COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Preface 2004

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 2004, the Committee provided advice on a wide range of chemicals which included 2,3-dichloropropan-1-ol, chromium picolinate, the effect of DNA repair on mutagenicity of genotoxic carcinogens and at low dose levels, malachite green/leucomalachite green and PAVA (pelargonyl vanillylamide)

The Committee was asked to contribute to two reviews in conjunction with its sister Committees on Toxicity (COT) and Carcinogenicity (COC). Advice was provided on the mutagenicity assessment of tobacco products (specifically tobacco-based Potentially Reduced Exposure Products) and the application of toxicogenomic methods to mutagenicity. 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 conduct of *in-vitro* micronucleus assay and the testing strategy advocated by the EC's Scientific Committee on Cosmetics and Non-Food Consumer Products (SCCNFP).

During 2004, the Committee also said farewell to Dr Robin Fielder who retired as scientific secretary to COM. I wish to record my thanks for his scientifically excellent contribution and commitment to the work of COM and public health during his 17 years of service for COM.

Professor P B Farmer Chair
MA DPhil CChem FRSC

2,3-Dichloropropan-1-ol (new mutagenicity data)

2.1 2,3-Dichloropropan-1-ol (2,3-DCP) is a member of a group of chemicals called chloropropanols. It was last considered by the COM and the COC in 2001, together with 1,3-dichloropropan-2-ol (1,3-DCP). COM concluded that it would be prudent to consider 1,3-DCP and 2,3-DCP as potentially genotoxic *in-vivo* but agreed that they should be tested for genotoxicity *in-vivo* using the approach set out in the COM guidelines. The COM had considered the results of *in-vivo* studies from a rat bone-marrow micronucleus test and a liver UDS assay conducted with 1,3-DCP at its May 2003 meeting and had reached a conclusion that there was no evidence for *in-vivo* mutagenicity in the tissues assessed. The Committee was asked to consider the data from two *in-vivo* mutagenicity assays (rat bone-marrow micronucleus test and a liver UDS assay) conducted with 2,3-DCP.

COM evaluation 2001

2.2 Members were aware that there was very little data on the absorption, distribution, and excretion of 2,3-DCP. Theoretically, 2,3-DCP could be metabolised to produce epichlorohydrin (and subsequently glycidol) and therefore there were structural alerts for genotoxicity and carcinogenicity.

2.3 The Committee noted 2,3-DCP was mutagenic in *Salmonella typhimurium* strains TA 100 and TA 1535 in a study with and without metabolic activation[1], and mutagenic in another Ames test. Positive results were also obtained for sister chromatid exchange with Chinese Hamster V79 cells both with and without metabolic activation. No *in-vivo* studies in mammals have been carried out.

2.4 The Committee concluded that in the limited studies conducted, 2,3-DCP was genotoxic *in vitro* with and without metabolic activation in bacterial and mammalian cells.

COM evaluation 2004

2.5 The Committee considered two new *in-vivo* genotoxicity studies at its February 2004 meeting. The Committee concluded that both the rat bone-marrow micronucleus test and the rat liver UDS test had been carried out to an acceptable standard and were negative. Thus the additional information recommended by the COM as being necessary to provide adequate reassurance that the mutagenic activity seen *in vitro* was not expressed *in vivo* had now been provided.

2.6 The Committee noted the uncertainties with regard to routes of metabolic activation of 2,3-DCP and agreed that the two new mutagenicity studies supported the view that reactive metabolites, if formed, did not produce genotoxicity *in vivo* in the tissues assessed.

2.7 The Committee concluded that 2,3-DCP can be regarded as having no significant genotoxic potential *in vivo*.

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

Chromium picolinate

2.9 The Food Standards Agency asked the COM to review the available information on the mutagenicity of trivalent chromium and specifically on chromium picolinate in order to support consumer advice and, if appropriate, to recommend what further studies would be required to draw definite conclusions. A US National Toxicology Program (NTP) carcinogenicity bioassay of chromium picolinate is underway but the final report is unlikely to be available for several years. Chromium picolinate is a widely available food supplement. The adverse effects of chromium had been reviewed by the Expert Group on Vitamins and Minerals (EVM). (http://www.food.gov.uk/multimedia/pdfs/vitmin2003.pdf) The reports of genotoxicity associated with chromium picolinate were noted and chromium picolinate was excluded from their recommendations for a safe upper level. Following the publication of the EVM report, the Food Standards Agency advised that consumers should use other forms of trivalent chromium supplements until more detailed advice is available.

