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 2005, the Committee provided advice on a wide range of chemicals which included furan, halonitromethanes, meglumine, proquinazid perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA).

The assessments of furan, PFOS and PFOA were undertaken in conjunction with its sister Committee on Carcinogenicity (COC). A successful joint meeting between COM and COC was held on the subject of the use of target organ mutagenicity data in carcinogen risk assessment and a publication for a peer reviewed journal is to be submitted soon. The COM also has an ongoing responsibility to provide Government Departments and Regulatory Authorities with advice on developments in procedures for the evaluation and risk assessment of mutagens. In this regard the Committee provided advice on the *cII* transgenic mutation assay and a comparison with data provided by the transgenic *lacI* and *lacZ* assays. A detailed consideration of potential areas of future consideration of mutagen risk assessment was undertaken including developments in the strategy for testing of mutagenicity, the role of methylation in transgenerational effects and evaluation of mixtures of mutagens.

The COM completed a major piece of work on the evaluation of biomonitoring studies of genotoxicity in pesticide applicators. The statement was forwarded to the Advisory Committee on Pesticides and was well received. A peer review publication is in preparation.

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Biomonitoring studies of genotoxicity in pesticide applicators

2.1 The Medical and Toxicology Panel (MTP) of the Advisory Committee on Pesticides (ACP) has asked the COM for advice on the biomonitoring studies of genotoxicity in pesticide applicators. The MTP had considered in April 2004 that there were sufficient numbers of reports of biomonitoring studies in the published literature which had been retrieved and evaluated by MTP to request an independent view from COM on the available studies. It was noted that pesticide active ingredients that are DNA reactive *in-vivo* mutagens are not approved for use in formulated pesticide products in the U.K.

2.2 The DH Toxicology Unit at Imperial College in collaboration with the COM secretariat drafted a series of review papers for the COM. The review considered published biomonitoring studies in the scientific literature up to December 2004. A comparison was undertaken with the literature search strategy used by the MTP in order to ascertain whether any published studies had been missed. A very good agreement between the MTP and COM literature searches was attained with only one study identified in the COM literature search which had not already been identified by MTP. The discussion papers can be accessed via the COM internet site under the "papers" section.
http://www.advisorybodies.doh.gov.uk/com/

2.3 The COM took its remit from the referral from MTP and agreed to consider data originating from applications of formulated pesticides and pesticide mixtures. COM did not consider studies where a single pesticide active ingredient with known mutagenic potential was used (e.g. methyl bromide fumigation). A total of 70 studies were subject to initial assessment and a review for the adequacy of the genotoxicity assessment. The COM discussed the exclusion/inclusion criteria at the October 2004 and February 2005 meeting and following a postal consultation after the February 2005 meeting. Following this procedure 24 studies selected by COM using the exclusion/inclusion criteria were subjected to an epidemiology overview. The full report of the epidemiology review can be accessed from the COM internet site (http://www.advisorybodies.doh.gov.uk/pdfs/mut0511.pdf). One objective of the epidemiology overview was to attempt a quality ranking of studies (for example study design, consideration of confounding, modelling of results and interpretation). The COM considered the epidemiology overview at its 26 May 2005 meeting. Members agreed that the review had highlighted and confirmed their views on the limitations of the data set. However members agreed to review the data in order to reach the most appropriate conclusions possible.

2.4 The COM considered the full published reports and a narrative summary of the selected studies.
http://www.advisorybodies.doh.gov.uk/pdfs/MUT0419.pdf
http://www.advisorybodies.doh.gov.uk/pdfs/mut051.pdf

2.5 The COM established criteria for assessment. In this regard the publication by Albertini et al (*Mutat Res* **463**, 111-72, 2000) were valuable. The COM also undertook a consideration of the variation in background frequency of genotoxicity indices used in the biomonitoring studies, the magnitude of response seen in studies of patients and nurses exposed to known *in-vivo* mutagenic cytostatic medicines, the potential influence of use of Personal Protective Equipment (PPE), and available exposure information from studies in pesticide applicators. Members agreed that consideration of statistical significance and magnitude of effects from adequately conducted studies was the most appropriate approach to evaluating the available data. Members confirmed that the available information was

severely limited and hence no definite conclusions could be drawn with regard to any U.K. agricultural applications of pesticides or to individual pesticide active ingredients. However the COM agreed that it would be appropriate to consider what conclusions could be derived from the selected studies.

2.6 Members considered that the factors which accounted for the variance in the indices of genotoxicity in these biomonitoring studies (chromosome aberrations and micronuclei predominantly in circulating blood lymphocytes) in nurses and cancer patients exposed to cytostatic medicines had not been fully evaluated. The biomonitoring indices of genotoxicity were observed to differ considerably in the control populations of different studies. Members concluded, therefore, that it was not possible to establish a minimum fold increase for biological importance that could be applied to studies of pesticide applicators based on the studies in nurses and cancer patients exposed to cytostatic medicines. Further, it was concluded that factors affecting variance in genotoxicity indices used in the biomonitoring studies of pesticide applicators which had been reviewed were not understood adequately. In this respect members considered it would be very difficult to infer causality for the small magnitude responses seen in the biomonitoring studies of pesticide applicators. There was a need for more data on the background variability in the general population of biomonitoring indices of genotoxicity, and on factors affecting variance, which was required before a proper assessment of studies could be made.

2.7 The COM noted that with regard to pesticide active ingredients that were currently approved for use in the U.K. and which were also classified with regard to mutagenicity under EC/67/548, carbendazim was used in 4/11 positive and 1/4 negative studies respectively. This represented a very small and incomplete amount of information. It was noted that benzimidazoles were used in a number of approved pesticide products since a risk assessment could be undertaken for the threshold related effects of these active ingredient on microtubule inhibition. The COM confirmed that the available information was severely limited and hence no definite conclusions could be drawn with regard to any U.K. agricultural applications of pesticides or to individual pesticide active ingredients. The COM concluded;

i) The COM review was based on 70 retrieved published studies of biomonitoring of genotoxicity in pesticide applicators. The evidence covered a large number of types of applications and a wide diversity of pesticide mixtures. The COM selected 24 studies from which conclusions could be drawn through the application of a quality screen of retrieved studies. An independent epidemiological overview of these 24 studies reported that all had significant design and evaluation faults. The COM agreed that any conclusions reached on this evidence would be limited by the poor quality of the available studies.

ii) The COM agreed that following a review of the 24 selected studies there was limited evidence supporting increased biomonitoring indices of genotoxicity in biomonitoring studies of pesticide applicators. The COM agreed that it was not possible to make any conclusions regarding exposure-response from the selected biomonitoring studies of genotoxicity because of the inadequacy and unreliability of exposure measurements and the generally small increases in response. The COM noted that there was no published study of pesticide applicators using pesticide mixtures in the U.K.

iii) The COM agreed that the factors which accounted for the variance in biomonitoring indices of genotoxicity (chromosome aberrations and micronuclei predominantly in circulating blood lymphocytes) in nurses and cancer patients exposed to cytostatic medicines and in pesticide applicators had not been fully evaluated. It was not possible to define a minimum increase in biomonitors of genotoxicity associated with cytostatic medicines from the available studies on nurses and cancer patients. Based on these observations and the large inter-study variation for the biomonitoring indices of genotoxicity in unexposed populations, the COM concluded that it would be very difficult to infer causality for the small magnitude responses seen in the biomonitoring studies of pesticide applicators. There was a need for more data on the background variability in the general population of biomonitoring indices of genotoxicity, and on factors affecting variance, which was required before a proper assessment of studies could be made.

iv) The COM agreed that it is very difficult to draw conclusions on what might be the most appropriate biomonitoring study for U.K. pesticide applicators. There was some very limited evidence to suggest that an appropriate study of floriculturalists using benzimidazoles (e.g. carbendazim) might represent a reasonable proposal. The COM concluded that in view of the large numbers of individuals which would be required in order to detect an effect in such a study it was most unlikely that a sufficiently large and appropriate exposure group could be identified.

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

Furan

2.9 Furan is used industrially primarily as a solvent or in the synthesis of commercial compounds. It is also found in air pollution, tobacco smoke and many foods and beverages. The COM had been asked for advice on the mutagenicity of furan by the COT who were considering research that would be helpful regarding the risk assessment of this contaminant. Members agreed there were no clear structural alerts for mutagenicity but metabolism mediated by CYP2E1 could lead to epoxide formation which was consistent with the finding of cis-2-butene-1,4-dial. It was noted that carbon dioxide was a major metabolite which was indicative that furan ring opening occurred. However there were no quantitative data on metabolism available.

COM evaluation

2.10 The COM considered that the available *in-vitro* mutagenicity tests of furan in bacteria had been adequately conducted. There was no evidence that furan was mutagenic in *Salmonella typhimurium* strains except for a weak positive response in one study in TA100 in the presence of exogenous metabolising fraction. The positive results in tests using Salmonella typhimurium *TA 104* with cis-2-butene-1,4-dial were consistent with this compound being a reactive aldehyde. Members noted the positive results reported in chromosomal aberrations tests in CHO cells in both the presence and absence of exogenous metabolic activation but commented that the concentrations where positive results had been identified (ca 125 mM and 250 mM) were considerably greater than the OECD upper limit of 10 mM for non cytotoxic test materials. There was no information on cytotoxicity in the NTP CHO test report.

2.11 Members considered that the L5178Y mouse lymphoma assay reported by the NTP had been conducted to standards acceptable at the time using plating in soft agar but that a trial had not been conducted in the presence of an exogenous metabolising fraction. Members agreed that in view of the positive results at non-cytotoxic doses this study should be considered as positive and that furan should be regarded as having *in-vitro* mutagenic activity.

2.12 The COM considered the available *in-vivo* mutagenicity studies. The key *in-vivo* bone marrow chromosomal aberration assay was undertaken as part of the NTP programme on furan. Members agreed that the data from this study could not be interpreted with certainty because of the confounding effects of toxicity at the dose level used. The COM agreed that negative results had been obtained in *in-vivo* liver UDS studies in rats and mice. Covalent binding studies using oral or intraperitoneal doses of radiolabelled 2,5-^{14}C-furan in rats were available but members agreed the data could not be interpreted due to the low specific activity radiolabelled furan used. The results of *in-vitro* studies of the reaction of cis-2-butene-1,4-dial with deoxynucleosides indicated reactions with 2'-deoxyadenosine, 2'-deoxyguanosine and 2'-deoxycytidine but not thymidine. Thus it had been proposed that cis-2-butene-1,4-dial might form DNA cross links. No definite conclusions could be drawn from the available studies of proto-oncogene mutation spectra in hepatocellular adenomas and carcinomas from control and furan treated mice.

2.13 The COM concluded that furan should be regarded as an *in-vitro* mutagen but there was insufficient evidence to reach a conclusion on the available *in-vivo* mutagenicity data. Members felt that adequate *in-vivo* mutagenicity testing would be useful. It was suggested this might include a bone marrow micronucleus test or DNA binding studies in cancer target organ tissues. It was noted that the critical carcinogenic effect was cholangiocarcinoma and there would be difficulties in isolating bile duct cells during DNA binding studies.

2.14 The COM advice was forwarded to COC and subsequently to COT.

Halonitromethanes

2.15 Halonitromethanes (HNM's) are a class of compound characterised by the presence of one or more halogen atoms together with a nitro moiety on a single central carbon. They differ from the halomethanes (HM's) by the presence of the nitro moiety. The HNM's had been identified as disinfection by-products (DBP) in drinking water in the US and had received a high priority ranking for investigation by the Environmental Protection Agency http://www.epa.gov/athens/publications/EPA_600_R02_068.pdf. The only available published data related to the *in-vitro* studies in Salmonella typhimurium strains and in *in-vitro* Comet studies, conducted using CHO cells. Some preliminary findings had been reported by the EPA to the DH Toxicology Unit regarding *in-vivo* testing of the HNM's in transgenic medaka fish. The COM was also aware of an NTP negative *in-vivo* bone marrow mouse micronuclei test with trichloronitromethane (TCNM; chloropicrin), but that there were no appropriate *in-vivo* mutagenicity data available for other HNMs.

