S Robjohns BSc MSc	*Scientific – Health Protection Agency*

Declaration of COM members interests during the period of this report

	Personal Interest		Non Personal Interest	
MEMBER	COMPANY	INTEREST	COMPANY	INTEREST
Professor P B Farmer (Chair)	Santander Bradford & Bingley Foreign & Colonial Friends Provident Health Effects Institute Torotrak ILSI HESI	Shareholder Shareholder Shareholder Research Committee Member Shareholder Committee Member	American Chemistry Council CEFIC	Research support and conference attendance expenses. Research Support
Dr C Allen	NONE	NONE	NONE	NONE
Dr B Burlinson	Huntingdon Life Sciences	Salary Employee Share Option Holder	NONE	NONE
Dr G Clare	Covance Allied Domecq AstraZeneca Diageo HBOS Marks & Spencer	Salary Shareholder Shareholder Shareholder Shareholder Shareholder	NONE	NONE
Dr J Clements	Covance	Salary Share Option Shareholder	NONE	NONE
Dr B M Elliott AstraZeneca	Syngenta	Salary Share Option Holder Shareholder	NONE	NONE
Dr D Gatehouse	Covance Friends Provident GlaxoSmithKline Share Option Holder Shareholder	Salary Consultant Shareholder Pension	NONE	NONE
Mrs R Glazebrook	BT Group Lloyds TSB National Grid	Shareholder Shareholder Shareholder	NONE	NONE

MEMBER	Personal Interest		Non Personal Interest	
	COMPANY	INTEREST	COMPANY	INTEREST
Professor N J Gooderham	Banco Santander	Shareholder	FSA	Research contract
	CENES	Shareholder	GlaxoSmithKline	CASE studentship
	Silence Therapeutics	Shareholder	FEMA (USA)	Research support
	Hargreaves Lansdown	Shareholder		
	Proctor & Gamble	Consultant		
Dr D P Lovell	National Grid Transco	Shareholder	AstraZeneca	Spouse Shareholder
			National Grid Transco	Spouse Shareholder
	Pfizer	Shareholder Share Options Pension		
Dr I Mitchell	Kelvin Associates	Associate Consultant		
	IM Enterprises	Director/Creditor		
	Chilfrome Enterprises	Director Pensioner		
	GlaxoSmithKline	Option and Shareholder Consultant	NONE	NONE
	Allergy Therapeutics	Shareholder		
	BG	Shareholder		
	Cadbury Schweppes	Shareholder		
	GEC	Shareholder		
	GSK	Shareholder		
	ICH	Shareholder		
	Mitchell & Butler	Shareholder		
	Pfizer	Shareholder		
	Real Good Food	Shareholder		
	Renishaw	Shareholder		
	Royal Dutch Shell	Shareholder		
	RTZ	Shareholder		
	Unilever	Shareholder		
	Vedanta	Shareholder		
	BP	PEP Holder		
	Centrica	PEP Holder		
	Green King	PEP Holder		
	Scottish & Southern	PEP Holder		
Dr E M Parry	Invesco	PEP Holder		
	Fleming	PEP Holder	NONE	NONE
	Legal & General	PEP Holder		
	Quintiles	Consultancy		

MEMBER	Personal Interest		Non Personal Interest	
	COMPANY	INTEREST	COMPANY	INTEREST
Prof D H Phillips	Aviva Banco Santander BG Group Bradford & Bingley Centrica National Grid ECETOC Servier Butler Jeffries (solicitors)	Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Honorarium Honorarium Honorarium	NONE	NONE