Public Health Issue

2.10 Hexavalent chromium compounds are established human carcinogens on the basis of both animal studies and epidemiological evidence of carcinogenicity (i.e. considered Group 1 carcinogens by the WHO International Agency for Research on Cancer (IARC) (http://monographs.iarc.fr/). Trivalent chromium compounds have lower toxicity, which is generally attributed to their lower solubility and to their very limited ability to cross cell membranes. Trivalent chromium compounds were considered by IARC to be not classifiable with regard to carcinogenicity in humans (i.e. Group 3).

2.11 Trivalent chromium may be the ultimate carcinogen responsible for the effect of hexavalent chromium since it is able to bind DNA directly. However, it is unclear whether the reduction of hexavalent to trivalent chromium and the subsequent oxidative damage, or the direct binding of trivalent chromium to DNA, or both is responsible.

COM consideration

2.12 The COM undertook a preliminary discussion of available mutagenicity data at its October 2003 meeting. A request for additional information was forwarded to the manufacturers who supply chromium picolinate to the food supplement industry.

2.13 The evaluation of the mutagenicity of chromium picolinate is complex and the available data are conflicting. Chromium picolinate has given positive results in some *in-vitro* mutagenicity tests. The mechanism by which this occurs is unclear. However, in these studies the test material had been synthesised in the laboratory concerned and an adequate specification was not available. The Committee considered that the *in-vitro* studies had given some indication of mutagenic activity and recommended that the critical tests should be repeated using commercial grade material (the previously tested material had been synthesised by the testing laboratory) before any definite conclusions could be drawn. Thus a further *in-vitro hprt* assay and an *in-vitro* chromosome aberration

study were recommended with chromium picolinate (both tests using CHO cells and to international standards) to determine whether the published results could be confirmed with a commercial grade sample. It was also recommended that the *hprt* assay should include a 48-hour incubation experiment in the absence of S-9 in addition to the standard time points of analysis.

2.14 Replication of the tests using commercial grade material in tests conducted to internationally accepted protocols gave negative results. The Committee expressed some reservations regarding the conduct of these studies (possible limitations in sensitivity) and in particular regarding the repeat *in-vitro* chromosome aberration study in CHO cells. However, overall it can be concluded that the balance of the data suggest that chromium picolinate should be regarded as not being mutagenic *in vitro*.

2.15 The available *in-vivo* tests in mammals with chromium picolinate are negative. In view of the negative *in-vitro* results with commercial grade chromium picolinate, there is no further requirement for *in-vivo* testing at the current time.

2.16 The ongoing US NTP carcinogenicity bioassays of chromium picolinate will provide important *in-vivo* data in the future. The in-life phase of the NTP bioassays is due to end in July 2004. These data should be considered when the full results of the bioassay are available.

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

Genotoxic carcinogens and DNA repair at low doses

2.18 The Committee on Carcinogenicity had asked for advice on the effect of chemical induced DNA repair at low doses with regard to genotoxic carcinogens.

Background to request

2.19 The COC had considered a horizon scanning paper by the DH Toxicology Unit at its June 2003 meeting. One aspect considered was hormesis (the occurrence of "U" shaped dose-response curves). During its discussions the COC considered a paper by Calabrese EJ and Baldwin LA (*Nature*, vol 421, 691-692, 2003), which provided an argument for the occurrence of hormesis (i.e. the occurrence of "U" shaped dose-response curves at low doses). The COC conclusions included the recommendation that the evidence for the induction of increased DNA repair (above background levels and with the potential to reduce the number of spontaneous mutagenic lesions) following exposure to very low levels of genotoxic carcinogens warranted further review, as a potential mechanism that could result in a "U" shaped dose response curve; they had therefore asked the COM for advice.

COM evaluation

2.20 The COM undertook an initial evaluation based on a preliminary position paper at its February 2004 meeting. The Committee considered that is was still prudent to assume that there is no threshold for mutation for *in-vivo* mutagens and where a potential threshold mechanism could be identified, appropriate evidence should be provided on a case by case basis. The COM considered that it was important to keep the issue of the absence of a threshold under review. They felt that it would be useful to carry out a literature search targeted on low dose effects of a few direct acting chemical mutagens on DNA adduct formation, mutation rates, and the significance of DNA repair mechanisms. The search should concentrate on low molecular weight compounds such as ethylene oxide and ethyl or methyl methanesulphonate, (EMS, MMS) for which there were considerable amounts of data available. Members agreed that bacteria would most likely demonstrate the most sensitivity to low doses of mutagens and had reservations as to whether mammalian cell systems would have sufficient sensitivity to detect evidence for an effect of DNA repair induction on the dose-response relationship for mutation.