COM evaluation

2.16 The strains tested included TA 98, TA 100, TA 104 and a strain RSJ100 which expresses rat glutathione transferase theta (GSTT1-1) known to activate halomethanes. Members agreed that the standard plate incorporation approach had been undertaken in this study used two plates per dose point (instead of the normal three) but that reproducible small increases in the number of revertant colonies had been reported. It was noted that HNMs might volatilise and therefore procedures to prevent evaporation in the bacterial mutagenicity tests might have been more appropriate. With regard to the Comet studies Members noted that HNMs tested were cytotoxic in CHO cells and that apoptosis induction had not been recorded in these studies. In addition none of the trials had included the addition of an exogenous metabolising fraction. However members agreed that HNMs were genotoxic in this test system.

2.17 Overall, in accordance with the COM strategy, HNM's should be regarded as *in-vitro* mutagens and potential *in-vivo* mutagens. It was considered that there was a need for appropriate *in-vivo* testing. The COM suggested:-
a) *in-vivo* rat liver UDS assay, followed by
b) site of contact COMET assay
If either of these studies yielded positive results, then the chemical under test should be considered to be an *in-vivo* mutagen.

2.18 A statement was published and is appended at the end of this report.

2.19 The COM was subsequently informed of a personal communication from Dr S Richardson (US EPA) at the October 2005 meeting. It was noted that the preliminary finding of DNA adducts in rat liver following *in-vivo* exposure to dibromonitromethane supported the view that this compound should be considered as having mutagenic potential *in-vivo*.

Joint COT/COC/COM review of nanomaterials

2.20 The risk assessment of nanomaterials was identified by COT/COC/COM as an area of interest during horizon scanning discussions in 2004. The Committee was aware of the publication of a report by the Royal Society and the Royal Academy of Engineering 'Nanoscience and nanotechnologies: opportunities and uncertainties' previously discussed by COT in September 2004. The UK Government's response to this report was published in February 2005. The COT, COC and COM were identified as relevant scientific committees to provide advice on the development of nanotechnology. The information presented to the committees was based on a hazard assessment document published by the Health and Safety Executive (HSE) with additional papers and abstracts identified by the secretariat. There were considerable limitations in the number of materials tested, and in the toxicology data available.

2.21 The COM considered some specific *in-vitro* mutagenicity data for particulate titanium dioxide and zinc oxide of various sizes including test materials described as being nanomaterials. The COM also considered some specific mutagenicity data on fullerenes. It was agreed that no definite conclusions on the influence of particle size on mutagenicity *in-vitro* could be drawn from the available information.

2.22 The COM considered that specific information on particle size was required to assess mutagenicity studies undertaken with nanomaterials. Thus the available information on titanium dioxide did not allow an assessment of the agglomeration/disagglomeration of particles in the vehicles used and it was not possible to conclude what particles had been tested. The COM agreed that it might be appropriate to provide imaging data on particle sizes in order to evaluate *in-vitro* mutagenicity tests nanomaterials.

2.23 A statement is appended at the end of the COT section of this report.

Meglumine

2.24 The COM provided advice on the mutagenicity of Flunixin, meglumine and Flunixin meglumine (a non-steroidal anti-inflammatory veterinary medicine)-meglumine (an excipient, exempt from EU regulations) in a statement COM/03/S2 in response to a request for advice from the FSA. The available data on these compounds in 2003 were relatively poor and the COM agreed the best approach was to consider the data on each entity separately. The evaluation of meglumine posed the greatest difficulty in that there were inadequate *in-vitro* data on this compound but some clear positive results had been documented in relatively old *in-vivo* bone-marrow assays using intraperitoneal administration of 2 doses of meglumine followed by harvest 6 hours after the last dose. A number of confirmatory *in-vivo* studies undertaken for the COM had yielded negative/inconclusive findings. The COM had concluded that there was a need for two adequately undertaken *in-vitro* studies (namely a mouse lymphoma assay and an *in-vitro* chromosome aberration assay in human peripheral blood lymphocytes) before conclusions could be reached.

2.25 The COM agreed that the two *in-vitro* studies submitted did not provide any evidence for a mutagenic effect and the assays had been adequately conducted.

2.26 A revised statement on Flunixin, meglumine and Flunixin meglumine is appended at the end of this report.

Proquinazid

2.27 Proquinazid is a novel fungicide being considered by the ACP under the plant protection Directive (91/414/EC). The data holder is DuPont Chemicals. Proquinazid (6-iodo-2-propoxy-3-propyl-3H-quinazoli-4-one) is intended for use in agriculture and viticulture providing control of powdery mildew in cereals and grapes (Blumeria graminis. Class Ascomycetes). It is a novel class of fungicide acting by inhibiting the development of the appressorial germ tube (which is responsible for penetration of the host) but the mechanism of action was unknown. The ACP deferred making a decision pending advice from the COC and COM with regard to the occurrence of cholangiocarcinoma in the rat carcinogenicity bioassay. The COC/COM have not been asked to review any other tumour reported in rodent carcinogenicity bioassays with proquinazid.

2.28 Members agreed there were no particular structural alerts for mutagenicity with regard to proquinazid but noted that a possibility for generation of metabolites with a quinone type structure which might potentially alert for free radical generation.

2.29 Members heard a presentation from the data holder (Du Pont Chemicals) and discussed a number of aspects of the conduct of the mutagenicity tests which had been submitted. The purpose of the presentation was to provide DuPont's rationale on the adequacy of the genetic toxicology data base for proquinazid and to specifically discuss the results of the mammalian cell *in-vitro* chromosome aberration assay in human lymphocytes and the *in-vitro* mammalian cell gene *hprt* mutation assay in CHO cells. DuPont sought the Committee's agreement that proquinazid is not genotoxic and further testing is unnecessary.

2.30 Members considered that the company had provided some relevant information and comments but had not been convinced that the *in-vitro* mutagenicity test package was adequate. Members agreed that a key element of any proposal regarding mechanism of proquinazid induced cholangiocarcinoma in the rat would require an evaluation of mutagenicity and hence it was important to complete the mutagenicity test package. The COM considered that a mouse lymphoma assay should be conducted and that it was advisable to also conduct a continuous 20 h exposure in a mammalian cell chromosomal aberration assay (in human lymphocytes) in the absence of exogenous metabolic activation. Both studies should be conducted to internationally accepted standards. These studies were required to provide full information on the mutagenicity evaluation of proquinazid.

COM post meeting consideration of additional mouse lymphoma assay

2.31 The data holder submitted on 12 July 2005 the results of a new mouse lymphoma assay undertaken with proquinazid. The assay included an exposure of 3 hour (in presence and absence of exogenous metabolic activation) and a continuous 24 h exposure in the absence of metabolic activation. The results suggested that proquinazid was not mutagenic in this assay. A full report was not available during the postal consultation with COM members. The COM considered the available data from the new mouse lymphoma assay by postal consultation and agreed there was no evidence for a mutagenic effect. It was also agreed that the full report should be considered by COM when available. The final report was submitted to COM members by postal consultation on the 22 August 2005. Members agreed that the submitted mouse lymphoma assay was acceptable and gave negative results.

2.32 The COM conclusions were forwarded to the COC. A statement is appended at the end of the COC section of this report.

Perfluorooctane sulfonate (PFOS) and Perfluorooctanoic acid (PFOA)

2.33 These compounds are multifluorinated chemicals based on an eight carbon linear molecule (some branched chain and lower alkane fluorinated carbon impurities may be present at low levels). PFOS and PFOA had unusual physico-chemical properties forming microdispersion micelles in aqueous systems. Members heard that the Environment Agency had recently concluded that PFOS meets the criteria for classification as a Persistent, Bioaccumulative and Toxic (PBT) substance. PFOS and PFOA have the potential to enter the food chain and could have a negative health impact on humans. The COT had concluded that the available toxicology data indicate that PFOS and PFOA do not demonstrate sufficient similarities to be assessed jointly. The COT had asked for advice on mutagenicity and carcinogenicity from the COM.

2.34 There were no apparent structural alerts for mutagenicity and the evidence from animal studies is that absorbed material is not metabolised. However there is evidence for target organ toxicity in repeat dose studies with both PFOS and PFOA in rats and monkeys and evidence for adverse effects on reproduction and the induction of endocrine target organ tumours with PFOS and PFOA and liver tumours in rats with PFOS. The mechanisms for these toxicological effects had yet to be evaluated by COC and COT.

PFOS

2.35 Members discussed the mutagenicity data on PFOS and agreed that the physico-chemical properties of PFOS considerably complicated the formulation of appropriate test solutions for mutagenicity studies. The use of dimethyl sulphoxide (DMSO) as a solvent using dose levels up to those which induced cytotoxicity or precipitation was agreed as acceptable. Members agreed that the *in-vitro* plate incorporation test using five strains of Salmonella typhimurium and the D4 strain of Saccharomyces cerevisiae gave negative results. Members agreed that the reverse mutation assay using Escherichia coli gave negative results. The difficulty in formulating an adequate suspension of the test material was noted for the *in-vitro* chromosomal aberration assay in human lymphocytes. Members agreed that this study had yielded negative results. Members agreed that the *in-vitro* UDS assay in rat liver primary hepatocytes gave negative results. Members commented on the difficulty in adequately formulating PFOS for oral dosing of mice in the bone-marrow micronucleus test. Members noted that only 1000 micronuclei had been evaluated at each dose level. However overall the study was considered to be acceptable and provided negative results.

2.36 The COM agreed that the studies undertaken with PFOS were acceptable and that PFOS should be regarded as not mutagenic.

PFOA

2.37 The COM discussed the data on PFOA. It was noted that mutagenicity assays had been conducted using aqueous solutions of sodium or ammonium perfluorooctanoate.

2.38 Members agreed that the plate incorporation bacterial mutagenicity tests using strains of *Salmonella typhimurium* and *Escherichia coli* using sodium or ammonium perfluorooctanoate were adequately undertaken and gave negative results. The COM noted that the *in-vitro* hprt assay in CHO cells using ammonium perfluorooctanoate had reported negative results.

2.39 Members considered that a mutagenic response had been documented in the *in-vitro* chromosomal aberration assay of sodium perfluorooctanoate in CHO cells in the presence of exogenous metabolic activation at the two highest concentrations. This finding had been reproduced in a confirmatory assay in the presence of exogenous metabolic activation at the highest concentration tested. No evidence for increased chromosome aberrations had been documented in the absence of exogenous metabolic activation. However it was unclear as to what extent the results reported from this *in vitro* study were due to cytotoxicity. An increase in chromosome aberrations was also documented in an assay using ammonium perfluorooctanoate at the highest dose tested in the presence of exogenous metabolic activation. There was clear evidence of cytotoxicity at this dose level in this particular assay. It was

agreed that sodium perfluorooctanoate, in the absence and presence of metabolic activation, had not induced chromosomal aberrations in cultured human whole blood lymphocytes when tested up to doses that were cytotoxic. Members commented that the available information indicated that absorbed PFOA was not metabolised by mammals.

2.40 No evidence for a mutagenic effect was found in a mouse bone marrow micronucleus assay where mice had been given a single oral gavage dose of up to 5000 mg/kg bw sodium perfluorooctanoate or 1990 mg/kg bw ammonium perfluorooctanoate. The test materials had been solubilised in deionised water. There was clear evidence of toxicity (mortality of both males and females) and reduced PCE/NCE ratios in animals dosed with 5000 mg/kg bw sodium perfluorooctanoate. A dose level of 1990 mg/kg bw ammonium perfluorooctanoate did result in one male death but did not have an effect on the PCE/NCE ratio. Members commented that the in-vivo bone marrow mouse micronucleus studies had been adequately conducted although there was no direct measure of exposure of the bone marrow to the test materials and also noted that absorbed PFOA was highly bound to proteins.

2.41 The COM discussed what further testing might be appropriate for PFOA salts and considered whether an investigation of in-vivo DNA binding or further evaluation of the mechanism of *in-vitro* chromosomal aberration assays were appropriate. Overall, the COM concluded that the results from the *in-vitro* chromosomal aberration assays with PFOA salts were likely to represent a cytotoxic response. It was agreed that a plausible *in-vitro* mechanism for the positive response in CHO cells was required to reassure the COM of this conclusion.

Horizon Scanning

2.42 The COM undertakes an annual exercise to identify areas of mutagenicity risk assessment, testing and evaluation which might be the subject of future consideration. The following areas of work were agreed which have been grouped into three areas of priority.

2.43 Areas with high priority were;

i) COM agreed evaluation of UDS v COMET was useful but noted the amount of data available in contract test houses and in the public domain would be limited. In the first instance the secretariat was asked to identify UDS positives and look for concordance with COMET (liver). It was agreed there might be very few data available. Some wider considerations of usefulness of the comet assay could be considered subsequent to this review.

ii) Members were very interested in methylation status changes in transgenerational effects and suggested that broadening to effects on histones as potential mechanisms for permanent changes to phenotype needed to be considered. COM agreed this was a major undertaking. (The original request comes from ACP/MTP) COM felt the current mutagenicity tests strategies would not address such compounds, there might be examples from the Medicines and Healthcare products Regulatory Agency (MHRA) (e.g. antisense DNA products).

iii) COM requested some joint working with COC to improve presentation of advice to general public, to try to get over misconceptions, e.g. all pesticides are genotoxic.

iv) COM agreed some additional work on the background information on variance of biomarkers of genotoxicity in populations.

2.44 Areas with medium priority were;

i) COM agreed that consideration of mitochondrial DNA as a cellular target for mutagens was a priority for future work.

ii) COM agreed that consideration of nitropyrenes and nitro PAHs was a priority for future work.

iii) COM agreed that a review of aspects of mutagenicity of mixtures would be valuable. After some long consideration of potential mechanisms of interaction, COM agreed to look at modulation of alkylating agent mutagenicity as a first start, to see if dose/effect addition rules were adequate.

iv) COM asked for a presentation on wider aspects of international validation of mutagenicity testing.

2.45 Areas of low priority were the potency indicator approach suggested by Sanner T and Dybing E (Basics in Clinical Pharmacology and Toxicology, 96, 131-139, 2005) and the proposed evaluation of *hprt* CHO assays held by PSD. It was agreed the review would be of value to PSD in assessing overall performance of this mutagenicity assay.

Test Strategies and Evaluation

cII transgenic mutation assay (comparison with transgenic *lacI* and *lacZ* assays)

2.46 The COM and COC had considered the available mutagenicity and carcinogenicity data on malachite green and leucomalachite green during 2004. (http://www.advisorybodies.doh.gov.uk/com/mglmg.htm) A number of mutagenicity studies using the *lacI* transgene in both rats and mice were reported. However one key piece of evidence came from the finding of an increased mutagen frequency in the liver in an *in-vivo* mutagenicity assay using the *cII* transgene in Big Blue mice fed a diet containing leucomalachite green. An overview of the development and use of *in-vivo* mutagenicity assays using the *cII* transgene was drafted for the February 2005 COM meeting. A general conclusion was reached on the basis of the information reviewed.

2.47 The COM concluded that the *cII* transgene was a useful *in-vivo* mutation assay to investigate potential mutagenic effects in cancer target organs in rodents. It was necessary to routinely sequence mutant colonies in order to evaluate the significance of results obtained with the *cII* transgene in rodents. The COM felt that the currently available data in the published literature did not support the routine use of the *cII* transgene for screening chemicals but suggested it was of value in targeted studies of mutagenicity in specific organs in rodents. The COM agreed to keep the use of the *cII* transgene under review along with other developments of *in-vivo* mutagenicity testing.

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

Joint meeting of COM/COC on use of target organ mutagenicity assays in carcinogen risk assessment

2.49 The COM and COC undertake routine horizon scanning exercises as part of their annual remit (see appended internet links at the end of this statement). The COM identified the use of *in-vivo* target organ mutagenicity studies as a subject for further consideration. The COM and COC agreed to hold a joint meeting on the use of data derived from *in-vivo* target organ mutagenicity studies in carcinogen risk assessment. An open meeting of the committees was held on the 9 June 2005. Attendees included a number of external experts who gave presentations and comments to the committees discussions.

2.50 The COM and COC agreed that data from adequately conducted *in-vivo* carcinogen target organ mutagenicity and genotoxicity studies (which included information from investigations using transgenic animals, the comet assay, and approaches to measuring DNA binding (e.g. postlabelling and radiolabel methods) can provide valuable information of use in the mode-of action of carcinogenic responses seen in rodents. Such studies can provide supporting information for use by regulatory authorities in carcinogen risk assessment on a case-by-case basis.

2.51 A brief statement is appended at the end of this report. A full write up is being prepared for publication in a peer review journal.

Ongoing reviews

2.52 A number of review papers are in preparation for 2006. These currently include;

- A review of biomonitoring for genotoxicity in patients who have undergone revision arthroplasty for hip replacement.
- A review of the utility of the rat liver UDS assay and comparison with data from the COMET assay.
- A review of the factors affecting the background frequency of micronuclei in peripheral blood lymphocytes in biomonitoring studies.

Statements

Review of Biomonitoring Studies of Genotoxicity in Pesticide Applicators

Background

1. The Medical and Toxicology panel of the Advisory Committee on Pesticides has asked the COM for advice on the genotoxicity in pesticide applicators. The referral statement is given below.

"To review investigations of mutagenicity and DNA adducts in pesticide applicators and workers exposed to pesticides (e.g. handling cut flowers) and factory (manufacturing) staff engaged in pesticide manufacture/formulation and produce a statement for the Advisory Committee on Pesticides and its Medical and Toxicology Panel (MTP). The review should include all studies identified by the MTP and any other relevant studies published, particularly those originating from the UK.

The review should consult COC epidemiologists with regard to the rigour of studies evaluated. this should include design, selection of controls, bias, confounding and use of multiple statistical comparisons.

The review should be initiated at the October 2004 COM meeting."

2. The referral from ACP and the MTP came about through the ongoing routine review of epidemiology literature undertaken by the MTP. The MTP had considered in April 2004 that there were sufficient numbers of reports of biomonitoring studies in the published literature which had been retrieved and evaluated by MTP to request an independent view from COM on the available studies. It is noted that pesticide active ingredients that are DNA reactive *in-vivo* mutagens are not approved for use in formulated pesticide products in the U.K.

Evidence reviewed

3. The DH Toxicology Unit at Imperial college in collaboration with the COM secretariat drafted a series of review papers for the COM. The review considered published biomonitoring studies in the scientific literature up to December 2004. A comparison was undertaken with the literature search strategy used by the MTP in order to ascertain whether any published studies had been missed. A very good agreement between the MTP and COM literature searches was attained with only one study identified in the COM literature search which had not already been identified by MTP. (A total of 70 biomonitoring studies of genotoxicity markers in pesticide applicators was identified.[1-70]). A listing of the discussion papers considered by COM during the review period (from the October 2004 to the October 2005 meetings) is given below. All of these review papers are draft discussion papers and do not necessarily represent the views of the COM. A detailed evaluation was undertaken for all of the studies. The discussion papers can be accessed via the COM internet site under the "papers" section. (http://www.advisorybodies.doh.gov.uk/com/)

i) *Biomonitoring studies from EU (MUT/04/19). Annex 1 (overview of literature), Annex 2 Summary of individual studies and IPCS guidelines on biomonitoring studies of genotoxicity. Annex 3 tabular summary according to occupation, Annex 4 tabular summary of statistical approaches used to analysis of data.*

ii) *Review of biomonitoring studies of pesticide applicators from Croatia (MUT/04/20)*

iii) *Further information and follow-up of review undertaken in October 2004 (MUT/05/1). (Draft exclusion criteria Annexes I and II, Draft inclusion criteria Annexes III and IV, Magnitude of response Annexes V and VI, Exposure patterns documented in studies).*

iv) *Submitted published papers (for February 2005 meeting) (MUT/05/6), Addendum 1 MUT/05/6 review of studies from rest of world, Addendum 2 to MUT/05/6, tabulation of rest of world studies by occupation.*

v) *Cytogenetic changes following cumulative exposure to pesticides (MUT/05/9)*

vi) *Revised criteria (MUT/05/10)*

vii) *Epidemiological overview (MUT/05/11)*

viii) *Discussion paper on evaluation of positive studies and control data (MUT/05/12) (Annex 1 Evaluation of positive response in biomonitoring studies of genotoxicity, Annex 2 Information on pesticide usage in UK).*

Pesticide applications considered in the review.

4. The papers retrieved identified a wide diversity of occupational pesticide exposures. The authors described investigations in occupational groups such as floriculturalists, green house workers, agricultural workers and farmers, pesticide sprayers and applicators (which included agricultural/horticultural, amenity, fumigators), production workers (e.g. manufacture of pesticides) and forestry workers. The extent of information provided on occupational exposure to pesticides (e.g. during handling, diluting, applying), the duration of exposure and use and adequacy of personal protective clothing varies considerably between the different accounts. The Committee considered it was difficult to evaluate such a diverse data set. It was agreed that the most appropriate approach would be to assess the adequacy of the studies with regard to investigation and evaluation of genotoxicity indices and with regard to overall adequacy of design, analysis and interpretation of results. With respect to overall adequacy, the COM sought an opinion from an independent epidemiologist.

Indices of genotoxicity used in the reviewed biomonitoring studies

5. A short overview of the indices of genotoxicity used in biomonitoring studies is provided to assist in evaluating the significance of findings. Almost all the studies considered in this review provided data for investigations using *in-vitro* culture of peripheral blood lymphocytes derived from blood samples. A

small number of studies used epithelial cells from the buccal cavity. The committee had access to the general guidance published by a WHO IPCS working group on use of genotoxicity indicators in biomonitoring studies.[71] The Committee agreed that in general the genotoxicity indices measured in samples (predominantly peripheral blood lymphocytes) including micronucleus formation, chromosomal aberrations, comet and, [32] P-postlabelled DNA adducts results indicate uptake and exposure to DNA damaging chemicals. The evidence suggested that there may be an increased risk of mutagenicity and also possibly carcinogenicity but it is not possible to be certain that there is a risk or to quantify this risk because of the poor quality of many of the studies and frequent contradictory findings.

Micronucleus frequency

Micronuclei are small, extranuclear bodies that arise from acentric chromosome fragments or from whole chromosomes that are excluded from the nucleus during mitotic cellular division. They can be a consequence of DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis, failure of any of the mitotic apparatus or alterations in cellular physiology and mechanical disruption [71,]. In most cases, the cytokinesis-block MN method is used, in which scoring only takes place in cells that have only divided once in culture.[16] Micronucleus analysis can be used for a number of cells, both in vitro and in vivo, including lymphocytes[5,9,72] and buccal epithelial cells.[49-50] Micronucleus induction is an indirect indicator of mutagenicity. It is unclear however, whether MN formation has a specific role in carcinogenesis.[71]

Chromosome aberrations

Structural chromosome aberrations arise from direct DNA breakage, replication on a damaged DNA template or inhibition of DNA synthesis and may involve both chromatids of the chromosome (chromosome-type CA), or only one chromatid of the chromosome (chromatid-type CA).[71,73] Chromosome aberration analysis has been commonly performed on human peripheral blood lymphocytes to assess DNA damage.[11,14,16,29,42,44] To ensure that only first-generation metaphase cells are scored for CA, bromodeoxyuridine is commonly added to the culture medium prior to DNA replication *in vitro* [29]. Both structural and numerical chromosome aberrations may cause alterations to the structure or arrangement of oncogene and tumour suppressor genes of somatic cells, and hence are involved in the induction of cancer in humans.[74]

Sister chromatid exchange

Sister chromatid exchanges arise from equal exchange of DNA replication products between two identical sister chromatids of a duplicated chromosome[75] They are thought to arise as a consequence of "error free" homologous recombinational repair or bypass of DNA lesions during replication on a damaged DNA template, possibly at the replication fork.[75] In the most commonly used method of SCE analysis, DNA replication is required for two consecutive cell cycles, hence bromodeoxyuridine is added to the culture medium and cells are scored in the second division metaphase.[14,61] Although the induction of SCE has been widely used as an indicator of DNA damage following exposure to pesticides,[12,26,32,36,45,61] the mechanism of formation and biological significance of SCEs are still unknown.[75]. The COM agreed that biomonitoring studies using SCE analysis were not informative with regard to evidence for genotoxicity.