2.21 A further detailed review paper was considered at the May 2004 meeting. This had considered information on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), EMS and MMS. In view of the amount of data on these three compounds, ethylene oxide had not been used as an example.

2.22 Most information was available on the O^6-methyltransferase (O^6-MT) repair system, mainly in bacterial systems but some information was also available in mammalian cells. Members agreed that these data provided some evidence for thresholds in the adaptive state that could be attributed to the induction of the O^6-MT. The difficulty in establishing unequivocal evidence for a threshold was discussed, as opposed to a no-observable-effect-level depending on the sensitivity of the assay. The importance of some knowledge of the underlying mechanism to support the biological plausibility of a threshold (as emphasised in the current COM guidance) was acknowledged.

2.23 Only limited data were available on systems other than the O^6-MT repair, and members considered that these did not provide any significant evidence regarding thresholds. In the general discussion it was noted that evidence regarding DNA adducts induced by bulky compounds such as aromatic amines was consistent with a linear dose response and no threshold. It may thus have been useful to have considered data on such compounds as well as simple alkylating agents. However, it was recognised that this would have introduced the added complication of the need for metabolic activation. It was also pertinent that the key issue was what happened to the DNA adduct with regard to error-prone or error-free repair, the latter being a potentially threshold related pathway.

2.24 Overall, the Committee agreed that there was no clear evidence for a J shaped dose response in any of the data considered. Data regarding the O^6-MT induction suggested that an *in-vivo* threshold was likely, but not proven. No conclusions could be drawn from the limited data on other DNA repair mechanisms.

2.25 When considering whether any further work would be appropriate it was important to consider if this would be cost effective. The difficulty in designing experimental studies with adequate sensitivity to provide definite answers needed to be recognised.

2.26 The Committee agreed that these data considered in this paper do not warrant any reconsideration of the Committee's current advice that it is prudent to assume that *in-vivo* mutagens do not have a threshold, unless there is good evidence to the contrary.

2.27 This advice was passed to the COC.

Malachite green/Leucomalachite green

Background to request for advice

2.28 Malachite green is a cationic triphenylmethane dyestuff used in a number of industries, including fish farming. It is used in freshwater fisheries for the treatment of external fungal and other infections, its main use being to stop fungal growth on the eggs. The Food Standards Agency had asked for a toxicology review from the COT in 1999 who had in turn asked for advice on the mutagenicity of malachite green and the lipophilic metabolite leucomalachite green from the COM. The COM had reached an interim conclusion based predominantly on limited evidence which indicated *in-vitro* mutagenic activity of these two compounds and the formation of DNA adducts in rats and mice with both compounds indicated that it would be prudent to consider malachite green and leucomalachite green as potential *in-vivo* mutagens.

2.29 The COM had considered preliminary data from *in-vivo* mutagenicity studies in 2003. The studies had been undertaken with leucomalachite green using transgenic rats (Big Blue™) and indicated that leucomalachite green had *in-vivo* mutagenic activity in the liver. The Committee agreed that there was no need to alter its previous conclusions on malachite green and leucomalachite green.

Advice requested in 2004

2.30 The COM was asked in 2004 to consider the full published reports of *in-vivo* mutagenicity studies undertaken for the US National Toxicology Programme. The COC was asked to review the carcinogenicity data. A joint COM/COC statement would be prepared.

2.31. The COM agreed to revise their 1999 conclusions regarding the mutagenicity of MG and LMG as follows:

Malachite green

i) There is one report of a *Salmonella* assay done to an acceptable protocol; MG oxalate (>90% pure) was shown to induce mutations in TA98 in the presence of an exogenous metabolic activation system. There is also some evidence of clastogenicity in Chinese Hamster Lung cells. MG has also been shown to produce DNA damage in CHO cells using the Comet assay. MG should thus be regarded as having mutagenic potential.

ii) Negative results were obtained in a poorly reported bone marrow micronucleus test in mice using a single oral dose of malachite green oxalate (>90% pure) at the MTD (75% of the LD50). Members agreed that the high dose level of 37.5 mg/kg was adequate, but it was difficult to assess the value of the negative results in the absence of appropriate information on bone marrow toxicity or data to show that malachite green and/or metabolites reached the bone marrow.

iii) ^{32}P post-labelling studies using a 28 day dietary exposure have indicated that MG induces DNA adduct(s) in the liver of both F344 rats and $B6C3F_1$ mice. This has been confirmed in a separate study using 16 weeks dietary exposure. MG did not induce micronuclei in peripheral lymphocytes nor *hprt* mutations in splenic lymphocytes in the 28 day study; the experimental design of these studies did not, however, optimise the chances of obtaining a positive response and no definite conclusions can be drawn.

iv) In view of the demonstration of DNA adduct formation in samples from both rats and mice, MG should be regarded as an *in-vivo* mutagen.

Leucomalachite green

i) Data are now available on the *in-vitro* mutagenicity of LMG from limited Salmonella and CHO/HGPRT assays, and also from a Comet assay in CHO cells. Negative results were obtained. However in view of the limitations of these studies, and the fact that no data are available on clastogenicity, it is not possible to make an adequate assessment of the mutagenic potential of LMG from these data.

ii) ^{32}P Post-labelling studies using a 28 day dietary exposure have indicated that LMG induces a low level of DNA adduct(s) in the liver of F344 rats; only a marginal response was however seen in $B6C3F_1$ mice, and no conclusions can be drawn from these data. This has been confirmed in a separate unpublished study in mice using 16 weeks dietary exposure; there was no evidence for DNA adduct formation in the liver. LMG did not induce micronuclei in peripheral lymphocytes nor *hprt* mutations in splenic lymphocytes in the 28 day study, but the experimental design of these studies did not optimise the chances of obtaining a positive response and no definite conclusions can be drawn.

iii) Gene mutation studies have indicated that LMG can induce mutations *in-vivo* in liver DNA. Studies in Big Blue F344 rats gave equivocal results. LMG produced an increase in *lacI* mutations only at the highest dose tested (543 ppm in diet) and at a single time point (16 weeks). No increase was seen after 32 weeks. Evidence was provided to suggest that this isolated positive may have been due to disproportionate expansion of spontaneous *lacI* mutations. However studies in female Big Blue B6C3F_1 mice indicated that LMG produced an increase in *cII* mutant frequency in liver DNA of mice treated with 408 ppm LMG in the diet. Furthermore it was shown that the spectrum of mutation seen in the DNA was distinct from that of the control mice. These data provide evidence of *in vivo* mutagenicity at the target site in the carcinogenicity bioassay.

iv) In view of the demonstration of the induction of mutations in liver DNA of female B6C3F_1 mice, LMG should be regarded as an *in-vivo* mutagen

2.32 The COC. provided advice on carcinogenicity (see section 3.47-3.51 of this annual report). There was no definite evidence for carcinogenicity in long term animal bioassays with malachite green. The overall conclusion was that it would be prudent to regard leucomalachite green as a genotoxic carcinogen. A statement is appended at the end of this annual report.

PAVA (pelargonyl vanillylamide)

2.33 In 2001 the Home Office requested advice from the COT on the health effects of a chemical incapacitant spray based on pelargonyl vanillylamide (PAVA), a synthetic equivalent of capsaicin, present in pepper. It is a sensory stimulant and is also used both as a food flavour (at up to 10 ppm) and as a topical rubifaciant in human medicine. The COT requested the advice of the COM on a package of mutagenicity data that had been commissioned by Sussex Police to support the health risk assessment of PAVA. The COM considered these mutagenicity data at its meeting in February 2002. The COM agreed that some further reassurance was necessary, in addition to the negative in vivo bone marrow micronucleus tests that had already been conducted. The Committee requested an *in vivo* liver UDS assay to provide this necessary reassurance.

2.34 Sussex Police commissioned the requested study and a full report was provided in Annex B. An *in vivo* liver UDS assay was carried out in rats using oral exposure and dose levels of 625 and 1250 mg/kg body weight. The top dose was the maximum tolerated dose, as determined from a range-finding toxicity study.

2.35 Members agreed that the study conformed to the relevant OECD test guideline (OECD Guideline No. 486: UDS test with mammalian liver cells *in-vivo*) and was adequate. 1,2- Dimethylhydrazine dihydrochloride was used as the positive control. There was no evidence of the induction of DNA repair by PAVA, as measured by unscheduled DNA synthesis in this assay. The positive control clearly induced DNA damage at both time points.

2.36 The COM agreed that the information sought by the Committee to conclude that PAVA was not expected to be an *in-vivo* mutagen had now been provided. No further data were required on the mutagenicity of this compound.

2.37 These conclusions were forwarded to the COT and were included in a COT statement in the COT section of this annual report.