Comet assay

The comet assay, or single cell gel electrophoresis technique is a more recent technique established as a sensitive method for detecting DNA single strand and double strand breaks, alkali-labile sites, DNA cross linking and incomplete excision repair events.[34,64,76,77] The comet assay can be carried out with a number of cells, both *in vitro* and *in vivo*, including peripheral blood leukocytes, bladder, liver, buccal, gastric and sperm cells. To date, peripheral blood lymphocytes are mainly used for human biomonitoring studies following occupational exposure to an array of chemicals.[34,53,64,77]. However, the relevance of the endpoint measured in the comet assay has yet to be established, as it is usually the result of a temporary strand breakage, which is repaired within a few hours under normal circumstances and may or may not become fixed as a mutation.[77]

DNA adducts

A DNA adduct is a chemical entity covalently bound to DNA[71], and is usually formed following the interaction of an electrophilic molecule with a nucleophilic site of DNA[78]. They are often the initial DNA lesion following exposure to a genotoxic chemical and may lead to mutation and altered gene function if not repaired. In epidemiological studies DNA adducts are particularly useful as they provide information on the exact chemical exposure of the individual.[78, 32] P-DNA postlabelling technique has been widely used to measure non-radioactive carcinogenic large DNA adducts in humans, due to it being a highly sensitive technique.[78]

The COM guidance on a strategy for testing chemicals for mutagenicity recognised that artifactual positives may be obtained in the cell assays that do not reflect intrinsic mutagenic activity. Factors such as hyperthermia, hypothermia or induction of erythropoiesis may produce MN or CA[79] or exercise immediately prior to sampling may lead to increased DNA damage measured by the comet assay.[80,81]

Overview of approach used by COM

6. A flow diagram outlining the approach used by COM is shown in Figure 1.

7. The COM took its remit from the referral from MTP and agreed to consider data originating from applications of formulated pesticides and pesticide mixtures. COM did not consider studies where a single pesticide active ingredient with known mutagenic potential was used (e.g. methyl bromide fumigation). The COM agreed to eliminate a number of publications arising from studies undertaken in Croatia because of irregularities in the reported data. A number of papers from Zeljezic and Garaj-Vrhovac from the laboratory of Mutagenesis, Institute for Medical Research and Occupational Health, Zagreb, Croatia were published during 2000-2 and were identified during literature searches.[65-70] It was noted that there were apparent discrepancies between the different publications in the reporting of the demographic data on exposed and controls and reporting of results of mutagenicity studies and no confidence could be attached to the results.

8. The remaining 65 studies were undertaken from all parts of the world but the literature search did not find any published study which had evaluated UK pesticide applicators. The 65 studies were subject to a review procedure for the adequacy of the genotoxicity assessment. The COM discussed the exclusion/inclusion criteria at the October 2004 and February 2005 meeting and following a postal consultation after the February 2005 meeting. The criteria and selected studies are outlined in Annex 1 to this statement.

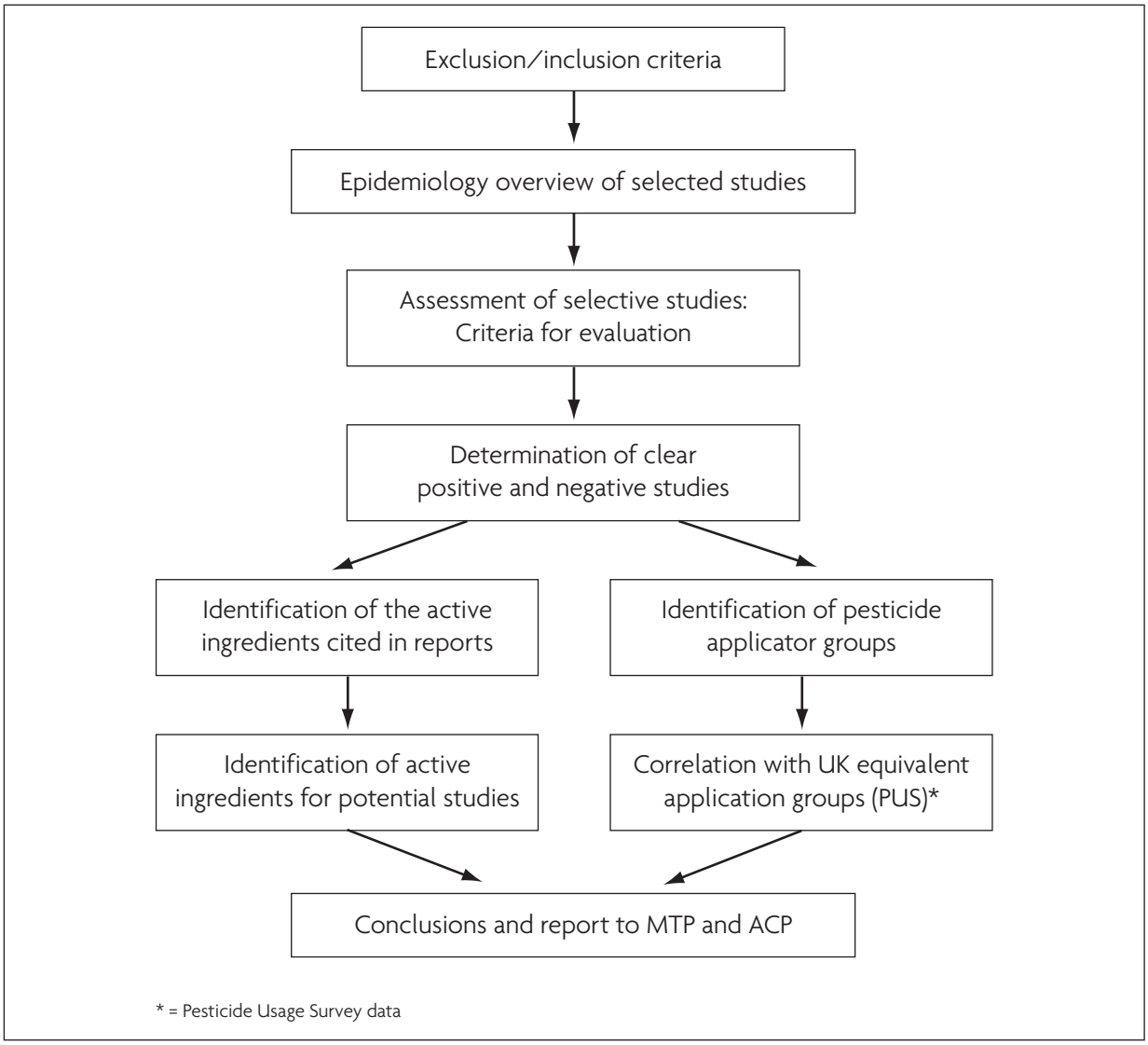

Figure 1: Flow Diagram of approach used by COM.

Epidemiology overview of biomonitoring studies

9. The 24 studies selected by COM using the exclusion/inclusion criteria were subjected to an epidemiology overview (see para 3 vii above). The full report can be accessed from the COM internet site (http://www.advisorybodies.doh.gov.uk/pdfs/mut0511.pdf). One objective of the epidemiology overview was to attempt a quality ranking of studies.

10. The Committee noted the conclusion reached in the epidemiology overview that all of the studies were limited in design, particularly with regard to measurement of exposure, study size, the assessment of selection and recruitment biases. Many of the studies provided information on demographics, medical history, lifestyle factors, potential occupational exposures to materials other than pesticides (e.g. solvents, radiation), and also information on type of pesticides used, duration and frequency of exposure and use of protective measures. However these data had generally not been used in the analyses reported and the majority of papers did not provide a specific analysis of individual pesticides. It was noted that the majority of studies were not sufficiently large to allow an evaluation of all the variables for which data might be available. Study designs were generally cross sectional, although a few had taken multiple samples (e.g. at different time points in a growing season). The time interval between exposure and sampling thus varied considerably between studies and this might affect the conclusions which could be drawn. There were limitations in the statistical approaches used in many of the studies. Thus for example many did not consider the form of the population distribution and made unsubstantiated assumptions that it was normal. The reporting of modelling was variable and in most cases was not adequate. It was noted that the papers tended to focus on statistical significance even when the absolute difference between groups was tiny. Overall it was not possible to identify any particular study that was clearly better in design and reporting than the other papers in the 24 studies identified by COM.

11. The COM considered the epidemiology overview at its 26 May 2005 meeting. Members agreed that the review had highlighted and confirmed their views on the limitations of the data set. However members agreed to review the data in order to reach the most appropriate conclusions possible.

COM Review of selected studies.

12. The COM considered the full published reports and a narrative summary of the selected studies.
http://www.advisorybodies.doh.gov.uk/pdfs/MUT0419.pdf
http://www.advisorybodies.doh.gov.uk/pdfs/mut051.pdf

Criteria for evaluation

13. The COM undertook an evaluation of the control data from the 24 studies for micronuclei and chromosomal aberrations in peripheral blood lymphocytes. Such an evaluation might aid in the assessment of the data from studies and help to decide what magnitude of response was suggestive of a positive result. Modelling of the data from the 24 selected studies suggested that MN data were normally distributed whilst there was evidence for a skewed distribution of CA as would be expected. Members felt the available data suggested that the distribution of chromosomal aberrations in human peripheral blood lymphocytes was consistent with an approximately binomial distribution, whilst distribution of micronuclei was much more dispersed than would be expected for binomial/Poisson distributions. It was considered that there might be bimodal or trimodal distributions indicating possible subpopulation effects and that more data would help to resolve the actual distribution of these indices in peripheral blood lymphocytes. The large overall variation in the negative control (reference) data (approximately 16 fold for micronuclei and chromosomal aberrations) suggested that it was not possible to define a single historical control range. Members agreed that statistical significance from adequately

conducted studies in combination with magnitude of response represented the most appropriate approach to evaluating the results of studies. Members agreed that the distribution of data should be assessed prior to consideration of the most appropriate statistical approach to analysis and that the effect of confounding factors should be clearly evaluated.

14. The COM compared the magnitude of response seen in the 24 studies of pesticide applicators with that reported for patients undergoing treatment with cytostatic medicines and nurses occupationally exposed to these medicines. Members were surprised at the small magnitude of response in the biomonitoring studies of nurses or patients exposed to cytostatic medicines. The mean fold increase in nurses (1.8, range 1.5-2.2) and in patients (mean 2.1, range 1.5-2.7) derived from studies for either micronuclei or chromosomal aberrations was similar to that reported for pesticide applicators in the studies reviewed by COM (1.7, range 0.8-5). Members noted that the higher maximum fold increase in pesticide applicators compared to nurses or patients exposed to cytostatic medicines might reflect differences in the extent of control for confounding factors between studies.

15. Members considered that the factors which accounted for the variance in the indices of genotoxicity in these biomonitoring studies (chromosome aberrations and micronuclei predominantly in circulating blood lymphocytes) in nurses and cancer patients exposed to cytostatic medicines had not been fully evaluated. The biomonitoring indices of genotoxicity were observed to differ considerably in the control populations of different studies. Members concluded, therefore, that it was not possible to establish a minimum fold increase for biological importance that could be applied to studies of pesticide applicators based on the studies in nurses and cancer patients exposed to cytostatic medicines. Further, it was concluded that factors affecting variance in genotoxicity indices used in the biomonitoring studies of pesticide applicators which had been reviewed were not understood adequately. In this respect members considered it would be very difficult to infer causality for the small magnitude responses seen in the biomonitoring studies of pesticide applicators. There was a need for more data on the background variability in the general population of biomonitoring indices of genotoxicity, and on factors affecting variance, which was required before a proper assessment of studies could be made.