Significance of environmental mutagenesis

2.38 At the COM October 2003 meeting a paper in Nature by Professor Thilly (Biological Engineering Division, MIT, Nat Genet. 2003 Jul;34(3):255-9.) had been tabled. One member had drawn the attention of the secretariat to this paper. It was agreed that detailed discussion of the significance of the paper would take place at the February 2004 meeting. The paper argued that the evidence for environmental mutagens causing cancer in humans is very weak and was limited to sunlight and ionising radiation. Thilly suggested that environmental risk factors for cancer act through non-mutational processes, and the genetic changes that cause cancer in humans arise from endogenous processes. The paper questioned the value of mutagenicity assay in screening for potential carcinogens.

2.39 Members agreed that the alternative hypothesis proposed by Thilly, namely that environmental risk factors for carcinogenesis acted not by mutation but by selecting particular kinds of preneoplastic cells previously initiated by spontaneous mutation resulting in a positive net growth rate, was interesting and warranted consideration. Regarding the most important environmental risk factor for lung cancer, cigarette smoking, Thilly pointed out that there was little evidence that smoking increased point mutations in bronchial epithelial cells or in peripheral T cells in humans, where the *hprt* locus had been examined. Members felt that the *hprt* assay in human lymphocytes may not be sufficiently sensitive or appropriate for detecting smoking induced mutations. Additionally, Thilly had not carried out a comprehensive review of this evidence and at least one report showing a significant increase in mutations in the *hprt* locus in smokers had been omitted.

2.40 The Committee noted that although Thilly accepted ionising radiation and sunlight as exceptions to his alternative hypothesis, there was a considerable body of evidence supporting the somatic mutation mechanism for chemical carcinogenesis that had not been taken into account. Further, Thilly had concentrated on only one type of mutation (gene mutation) and had not considered other examples, such as structural or numerical chromosome changes. It was widely accepted that clastogenic events, for example, are seen in smokers, and that it is crucial to control for smoking habits in any cytogenetics study in humans. No mention was made of the dominantly inherited conditions (and the changes in genetic material involved) that predispose to cancer in humans. Thilly had ignored the fact that essentially all clear *in-vivo* mutagens in somatic cells had also been shown to be carcinogens in animals when adequately tested. The fact that the majority of chemicals recognised as being carcinogenic in humans are also *in-vivo* mutagens had also been ignored.

2.41 Members concluded that arguments in the Thilly paper were not convincing and there was no justification to depart from the somatic mutation theory of chemical induced cancer or to change the COM recommended strategy for testing chemicals for mutagenicity.

Reassessment of toxicology of tobacco products

2.42 The Committees (COT/COC/COM) were asked to provide advice on the toxicological assessment of tobacco products with reference to the assessment of Potentially Reduced Exposure Products (PREPS) and in particular tobacco-based PREPS which are smoked. The Committees agreed that it was important to state that the ideal way forward to reduce risks and hazards of tobacco smoke was to encourage smokers to stop or people not to start in the first place and any attempt to reduce toxicity should not be allowed to detract from that. Members acknowledged that the primary remit of the Committees' discussions was to provide advice based on the information provided in the discussion papers.

2.43 The COM concluded that, using suitable protocols, it was possible to compare mutagenicity *in-vitro* of different PREPS which could be useful to assess hazard. However, the results of such *in-vitro* tests had no predictive value for risk of *in-vivo* mutagenicity or cancer. No conclusions could be drawn on the approaches using toxicogenomic methods. The available biomonitoring approaches were too limited to draw any conclusions regarding a comparison of PREPS.

2.44 A statement providing details of all the conclusions reached by COT/COC/COM is appended to the COT section of this annual report.

Review of Committee Procedures

2.45 The Committee's publication scheme (prepared in accordance with Freedom of Information Act 2000) is available on the COM internet site (http://www.advisorybodies.doh.gov.uk/foi/publicationscheme.htm). The COM meetings are now held in open session. The procedures adopted by COM are equivalent to those used by COT. Details can be found on the COM internet site. (http://www.advisorybodies.doh.gov.uk/foi/open.htm)

Horizon scanning

2.46 The COM undertakes 'Horizon scanning' exercises at regular intervals to identify new and emerging issues which have the potential to impact on public health and which might require COM advice. Members considered a paper which focussed on target organ mutagenesis and carcinogenicity risk assessment, low dose DNA adducts, mutagenicity of micronised chemicals and effects of micronutrients on mutagenicity.

2.47 Target organ mutagenesis in relation to identifying cancer risk had been identified as a potential topic for future consideration following recent COM/COC evaluations of chloropropanols and malachite green. Key mutagenic data from cancer target organs can potentially assist in carcinogen risk assessment. However, tumours may be observed in an organ that has not been assessed during *in-vivo* mutagenicity tests. The development of a variety of methodologies, such as Muta Mouse™ and Big Blue™ transgenic rodent assays, the Comet assay, mutation spectra data and microarray analysis, may help identify target organs. Thus, there was a potential to define more closely whether observed tumours were attributable to mutagenic events.

2.48 Members agreed that the link between target organ mutation and cancer had not been investigated in detail at present. The committee considered that factors that determined target organs for carcinogenicity were poorly understood and the process was complex. The COM agreed that it would be valuable to have a joint COM/COC meeting on the topic. A number of independent experts would be invited to attend.

2.49 Techniques to assess DNA adducts had improved in recent years and highly sensitive methods such as ^{32}P-postlabelling were available. The committee noted that ILSI/HESI had looked at the use of low dose DNA adducts in risk assessment at its meeting in April 2004. The COM agreed to await a review on this topic by a subgroup of ILSI/HESI before considering the matter further.

2.50 The COM agreed that genotoxicity testing of nanotechnology products was potentially important. There were a few examples where micronised particles of traditionally accepted non-mutagenic chemicals had given positive results in *in-vitro* tests (e.g. zinc oxide and titanium dioxide). Aspects to consider included whether nanoparticles could be more readily absorbed, whether conventional mutagenicity tests were suitable for particles and whether any genotoxicity could be secondary to toxic effects such as the generation of reactive oxygen species. Members agreed that a periodic review of this subject would be useful.

Test Strategies and Evaluation

In-vitro micronucleus assay

2.51 The Committee provided advice on the evaluation of the *in-vitro* micronucleus test. Members agreed that for observational purposes the unit was the cell; it is the number of cells with micronuclei that is recorded. However, the major source of variability is likely to be the culture, and this should be considered in the analysis. The following wording was agreed. 'The observational unit is the cell, but the unit for statistical analysis is the culture.' This advice would be forwarded for inclusion in the draft proposals for an OECD guideline.

SCCNFP Recommendations On A Strategy For Testing Cosmetic Ingredients For Mutagenicity

2.52 The Committee were asked to comment on the strategy recently proposed by the EC's Scientific Committee on Cosmetics and Non-Food Consumer Products (SCCNFP) for permitted cosmetic ingredients (Opinion SCCNFP/0755/03 adopted April 2004 http://europa.eu.int/comm/health/ph_risk/committees/sccp/sccp_en.htm). In particular the strategy proposed for hair dyes, which is based on their opinion of June 2003, is of particular concern as this differs from the COM recommended strategy both in the number of *in-vitro* tests being proposed, and the importance placed in the SHE cell transformation assay. The Committee were asked whether they were aware of any data that would warrant re-consideration of their current views that the SHE cell transformation assay should not be used for regulatory screening of chemicals for potential carcinogenicity, as outlined in their statement of April 2002.

2.53 The Committee agreed that the SCCNFP's general recommendations for *in-vitro* screening of cosmetic ingredients, based on an *in-vitro* gene mutation test in mammalian cells and an *in-vitro* micronucleus test were reasonable. However, the proposals for testing hair dye ingredients based on the SCCNFP's June 2003 recommendations, gave rise to concern. The requirement for both a metaphase analysis for clastogenicity and an *in-vitro* micronucleus test introduced unnecessary redundancy; the Committee has always felt that, as regards the clastogenicity end-point, these 2 assays should give equivalent data. Furthermore the Committee felt that there was little to be gained by the additional *in-vitro* assay for DNA damage.

2.54 Regarding the SHE Cell Transformation Assay, members were aware that considerable work was being carried out on the development of this assay, much of which would be discussed at a meeting of the Environmental Mutagen Society (EMS) later in the year. It was agreed that the COM should consider these data when they are published, with a view to revising their statement on this assay if necessary.

COT/COC/COM review of Toxicogenomics

2.55 The COT/COC/COM held a joint symposium on the use of genomics and proteomics in toxicology in October 2001. The Committees agreed to further consider toxicogenomics as part of the horizon scanning exercise initiated at the February 2004 COT meeting. It was noted that there was a considerable increase in the number of publications using toxicogenomic approaches. A number of discussion papers were subsequently prepared for the Committees which reviewed the available published literature. The data from 50 studies were considered during the review which also included available information from the HESI (Health and Environmental Sciences Institute of the International Life Sciences Institute (http://www.ilsi.org/)) collaborative scientific program on toxicogenomics. The current review considered information on use of metabonomics in toxicology for the first time. The COT requested a further paper and presentation on the use of statistics/bioinformatics in toxicogenomics. A presentation was given by Dr David Lovell (University of Surrey) to the COT at its meeting on the 7 September 2004.