16. Members agreed that consideration of statistical significance and magnitude of effects from adequately conducted studies was the most appropriate approach to evaluating the available data. Members confirmed that the available information was severely limited and hence no definite conclusions could be drawn with regard to any U.K. agricultural applications of pesticides or to individual pesticide active ingredients. However the COM agreed that it would be appropriate to consider what conclusions could be derived from the selected studies.

Consideration of available data on exposure from selected studies

17. The Committee noted the limited information on exposure. The only direct exposure measurements were reported in the study by Garry et al [18] for exposure to 2,4-dichlorophenoxyacetic acid. Information had been provided on the identity of pesticides which applicators had used in 15 (11 reporting positive results and 4 reporting negative results) out of the 24 studies reviewed.[5,7,11,14-16,18,20,22,31,33,35,45,48,52] This information had been reviewed in the context of information from the Pesticide Usage Survey regarding information on use over the period from 1993 up to 2002 and also with regard to the available information on classification status under Directive EC/67/548 with regard to mutagenicity which had

been provided by HSE. Members noted that apart from the study published by Garry et al[18] the magnitude of pesticide exposure in these studies had not been recorded and information on use of personal protective clothing had not been documented in many reports.

18. A number of the published papers selected by the COM had reported information which supported the view that the lack of protective clothing used by pesticide applicators/workers was associated with evidence of increased genotoxicity indices in biomonitoring studies.[11,15,22,32,45] It was noted that there was some limited evidence to suggest that work practices in green house (such as avoiding use of protective clothing in humid conditions and on re-entry after pesticide applications) might be potential sources of pesticide exposure.[32,61,82] It was also uncertain to what extent the application practices cited in the published reports were relevant to UK agricultural practice. In addition data from 2003 onwards on pesticide use was not available at the time of the COM consideration.

19. Using information from the Pesticide Usage Survey, the amount of metam sodium and carbendazim increased over part of the period 1993-2001 in floricultural and green house applications. The area of outdoor bulbs and flowers sprayed with carbendazim was reported to increase. Increases in the use of bifenthrin, metam sodium and thiram were reported in agricultural practices. The Committee noted that with regard to pesticide active ingredients that were currently approved for use in the U.K. and which were also classified with regard to mutagenicity under EC/67/548, carbendazim was used in 4/11 positive and 1/4 negative studies respectively.[5,22,31,35,52] This represented a very small and incomplete amount of information. It was noted that one of these studies had used micronucleus or chromosomal aberration analyses which could potentially be affected by spindle inhibitors such as benzimidazoles.[31] However it is unclear whether the results derived from the comet assay[22,35] could be related to benzimidazoles and there is no evidence for direct binding of benzimidazoles to DNA and hence the results of the remaining DNA-adduct study[52] were unlikely to be related to benzimidazole exposure. It was noted that there were a number of other classified mutagens listed in the positive studies which were not approved for use in the U.K.

20. Members considered the Bolognesi et al 2004 study in detail as this was the only available study with provided data on the specific aneuploidy inducing effects of benzimidazoles.[5] Members confirmed that this study should be considered as negative using the criteria agreed by the COM during the review of studies of pesticide applicators. Members agreed this was one of the better studies which had been considered as the investigators had attempted to use a specific index of exposures to tubulin inhibitors such as carbendazim and benomyl, although there were limitations in the dosimetric accuracy of the index of exposure, in the small number of individuals studied and in the lack of correction for multiple comparisons used in the statistical analysis. Members considered that the results of the centromere specific investigations were based on a very small number of individuals and no interpretation of biological significance could be placed on the data. The data provided in this study were consistent with the general conclusions on interpretation given below in paragraphs 22 and 23.

Consideration of data presented on duration of exposure

21. There were limited data on estimated duration of exposure to pesticides in 6 out of the 24 selected studies.[5,7,22,30,44,45] Four of these studies reported a positive correlation between duration of exposure and increased indices of genotoxicity[7,22,44,45] whereas the remaining two studies reported a negative

correlation.[5,30] The stratification into groups according to exposure was generally based on periods of 10 years or more. No rationale was given in the studies for the stratification of exposure groups according to duration of exposure. The magnitude of increased indices of genotoxicity with duration of exposure was small in all of the studies. The Committee was aware that biomarkers of genotoxicity (such as chromosome aberrations and micronuclei) increase in frequency with age and that this potential confounding factor had not been considered adequately in the analyses reported. The Committee agreed it was not possible to draw any definite conclusions based on these data, although it is noted that three out of four of the studies which reported on duration of exposure and which also reported a positive response had documented evidence for exposure to benzimidazole pesticides.

Consideration of use of Personal Protective Equipment (PPE)

22. None of the selected studies specifically investigated the effect of use of PPE on biomonitoring indices of genotoxic effects in pesticide applicators. The extent of PPE usage, where reported, varied considerably. Thus in some reports, no PPE was used,[29,30] whilst other reports describe conditions in which most pesticide applicators use PPE.[5,47] Several studies reported significant increases in chromosome aberrations and micronuclei correlated with a lack of PPE use during pesticide application.[11,15,31,33] It is noted that floriculturalists might report using PPE, but some investigators note that due to humid conditions within greenhouses appropriate PPE is not always worn. The Committee noted that a correlation between the lack of use of PPE and increased biomonitoring indices of genotoxicity but concluded that no definite conclusions could be reached with regard to exposure to pesticides based on the available data.

COM Interpretation of available data

23. The Committee was aware of the guidance available for the conduct on biomonitoring studies of genotoxicity from Albertini et al [71]but agreed that having considered a large data set of studies on pesticide applicators that more research and guidance on the factors affecting the background variance of biomonitoring indices of genotoxicity was required before such studies could be fully interpreted particularly with regard to the significance of the small magnitude of response seen in the available studies.

24. The COM discussed whether any proposed study should focus on an occupational group (such as floriculture) or on specific pesticides. Members acknowledged that the evidence was limited particularly with regard to the design, conduct, reporting and analysis of the available studies both with respect to identifying either an occupational category or specific pesticides. It was noted that a limitation in the available published literature concerned relevant information on the mutagenicity of mixtures of pesticides. Members were aware of the COT report on mixtures of pesticides (the WIGRAMP report http://www.food.gov.uk/science/ouradvisors/toxicity/COTwg/wigramp/).[83] The COT working group had identified benzimidazoles as a possible common mechanism group of compounds for further evaluation. The Committee considered that although a UK study based on use of carbendazim in floriculture represented a reasonable proposal, there were considerable difficulties in undertaking and interpreting such a study. In particular it was agreed that more background information on the factors affecting the variance of biomonitoring indices of genotoxicity in unexposed populations would be required before a study of specific pesticide exposures was undertaken. If appropriate

background information were available, then it was agreed that a longitudinal study where individuals acted as their own controls would be most appropriate. It was agreed that there was supporting evidence that biomonitoring for urinary excretion of 5-hydroxy-2-benzimidazole (5HBC) could be used to assess exposure and uptake of carbendazim.[84] Members commented that the application of Personal Protective Equipment (PPE) specified for use of carbendazim products (such as in floriculture) in the U.K. meant that it was unlikely that any increase in genotoxicity could be detected with any reliability. Any such study would have to be very large and there were doubts as to whether an appropriate exposure group could be identified.

COM conclusions

25. The Committee was aware that no DNA reactive *in-vivo* mutagens were used as active ingredients in approved formulated pesticide products in the U.K. It was noted that benzimidazoles were used in a number of approved pesticide products since a risk assessment could be undertaken for the threshold related effects of these active ingredient on microtubule inhibition. The Committee confirmed that the available information was severely limited and hence no definite conclusions could be drawn with regard to any U.K. agricultural applications of pesticides or to individual pesticide active ingredients. The COM concluded;

    i) The COM review was based on 70 retrieved published studies of biomonitoring of genotoxicity in pesticide applicators. The evidence covered a large number of types of applications and a wide diversity of pesticide mixtures. The COM selected 24 studies from which conclusions could be drawn through the application of a quality screen of retrieved studies. An independent epidemiological overview of these 24 studies reported that all had significant design and evaluation faults. The COM agreed that any conclusions reached on this evidence would be limited by the poor quality of the available studies.

    ii) The COM agreed that following a review of the 24 selected studies there was limited evidence supporting increased biomonitoring indices of genotoxicity in biomonitoring studies of pesticide applicators. The Committee agreed that it was not possible to make any conclusions regarding exposure-response from the selected biomonitoring studies of genotoxicity because of the inadequacy and unreliability of exposure measurements and the generally small increases in response. The Committee noted that there was no published study of pesticide applicators using pesticide mixtures in the U.K.

    iii) The Committee agreed that the factors which accounted for the variance in biomonitoring indices of genotoxicity (chromosome aberrations and micronuclei predominantly in circulating blood lymphocytes) in nurses and cancer patients exposed to cytostatic medicines and in pesticide applicators had not been fully evaluated. It was not possible to define a minimum increase in biomonitors of genotoxicity associated with cytostatic medicines from the available studies on nurses and cancer patients. Based on these observations and the large inter-study variation for the biomonitoring indices of genotoxicity in unexposed populations, the Committee concluded that it would be very difficult to infer causality for the small magnitude responses seen in the biomonitoring studies of pesticide applicators. There was a need for more data on the background variability in the general population of biomonitoring indices of genotoxicity, and on factors affecting variance, which was required before a proper assessment of studies could be made.

iv) The COM agreed that it is very difficult to draw conclusions on what might be the most appropriate biomonitoring study for U.K. pesticide applicators. There was some very limited evidence to suggest that an appropriate study of floriculturalists using benzimidazoles (e.g. carbendazim) might represent a reasonable proposal. The Committee concluded that in view of the large numbers of individuals which would be required in order to detect an effect in such a study it was most unlikely that a sufficiently large and appropriate exposure group could be identified.

October 2005.

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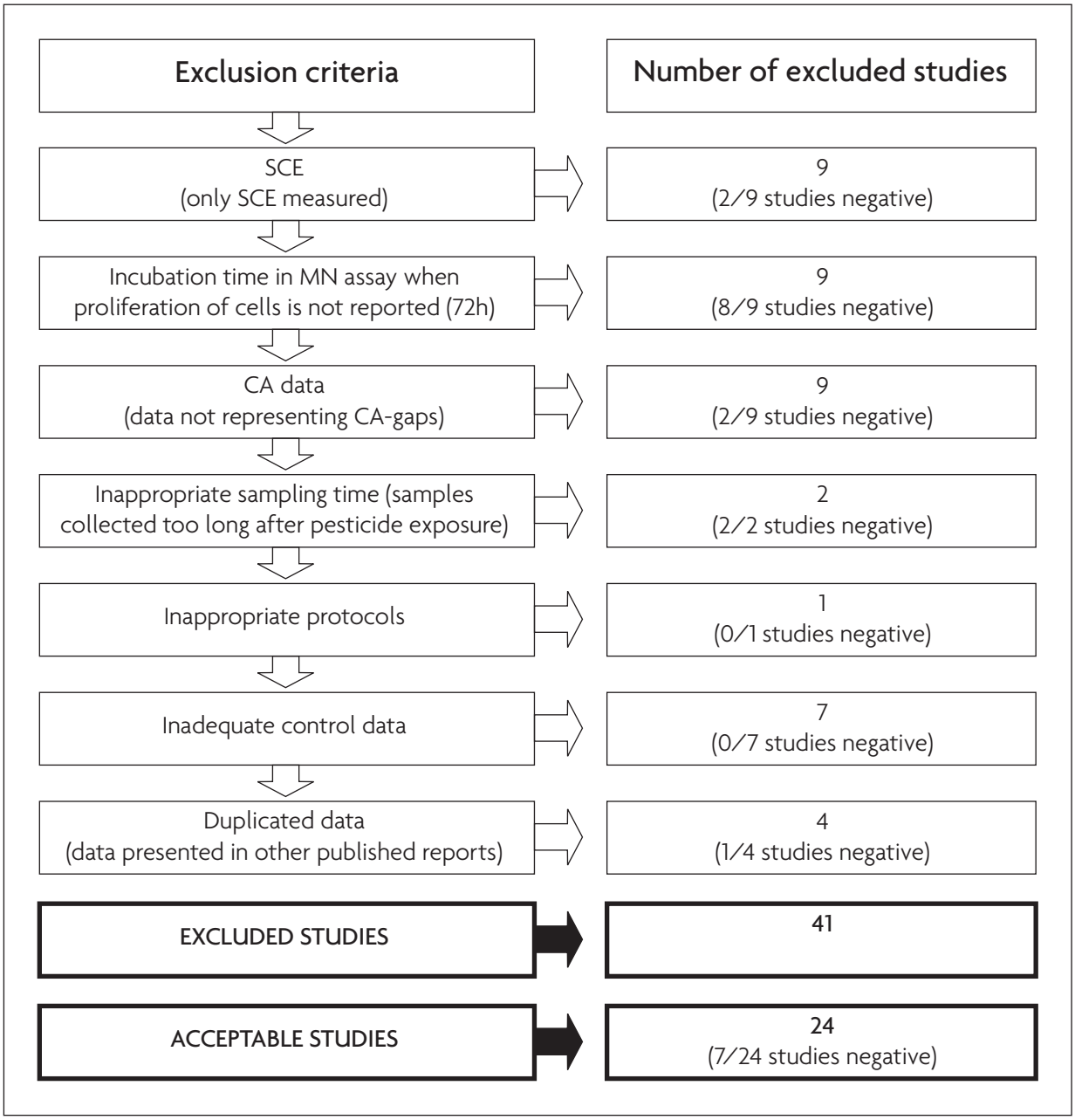
Annex 1 to Draft Working Paper on Biomonitoring Studies of Genotoxicity in Pesticide Applicators July 2005

Flow chart of excluded studies.