[The Committees used the following definitions for the methods used in Toxicogenomics. Transcriptomics refers to gene expression as measured through cDNA or oligonucleotide or cRNA microarray based approaches, proteomics refers to determination of protein levels through gel or solid phase approaches and metabonomics refers to measurement of metabolites in tissues, plasma or urine.]

2.56 The COM reached the following conclusions after discussions held at its February and May 2004 meetings:

i) No conclusions can be drawn from the preliminary results of the ILSI/HESI trial of mutagenesis in mouse lymphoma L5178Y $tk^{+/-}$ cells. Further information on the detailed results from this trial and validation of the findings would be needed before conclusions can be drawn.

ii) Mutagenicity may be associated with changes in expression of relatively few genes which might be potentially difficult to identify in high density arrays. The COM agreed there were considerable difficulties in developing *in-vitro* mutagenicity screening assays using toxicogenomic approaches with regard to selection of appropriate microarray platform, confirmation of microarray results using quantitative measures of mRNA levels, identification of appropriate fold change in gene expression, and development of appropriate statistical/bioinformatics approaches for assessment of studies. However it was possible that valid approaches to screening for mutagens might be developed in the future.

iii) The COM identified the need for more research on time dependent changes in gene expression using mutagens and the application of integrated toxicogenomic approaches to evaluating changes in protein and metabolic pathways in response to exposure to mutagens. No adequate proteomic/metabonomic studies of mutagens had currently been identified.

iv) The COM reviewed a number of published papers which presented data using mouse lymphoma L5178Y $tk^{+/-}$ cells and agreed that no clearly defined pattern of gene expression changes which could logically be associated with mutagenesis had been identified. The COM reviewed a recent study which had used HepG2 cells and agreed that the authors had been able to distinguish between genotoxic and non-genotoxic carcinogens but only when a number of genotoxic compounds (predominantly methylating agents) were excluded. Overall this latter study provided some useful information but there was a need for considerable additional research involving multiple dose levels and sampling times before conclusions could be reached.

v) The Committee considered that the limited available in-vivo studies using four hepatocarcinogens did provide some preliminary results which suggested genotoxic responses in gene expression could be identified *in-vivo*.

vi) One preliminary investigation provided evidence to suggest that transcriptomics could provide information to aid in the interpretation of conventional *in-vitro* clastogenicity assays to assist in the evaluation of mutagenic or cytotoxic responses in these tests.

2.57 A statement providing details of all the conclusions reached by COT/COC/COM is appended to the COT section of this annual report.

Ongoing reviews

Biomonitoring studies for genotoxicity in pesticide applicators

2.58 The committee has been asked by the Medical and Toxicology Panel (MTP) of the Advisory Committee on Pesticides (ACP) to undertake a review of the available studies of genotoxicity in pesticide applicators and workers exposed to pesticides, particularly focussing on pesticide sprayers/applicators, floriculturists/greenhouse workers, agricultural workers and farmers and forestry workers. Advice from COM would be submitted to PSD as the registration authority for agricultural pesticide products and subsequently to the MTP and ACP. An initial overview paper of studies published in the European Union was considered at the October 2004 meeting. A further paper will be presented to the February 2005 meeting.

Statements

2,3-Dichloropropan-1-ol

Chromium picolinate

Malachite green/leucomalachite green

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

Introduction

1. 2,3-dichloropropan-1-ol (2,3-DCP) is a member of a group of chemicals called chloropropanols, which includes 3-monochloropropane-1,2-diol (3-MCPD) and 1,3-dichloropropan-2-ol (1,3-DCP). 2,3-DCP and 3-MCPD can be present as process contaminants in some polyamine flocculants used in water treatment and may therefore potentially be present in drinking water. 1,3-DCP is found as a process contaminant in food stuffs where acid-hydrolysed vegetable protein has been used as an ingredient in soy sauce or similar oriental sauces. It is not currently known if 2,3-DCP is present in food. 2,3-DCP was last considered by the COM in 2001. COM concluded that it would be prudent to consider 2,3-DCP as potentially genotoxic *in vivo* but agreed that it should be tested for genotoxicity *in vivo* using the approach set out in the COM guidelines.

COM evaluation 2001

2. Members were aware that there was very little data on the absorption, distribution, and excretion of 2,3-DCP. Theoretically, 2,3-DCP could be metabolised to produce epichlorohydrin (and subsequently glycidol) and therefore there were structural alerts for genotoxicity and carcinogenicity.