The flow chart demonstrates how many studies were excluded from further analysis due to various selection criteria, and the data reported in such studies.



Annex 2 to COM Statement on Biomonitoring Studies of Genotoxicity in Pesticide Applicators

Data reported in selected studies

Author	Results	Data for controls	Data for exposed subjects	Fold increase over controls
[a]Bolognesi *et al.*, 1993b	+ MN	MN frequency = 6.67 ± 3.12	MN frequency = 8.57 ± 5.02 (mean / 1000 cells ± SD) RR = 1.25; 95 % CI = 1.11 – 1.41	**1.3-fold increase**
[a]Bolognesi *et al.*, 2004	– MN	Total MN (C+MN) = 2.18 ± 6.31 Total MN (C-MN) = 1.32 ± 3.38	Total MN (C+MN) = 2.79 ± 12.21 Total MN (C-MN) = 1.56 ± 6.00 (mean / 1000 cells ± SD)	**1.2-fold increase** **1.3-fold increase**
[a]Carbonell *et al.*, 1995	+ CA	Spring/summer Cells with aberrations = 4.56 ± 2.53% Chromatid-type aberrations = 3.14 ± 2.76% Chromosome-type aberrations = 1.90 ffl 1.51 % Total aberrations = 5.04 ± 2.85 % Autumn/winter Cells with aberrations = 3.39 ± 2.4% chromatid-type aberrations = 2.57 ± 2.0% Chromosome-type aberrations = 1.32 ± 1.96 % Total aberrations = 3.90 ± 3.23 %	Spring/summer Cells with aberrations = 6.27 ± 2.96% Chromatid-type aberrations = 5.31 ± 3.12% Chromosome-type aberrations = 1.63 ffl 1.56% Total aberrations = 6.93 ± 3.5% Autumn/winter Cells with aberrations = 3.69 ± 2.14% chromatid-type aberrations = 2.49 ± 0.56% Chromosome-type aberrations = 1.21 ± 1.83 % Total aberrations = 3.70 ± 2.15% (mean ± SD)	1.4-fold increase 1.7-fold increase 0.9-fold increase **1.4-fold increase** 1.1-fold increase 1.0-fold increase 0.9-fold increase **1.0-fold increase**
[a]De Ferrari *et al.*, 1991	+ CA + SCE	Chromatid-type aberrations = 4.44 ± 3.06 Chromosome-type aberrations = 1.08 ± 1.28 Complex rearrangements = ‹0.02 Total aberrations = 5.52 ± 4.12	Exposed subjects Chromatid-type aberrations = 7.46 ± 6.22 Chromosome-type aberrations = 2.72 ± 1.58 Complex rearrangements = 0.12 ± 0.12 Total aberrations = 10.30 ± 7.18 Exposed subjects with bladder cancer; Chromatid-type aberrations = 5.07 ± 3.90 Chromosome-type aberrations = 2.65 ± 02.26 Complex rearrangements = 0.30 ± 0.62 Total aberrations = 8.02 ± 4.98 (Mean / 100 metaphases ± SD)	1.7-fold increase 2.5-fold increase 6.0-fold increase **1.9-fold increase** 1.1-fold increase 2.5-fold increase 15.0-fold increase **1.5-fold increase**

Author	Results	Data for controls	Data for exposed subjects	Fold increase over controls
[b]Dulout *et al.*, 1985	+ CA	Abnormal cells = 2.65 ± 1.01 % Gaps = 1.56 ± 2.09 Chromatid breaks = 1.70 ± 0.74 Chromosome breaks = 0.54 ± 0.62 Dicentric chromosome and ring chromosome = 0.10 ± 0.21	Abnormal cells = 2.71 ± 0.36% Gaps = 2.43 ± 1.62 Chromatid breaks = 1.51 ± 1.26 Chromosome breaks = 0.95 ± 1.08 Dicentric chromosome and ring chromosome = 0.43 ± 0.84 (CA / 100 cells – gaps ± SD)	1.0-fold increase 1.6-fold increase 0.9-fold increase 1.8-fold increase 4.3-fold increase
[a]Falck *et al.*, 1999	+ MN	MN frequency 0.5 µg/ml BrdU = 7.4 ± 3.1 1 µg/ml BrdU = 7.4 ± 3.1	0.5 µg/ml BrdU = 7.8 ± 2.4 1 µg/ml BrdU = 8.0 ± 2.7 (mean / 1000 cells ± SD)	1.1-fold increase 1.1-fold increase
[b]Garry *et al.*, 1996	+ CA	Rearrangement frequency = 0.4 ± 0.57	Rearrangement frequency Fumigant = 1.4 ± 1.44 Insecticide = 1.4 ± 1.28 Herbicide = 1.0 ± 1.34 (mean ± SD)	3.5-fold increase 3.5-fold increase 2.5-fold increase
[b]Garry *et al.*, 2001	+ CA	Translocations/inversions/ deletions = 0.65 ± 1.12	Translocations/inversions/ deletions Low volume (1-100 gall) = 1.20 ± 1.13 Mid-range (100-1000 gall)= 1.00 ± 1.13 Heavy (›1000 gall) = 2.22 ± 1.14	1.9-fold increase 1.5-fold increase 3.4-fold increase
[b]Gomez-Arroyo *et al.*, 2000	+ MN	MN frequency = 0.38 ± 0.021	MN frequency = 1.01 ± 0.03 (mean / 100 cells ± SD)	2.7-fold increase
[b]Grover, *et al.*, 2003	+ comet	Smokers Comet tail length = 7.03 ± 11.46 Non-smokers Comet tail length = 10.34 ± 13.25	Smokers Comet tail length = 18.26 ± 9.76 Non-smokers Comet tail length = 19.75 ± 14.48	2.6-fold increase 1.9-fold increase
[b]Hogstedt *et al.*, 1980	– CA	Cell with aberrations = 4.6%	Cell with aberrations = 4.2%	0.9-fold increase
[a]Kourakis *et al.*, 1992	+ CA	Chromosome-type aberrations = 0.2 ± 0.37 chromatid-type aberrations = 0.34 ± 0.60 Total aberrations = 0.54 ± 0.90%	Chromosome-type aberrations = 1.34 ± 1.62 chromatid-type aberrations = 0.80 ± 0.81 Total aberrations = 2.14 ± 1.62 % (mean / 100 metaphases ± SD)	4.6-fold increase 6.1-fold increase 5.0-fold increase

Author	Results	Data for controls	Data for exposed subjects	Fold increase over controls
[a]Lander *et al.*, 2000	+ CA	Pre-season Chromatid-type aberrations-gaps = 1.03 ± 0.82 Chromosome-type aberrations-gaps = 0.28 ± 0.45 Total aberrations-gaps = 1.31 ± 0.85%	Pre-season; Chromatid-type aberrations-gaps = 0.87 ± 0.95 Chromosome-type aberrations-gaps = 0.45 ± 0.74 Total aberrations-gaps = 1.32 ± 1.23 %	0.84-fold increase 1.6-fold increase **1.0-fold increase**
			Post-season Chromatid-type aberrations-gaps = 1.04 ± 0.99 Chromosome-type aberrations-gaps = 0.34 ± 0.56 Total aberrations-gaps = 1.37 ± 1.20 % (Mean ⁄ 100 metaphases ± SD)	1.2 (1.0)-fold increase 0.8 (1.2)-fold increase **1.0 (1.0)** -fold increase compared with pre-season (compared with controls)
[a]Lebailly *et al.*, 1998	+ comet	Beginning of spraying season = 30 Beginning of spraying season = 30	Middle of spraying season = 43 End of spraying season = 36	**1.4-fold increase** **1.2-fold increase**
[a]Lebailly *et al.*, 1998b	+ comet	DNA damage Before spraying Mixture of pesticides = 48% Herbicides on wheat = 30% Fungicides on wheat = 43% Fungicides & insecticides on peas = 36%	DNA damage After spraying Mixture of pesticides = 56% Herbicides on wheat = 28% Fungicides on wheat = 35% Fungicides & insecticides on peas = 39%	**1.2-fold increase** **0.9-fold increase** **0.8-fold increase** **1.1-fold increase**
		Tail moment Before spraying Mixture of pesticides = 3.21 Herbicides on wheat = 2.30 Fungicides on wheat = 3.64 Fungicides & insecticides on peas = 2.39	Tail moment After spraying Mixture of pesticides = 3.92 Herbicides on wheat = 1.93 Fungicides on wheat = 3.58 Fungicides & insecticides on peas = 3.16 (mean)	**1.2-fold increase** **0.8-fold increase** **0.9-fold increase** **1.3-fold increase**
[a]Lebailly *et al.*, 2003	– comet	DNA damage Morning before pesticide use = 10% (2-21%) Tail moment Morning before pesticide use = 4.35 ± 1.11 (2.16-5.85)	DNA damage Evening after pesticide use = not measured Following morning = 13% (5-49%) Tail moment Evening after pesticide use = not measured Following morning = 4.80 ± 2.57 (3.18-12.76) (Mean ± SD)	**1.3-fold increase** (compared to before pesticide use) **1.1-fold increase** (compared to before pesticide use)

Author	Results	Data for controls	Data for exposed subjects	Fold increase over controls
[a]Munnia *et al.*, 1999	+ DNA adducts	DNA adducts = 2.17 x 10^9 ± 5.75 RAL	DNA adducts = 8.50 x 10^9 ± 14.95 RAL (Mean ± SD)	**3.9-fold increase**
[a]Mustonen *et al.*, 1986	– CA	Aberrant metaphases-gaps Non-smokers; 1.5 ± 0.73 Smokers; 1.9 ± 1.2	Aberrant metaphases-gaps Non-smokers; 1.2 ± 1.5 Smokers; 1.8 ± 1.26 (Mean ± SD)	**0.8-fold increase** **1.0-fold increase**
[b]Paldy *et al.*, 1987	+ CA	Chromosome aberrations = 1.1 ± 0.36	Years of exposure Chromosome aberrations 0-5 years = 2.96 ± 0.36 6-10 years = 3.55 ± 0.75 11-15 years = 4.28 ± 0.76 (Sum of aberrations / 100 cells without gaps ± SD)	**2.7-fold increase** **3.2-fold increase** **3.9-fold increase**
[a]Pasquini *et al.*, 1996	+ MN	MN frequency = 13.30 ± 5.35 Overall MN frequency = 13.30 ± 5.35	MN frequency (>19 year) = 18.30 ± 7.22 Overall MN frequency = 15.98 ± 7.65 (Mean / 1000 cells ± SD)	1.37-fold increase **1.2-fold increase**
[a]Pastor *et al.*, 2001b	– MN	MN frequency = 16.38 ± 12.19	MN frequency = 12.20 ± 6.58	**0.7-fold increase**
[a]Pastor *et al.*, 2002a	– MN	MN frequency = 10.3 ± 7.06	MN frequency = 10.22 ± 7.06	**1.0-fold increase**
[a]Peluso *et al.*, 1996	+ DNA adducts	DNA adducts = 9	DNA adducts = 42[44]	**4.7-fold increase**
[a]Piperakis *et al.*, 2003	– comet	DNA damage Male non-smokers = 82.3 ± 14.1 Female non-smokers = 81.1 ± 16.12	DNA damage Male non-smokers = 83.2 ± 14.02 Female non-smokers = 82.1 ± 13.14 (mean ffl SD)	**1.0-fold increase** **1.0-fold increase**

[a]Studies from EU [b]Studies from rest of world

Mean fold increase of positive studies over controls ± SD (SE) = 1.73 ± 1.07 (0.15)

Figures in bold denote the total or mean fold increase of the study.

Halonitromethanes (HNMs)

COM/05/S3 – June 2005

Background to COM review

1. Halonitromethanes (HNM's) are a class of compound characterised by the presence of one or more halogen atoms together with a nitro moiety on a single central carbon. They differ from the Halomethanes (HM's) by the presence of the nitro moiety. The HNM's have recently been identified as disinfection by-products (DBP) in drinking water in the US (Richardson *et al* 1999) and had received a high priority ranking for investigation by the Environmental Protection Agency (Weinberg *et al* 2002 http://www.epa.gov/athens/publications/EPA_600_R02_068.pdf). The current data review was initiated following the submission of one of papers published on the HNM's by a member of the COM.

2. Members were told that the only available published data related to the *in-vitro* studies in the three publications (Plewa *et al* 2004, Kundu *et al* 2004a, b). Some preliminary findings had been reported by the EPA to the DH Toxicology Unit regarding *in-vivo* testing of the HNM's in transgenic medaka fish. Members noted the availability of an NTP negative *in-vivo* bone marrow mouse micronuclei test with trichloronitromethane (TCNM; chloropicrin), but that there were no appropriate *in-vivo* mutagenicity data available for other HNMs.

Data considered by the COM

3. The COM discussed the *in-vitro* COMET studies, conducted using CHO cells (Plewa *et al* 2004). Members noted that HNMs tested were cytotoxic in CHO cells and that apoptosis induction had not been recorded in these studies. In addition none of the trials had included the addition of an exogenous metabolising fraction. However members agreed that HNMs were genotoxic in this test system and that the rank order reported by the study authors was a reasonable guide to relative potency for *in-vitro* DNA damage. It was noted that the data precluded a determination of absolute potency of HNMs compared to the positive controls used in the study but the data were consistent with HNMs being more potent in the test system than ethyl methanesulphonate.

4. The COM reviewed the available bacterial mutagenicity data obtained from plate incorporation trials using *Salmonella typhimurium* strains both in the presence and absence of an exogenous metabolising fraction (Kundu *et al* 2004a). The strains tested included TA 98, TA 100, TA 104 and a strain RSJ100 which expresses rat glutathione transferase theta (GSTT1-1) known to activate halomethanes. Members agreed that the standard plate incorporation approach had been undertaken in this study used two plates per dose point (instead of the normal three) but that reproducible small increases in the number of revertant colonies had been reported. It was noted that HNMs might volatilise and therefore procedures to prevent evaporation might have been more appropriate. In a further study (Kundu *et al* 2004b) trials had been undertaken using pre-incubation in screw-capped glass vials using *Salmonella typhimurium* strain TA 100 at 37^0C for 30 minutes followed by plate incorporation assessment of revertant colonies. Members felt that some residual loss of HNMs could still have occurred and that it

would have been appropriate to undertake pre-incubation at a lower temperature of 30^0C. Overall members considered that the estimates of relative potency for *in-vitro* mutagenesis in Salmonella should be interpreted with caution.

COM evaluation of the data

5. Members considered the potential mechanisms of HNM induced mutagenicity in *Salmonella typhimurium* strains and agreed this included the potential for both direct acting and metabolically activated mutagenic responses. This might include an oxidative pathway but it was evident that there were possible differences between the nine chemicals in the HNM group. Whether direct activity was due to HNM carbocation formation (loss of the halogen resulting in a reactive, positively charged carbon) and DNA alkylation in an SN1 reaction or alternatively SN2 substitution (in which nucleophilic attack precedes halogen loss), was not clear. Members felt that, unlike the halomethane group of compounds, there was no convincing evidence for a glutathione mediated pathway with HNMs. Members observed that it was difficult to derive clear conclusions regarding mechanism and potency in the bacterial mutagenicity tests, but overall brominated HNMs appeared from the limited data to be more potent *in-vitro* mutagens than chlorinated HNMs.

6. The COM discussed the potential testing strategy that might be applied to HNMs and agreed that each individual HNM needed to be tested. It was agreed that the standard COM strategy was appropriate in this instance but should be modified so that the *in-vivo* rat liver UDS assay was the first *in-vivo* test. Members felt that a liver UDS assay would be more appropriate than the more usual *in-vivo* micronucleus test because of the likelihood that potential direct acting chemicals or reactive intermediates would not reach the bone marrow intact. Also, due to the presence of potentially direct acting mutagens a site of contact assay was considered appropriate (for example the COMET assay in the stomach). Members felt that an *in-vitro* liver UDS assay would be a useful pre-screen.

COM overall conclusions

7. Overall, in accordance with the COM strategy, HNM's should be regarded as *in-vitro* mutagens and potential *in-vivo* mutagens. It was considered that there was a need for appropriate *in-vivo* testing. The COM suggested:-
    a) *in-vivo* rat liver UDS assay, followed by
    b) site of contact COMET assay

8. If either of these studies yielded positive results, then the chemical under test should be considered to be an *in-vivo* mutagen.

    This resulting statement could be shared with the EPA.

June 2005
COM/05/S3

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Flunixin, Meglumine and Flunixin Meglumine

COM/05/S1 – March 2005

Background to COM review

1. Flunixin in the form of the meglumine salt is a non-steroidal anti-inflammatory drug (NSAID) and a non-narcotic analgesic drug with antipyretic activities. It is used in veterinary medicine (including food-producing animals) but is not used in human medicine. Flunixin meglumine dissociates *in vivo* to Flunixin and meglumine. In 1997, the European Medicine Evaluation Agency's (EMEA) Committee on Veterinary Medicinal Products (CVMP) considered the safety of Flunixin meglumine as part of its review of old veterinary medicinal products, operating under Council Regulation 2377/90, which covers the marketing authorisation of pharmacologically active substances used in veterinary medicines.

2. With regard to meglumine, the CVMP considered that it could be regarded as an excipient that had the purpose of increasing the solubility of Flunixin. The CVMP concluded that "meglumine used as an excipient at up to 1.5 mg/kg bw does not fall within the scope of Council Regulation (EEC) number 2377/90".

3. The Food Standards Agency is concerned about the possible mutagenicity of Flunixin meglumine, meglumine and Flunixin. The Food Standards Agency considered that it would be useful to seek the opinion of the COM.

4. The COM undertook an initial review of the mutagenicity data on Flunixin meglumine, Flunixin and meglumine during 2002/2003. A statement was published in March 2003 (www.advisorybodies.doh.gov.uk/com/flunmeg). The COM requested additional *in-vitro* mutagenicity data on meglumine, which is considered in this statement.

Assessment of Mutagenicity data – Flunixin

5. In the statement COM/03/S2, the COM agreed that there was limited evidence that Flunixin was mutaganic *in-vitro*, as data were inadequate, but there was no evidence to suggest that Flunixin had mutagenic potential *in vivo*, as a bone-marrow assay was carried out in mice that was considered adequate by the COM. Members, however, considered that the issue of the inadequate *in-vitro* data should be raised with the CVMP.

Assessment of Mutagenicity data – Meglumine

6. In the statement COM/03/S2, negative results were reported in an old plate incorporation assay in a limited number of *Salmonella typhimurium* strains. A positive result had been reported in a bone-marrow micronucleus assay in BSI mice using intraperitoneal administration of 500 or 1000 mg/kg bw suspended in 0.25% methylcellulose (two doses given 24 hours apart and harvest 6 hours after last dose). A repeat test using 1000 mg/kg bw was also positive. Members queried how the repeat test could

have been undertaken on the same day as the initial test. It was noted that negative results were obtained in a separate *in-vivo* micronucleus assay using intraperitoneal administration of two doses given 24 hours apart at up to 600 mg/kg bw of meglumine to CD1 mice. Members noted that a clear positive control had only been seen in males and not females in this latter assay and that the sampling regimen (24 and 48 hours after last dose) differed from the study in BS1 mice. A number of *in-vivo* bone marrow micronucleus assays were undertaken for the Committee using a variety of single/double intraperitoneal dosing regimens at dose levels of 500 mg/kg bw and 1000 mg/kg bw and sampling for micronuclei at 6 or 24 h after the last dose. There was some evidence for an effect 6 h after the second dose in one study using Alpk:ApfCD-1 mice, but no definite conclusions could be reached as the result was not repeatable, there was considerable individual animal variation and the observed effect could have been complicated by toxicity.

Assessment of Mutagenicity data – Flunixin meglumine

7. In the statement COM/03/2 the Committee agreed that there was limited evidence that Flunixin meglumine was an *in-vitro* mutagen. However, although negative results had been documented in an *in-vivo* micronucleus assay in BS1 mice, members were concerned about adequacy of the selection of dose levels. Therefore the inconclusive *in-vivo* data on meglumine suggested that a definite conclusion regarding Flunixin meglumine could not be reached.

COM Evaluation

8. The Committee noted the poor quality of mutagenicity data on flunixin, meglumine and Flunixin meglumine. Members noted that while negative results obtained in carcinogenicity bioassays with Flunixin meglumine in the rat and the mouse provided some reassurance with regard to flunixin, they were not informative with regard to meglumine.

9. The Committee was aware of several *in-vivo* bone-marrow micronucleus assays using meglumine but noted that the results were inconsistent. Two studies using BS1 and Alpk:ApfCD-1 mice reported a mutagenic effect following a treatment regime of two doses of meglumine (24 hour apart) with a sampling time of 6 hours after the second dose (i.e. 30 hours after the first dose). However this positive result was not repeated in two bone marrow micronucleus assays in mice using an equivalent treatment regime.

10. The Committee requested additional *in-vitro* assays with meglumine to properly assess whether meglumine had mutagenic potential *in vitro* before considering the need for any further *in-vivo* mutagenicity studies. Thus data were required from an *in-vitro* chromosomal aberration assay and a mouse lymphoma assay, which together with the data currently available for a test in bacteria using Salmonella, would complete the *in-vitro* package to modern standards given in the COM guidance. If these were negative, there would be no concerns regarding meglumine.

Data submitted in February 2005

Mouse Lymphoma Assay

11. A Mouse Lymphoma Assay was undertaken that conformed to the OECD Guideline 476[1] and ICH S2B Guideline.[2,3]

12. Meglumine was not mutagenic, at the concentrations tested in L5178Y mouse lymphoma cells, either with or without exogenous metabolic activation.

Chromosome Aberration Assay

13. A cytogenetics test was undertaken which conformed to OECD Guideline 473.[4,5]

14. Meglumine, at the concentrations tested, did not significantly increase chromosome aberrations or decrease the mitotic index in human blood lymphocytes *in vitro*, either with or without exogenous metabolic activation.

COM conclusion and recommendations

15. The COM confirmed the following general conclusions on the mutagenicity of flunixin, Flunixin meglumine and meglumine

    i) The mutagenicity data previously submitted to the Committee on flunixin, Flunixin meglumine and meglumine are relatively old and the studies have not been conducted to contemporary standards.

16. The COM reaffirmed that it is difficult to draw any definite conclusions on the mutagenicity of these chemicals. The prudent conclusions reached for flunixin and Flunixin meglumine were reaffirmed.

    ii) For Flunixin (a non-steroidal anti-inflammatory veterinary medicine) there was limited evidence that Flunixin was mutagenic *in-vitro* but there was no evidence to suggest that flunixin had mutagenic potential *in-vivo*.

    iii) For Flunixin meglumine, (Flunixin in the form of the meglumine salt) there is limited evidence for a mutagenic effect *in-vitro*. In addition the inconclusive *in-vivo* data on meglumine indicate that a definite conclusion regarding Flunixin meglumine cannot be reached.

17. The COM agreed a revised conclusion for meglumine using the new information from the *in-vitro* mutagenicity studies considered in this statement.

iv) For meglumine, the available *in-vivo* mutagenicity data are inconsistent with some positive and negative results. The new *in-vitro* mutagenicity tests (namely a mouse lymphoma assay and a chromosome aberration assay using human blood lymphocytes) were conducted to internationally accepted standards. There was no evidence for a mutagenic effect in either of these two assays either in presence of absence of exogenous metabolic activation. The COM concluded that there was no further requirement for testing of meglumine.

March 2005
COM/05/S1

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STATEMENT ON JOINT COC AND COM MEETING 9 June 2005

Use of target organ mutagenicity data in carcinogen risk assessment

COM/05/S6 AND COC/05/S3 – October 2005

Background

1. The COM and COC undertake routine horizon scanning exercises as part of their annual remit (see appended internet links at the end of this statement). The COM identified the use of *in-vivo* target organ mutagenicity studies as a subject for further consideration. The COM and COC agreed to hold a joint meeting on the use of data derived from in-vivo target organ mutagenicity studies in carcinogen risk assessment. An open meeting of the committees was held on the 9 June 2005. Attendees included a number of external experts who gave presentations and comments to the committees discussions. This brief statement has been drafted to record the main outcomes of the meeting. A full write up is being prepared for publication in a peer review journal.

Introduction to current review

2. The interpretation and consequent risk assessment of rodent carcinogenicity data is currently aided by the evaluation of a battery of mutagenicity data.[1] However there are examples of compounds for which equivocal data, or lack of concordance between mutagenicity and carcinogenicity data make it difficult to complete the risk assessment. This is of particular importance when the organ in which tumours are found is not one of those assessed during in vivo genotoxicity tests. Recent developments have facilitated the identification of target organ mutagenicity thus offering the potential to more closely define whether tumours seen are attributable to specific mutagenic events.[2]

Muta®mouse and Big Blue transgenic rodent assay systems

3. An increasing number of rodent carcinogens are being investigated using the Big Blue or Muta™Mouse transgenic systems. (For example the COM and COC have recently evaluated transgenic mutation assays as part of the evaluation of malachite green and leucomalachite green.[2] An important contribution of these assays is that any tissue can be evaluated for the presence of mutations following the administration of a chemical by any exposure route. By demonstrating carcinogen target organ mutagenesis, it can then be inferred that conditions are favourable for DNA reactivity therein (e.g. the occurrence of site-specific metabolism). Site of contact mutagenesis can also be readily studied.[4] Additionally, sequencing of both the lac genes and cII is now commonplace and this provides information on the more precise nature of the induced mutations. It is considered that these analyses will contribute to the understanding of target organ tumourigenesis and subsequent risk assessment.[5] There are several examples in the published literature of how data from Big Blue or Muta™Mouse have aided carcinogen risk assessment.[6-8]

The Comet assay

4. The comet assay is now well established as a supplementary assay to the standard battery of genotoxicity tests and can be used to assist in evaluating chemical which have given equivocal results in other *in-vivo* mutagenicity tests or to investigate the potential mechanisms of tumourigenic responses.[9,10] Guidelines and recommendations for performing the assay have recently been developed.[11] The principle concerns that may arise in respect of the use of the comet assay are the relevance of the measured endpoint to the carcinogenic process, and the robustness and sensitivity of the method.

DNA binding approaches

5. The COM considered the measurement of DNA binding by a number of methods (e.g. postlabelling, radioimmunochemical and HPLC/mass spectrometry) in 1996.[12] The COM agreed that these methods could provide useful data on exposure to and uptake of DNA reactive chemicals and metabolites. More recently the COC has considered the application of Accelerator Mass Spectrometry (AMS) in the detection of DNA binding in biological samples.[13] AMS is the most sensitive technique for measuring the formation of adducts with DNA. AMS technology allows the accurate measurement of very low levels of radiolabelled chemicals (particularly ^{14}C) in biological samples at around 10^{-21} to 10^{-18} mole. The COC considered that one potential application of AMS was in hazard identification. AMS has provided evidence for a lack of DNA binding of 2-phenylphenol and its metabolites in rat bladder which has been important information in concluding a non-genotoxic mechanism for the carcinogenic effect of 2-phenylphenol and its sodium salt in rat bladder.[14.]

Overview of joint COM/COC meeting on target organ mutagenicity studies

6. The symposium was attended by Committee members, relevant officials from government agencies and delegates from industry and academia, took the form of introductory presentations followed by round-table discussion groups. A programme was published.[15]

Overview of presentations

7. Professor John Heddle (York University, Toronto, Canada) gave the first presentation and provided a comprehensive overview of the usefulness of the transgenic mouse and rat mutation assays in carcinogenicity risk assessment. His principal observations were that the transgenes lac I and lac Z, are neutral genes which persist and accumulate mutations linearly over time, ensuring consistency in response which has allowed for the development of optimum protocols, notably the establishment of expression periods. The Committee noted the relevance of persistent mutations in transgenes to the initiation-promotion model of carcinogenesis.

8. Professor David Phillips (Institute of Cancer Research, Sutton) gave a brief synopsis on the use of DNA adduct detection methodologies in providing evidence of genotoxic mechanisms of action. These techniques are constantly being improved. DNA adduct formation represents mutagenic and carcinogenic potential, but it is recognised that the presence of DNA adducts in a particular tissue does not necessarily correlate with tumour induction.

9. Dr Brian Burlinson (HLS) summarised recent developments in the use of the COMET assay, which was gaining in popularity as a second tissue in vivo mutagenicity assay, to supplement the in-vivo bone marrow assay for chromosomal aberrations or micronuclei. One advantage was that a number of tissues including any tumour target organ could be included in any study. It was noted that a draft OECD guideline was currently under consideration.

10. Dr Phil Carthew (Unilever) presented the pathologists view of the usefulness of target organ mutagenicity data. He pointed out that lack of concordance between mutation frequency, DNA adducts and tumour burden indicate a need to understand more definitively the importance of other steps in the carcinogenic process. However, it is anticipated that data from transgenic mutation assays will be able to provide answers to critical questions, principally with view to hypothesis testing and mode of action evaluation.

Group discussions

11. Delegates then split into two groups for the ensuing discussions. Group 1 considered methodological developments, robustness, sensitivity and target organ specificity of the assays under scrutiny, whilst Group 2 addressed specifically the use of target organ mutagenicity data in carcinogenicity risk assessment through questions such as 'how can these data be used to understand the aetiology/ pathogenesis of rodent tumours?' and 'are these data likely to be more useful for the evaluation of some tumours types and/or target organs?'.

Conclusions from Group discussions

12. Group 1 (methods) derived conclusions on the use of the assays considered during the symposium with regard to the screening of chemicals for potential in-vivo mutagenesis. It was concluded that the transgenic mutation assays were sufficiently robust for general use and that moves should be made to optimise assay conditions and develop protocol guidelines. There were some concerns regards overall sensitivity and this may be particularly relevant when using unusual tissues or chemicals about which little is known (e.g. nature of DNA damage, ADME profile). Nevertheless, it was felt that the limitations of transgenic mutation assays were fairly well understood and this added to a general feeling of confidence in their use. However, it was recognised that the expense of conducting the transgenic assays meant that it was likely that the COMET assay would be used more frequently, even though this was more suited to detecting clastogens and potentially less sensitive at identifying point mutagens. The COMET assay was considered to be satisfactorily validated in most tissues. DNA adducts were not considered useful in a risk assessment scenario, and more likely to be of value for weight of evidence approaches to understanding carcinogenic mechanisms.

13. Group 2 (risk assessment) derived conclusions on the use of target organ mutation assays in carcinogen risk assessment, i.e. as part of mode-of-action assessments. It was concluded that the in-vivo target organ mutagenesis/ genotoxicity assays provided important information for the mode-of-action evaluations for rodent cancer target organs and thus contribute to carcinogen risk assessment. It was considered important to ensure adequate conduct of studies particularly with regard to information on target cell exposure and potential modes of action. In general, positive results which are not due to high dose cytotoxicity, inflammation or reactive oxygen DNA damage support a non-threshold genotoxic

mode of action and that negative results need to be interpreted with regard to the sensitivity of the study used. Clearly, concordant results for several in-vivo approaches increase the confidence of conclusions reached.

Overall conclusion

14. The COM and COC agreed that data from adequately conducted in-vivo carcinogen target organ mutagenicity and genotoxicity studies (which included information from investigations using transgenic animals, the comet assay, and approaches to measuring DNA binding (e.g. postlabelling and radiolabel methods) can provide valuable information of use in the mode-of action of carcinogenic responses seen in rodents. Such studies can provide supporting information for use by regulatory authorities in carcinogen risk assessment on a case-by-case basis.

October 2005
COM/05/S6
COC/05/S3

(COM/ COC horizon scanning papers for 2004;
http://www.advisorybodies.doh.gov.uk/pdfs/MUT0422.pdf
http://www.advisorybodies.doh.gov.uk/pdfs/cc0432.pdf)

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2005 Membership of the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment

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Mr J Battershill BSc MSc

Declaration of COM members interests during the period of this report

Member	Personal interest		Non personal interest	
	Company	Interest	Company	Interest
Prof P B Farmer (Chairman)	Abbey National Bradford & Bingley Celltech Foreign & Colonial Friends Provident Health Effects Institute Torotrak	Shareholder Shareholder Shareholder Shareholder Shareholder Research Committee Member Shareholder	American Chemistry Council CEFIC	Research Support 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 D Gatehouse	Covance Friends Provident GlaxoSmithKline	Salary Consultant Shareholder Pension Share Option Holder Shareholder	NONE	NONE

Member	Personal interest		Non personal interest	
	Company	Interest	Company	Interest
Mrs R Glazebrook	Dr Foster Ltd	Salary	NONE	NONE
	BT Group	Shareholder		
	Lloyds TSB	Shareholder		
	National Grid	Shareholder		
Dr N Gooderham	Abbey National	Shareholder	GlaxoSmithKline	BBSRC Collaborative Studentship
	Friends Provident	Shareholder		
	Game	Shareholder		
	ML Laboratories	Shareholder		
	Northern Rock	Shareholder		
	Proctor & Gamble	Consultant		
	Protherics	Shareholder		
	Sunderland AFC	Shareholder		
Dr I Mitchell	Kelvin Associates	Associate	NONE	NONE
		Consultant		
	IM Enterprises	Director/Creditor		
	GlaxoSmithKline	Pensioner		
		Option and		
		Shareholder		
		Consultant		
	Bass	Shareholder		
	Cable & Wireless	Shareholder		
	Cadbury Schweppes	Shareholder		
	Renishaw	Shareholder		
	Pfizer	Shareholder		
	RTZ	Shareholder		
	Shell	Shareholder		
	Unilever	Shareholder		
	Vodafone	Shareholder		
	Whitbread	Shareholder		
	British Telecom	PEP Holder		
	Centrica	PEP Holder		
	Scottish Power	PEP Holder		
Dr E M Parry	Invesco	PEP Holder	NONE	NONE
	Fleming	PEP Holder		
	Legal & General	PEP Holder		
Prof D Phillips	Aviva	Shareholder	NONE	NONE
	Banco Santander	Shareholder		
	BG Group	Shareholder		
	Bradford & Bingley	Shareholder		
	Centrica	Shareholder		
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	National Grid	Shareholder		
	Takeda	Consultant		