3. The Committee noted 2,3-DCP was mutagenic in *Salmonella typhimurium* strains TA 100 and TA 1535 in a study with and without metabolic activation[1], and mutagenic in another Ames test[2]. Positive results were also obtained for sister chromatid exchange with Chinese Hamster V79 cells both with and without metabolic activation[3]. No *in-vivo* studies in mammals have been carried out.

4. The Committee concluded that in the limited studies conducted, 2,3-DCP was genotoxic *in-vitro* with and without metabolic activation in bacterial and mammalian cells.

COM evaluation 2004

5. The Committee considered two new *in-vivo* genotoxicity studies at its February 2004 meeting. These comprised a rat bone-marrow micronucleus test and a rat liver unscheduled DNA synthesis (UDS) assay, both of which are widely used to assess genotoxicity *in vivo*.

Rat in-vivo bone-marrow micronucleus test[4]

6. The assay followed the current OECD guideline (No 474). The highest dose used in the study was selected so that it would produce some signs of toxicity, but not severe effects, based on the results of a range-finding study. In the main study, 1,3-DCP was administered once daily for two consecutive days to groups of six male Han Wistar rats at doses of 70, 140 and 280 mg/kg. Bone marrow was harvested 24 hours after the final dose. A single sex study was considered adequate because no substantial difference in toxicity was observed between males and females in the range-finder.

7. Clinical signs of toxicity, including lethargy, eye closure and piloerection were observed at 140 and 280 mg/kg bw. A dose related decrease in the mean ratios of polychromatic to normochromatic erythrocytes (PCE/NCE) compared to vehicle control was documented indicating that the test material was toxic to the bone marrow.

8. There were no statistically significant increases in micronucleus frequency at any dose of 2,3-DCP. The positive control agent, cyclophosphamide, produced a clear increase in micronuclei.

Rat liver in-vivo UDS assay[5]

9. The UDS assay protocol conformed to the current OECD guideline (No 486). Based on the results of the range-finder for the micronucleus study, the study was conducted in male rats and the highest dose was 280 mg/kg. Single doses of 110 and 280 mg/kg were administered to groups of four male Han Wistar rats. Hepatocytes were isolated for analysis for UDS by the autoradiographic technique after 12-14 hours in the first study (3 rats per dose group) and at 2-4 hours in the second study (3 rats per dose group).

10. The investigators reported notable weight loss in animals dosed with 280 mg/kg bw at 12-14 hours post-dose. It was noted that for a one animal, at 110 mg/kg bw in the 12-14 hour trial, the number of cells scored was below the recommended number. However there were sufficient cells scored from all other animals in the study. There was clear evidence of toxicity at the top dose level used. There was no evidence of an increase in Net Nuclear Grain counts in treated animals at either time point. The positive control compounds 2-AAF and DMN both gave clear positive results.

COM discussion

11. Members agreed that the two new studies met the previously stated requirement that 2,3-DCP should be tested for genotoxicity *in-vivo* using the approach set out in the COM guidelines. The studies were adequately conducted and gave clear negative results, and therefore Members considered that these studies provided evidence that 2,3-DCP was not an *in-vivo* mutagen. Members then gave consideration as to possible mechanisms whereby mutagenic activity observed *in vitro* was not expressed *in vivo*.

12. Members agreed that 2,3-DCP was metabolised to 2,3-dichloroacetaldehyde and from this to the corresponding acid. One research group had provided some *in vitro* data to suggest that induction of CYP2E1 resulted in 2,3-DCP mediated hepatotoxicity and glutathione depletion[6]. Members noted the findings of Koga *et al*[7] which suggested dechlorination/ hydroxylation occurred but agreed these authors had not provided evidence for the formation of an epoxide. Members concluded that there were insufficient data to draw conclusions on the metabolic activation of 2,3-DCP but overall the evidence suggested metabolic activation of 2,3-DCP differed from 1,3-DCP.

13. The COM considered that the metabolism of 2,3-DCP had not been fully elucidated. Metabolic activation *in vivo* to active metabolites had been postulated but had not been proven. The Committee agreed that 2,3-DCP was not mutagenic in the two tissues assessed which provided some assurance that active metabolites were not formed *in-vivo*.

Conclusions

14. The Committee concluded that both the rat bone-marrow micronucleus test and the rat liver UDS test had been carried out to an acceptable standard and were negative. Thus the additional information recommended by the COM as being necessary to provide adequate reassurance that the mutagenic activity seen *in vitro* was not expressed *in vivo* had now been provided.

15. The Committee noted the uncertainties with regard to routes of metabolic activation of 2,3-DCP and agreed that the two new mutagenicity studies supported the view that reactive metabolites, if formed, did not produce genotoxicity *in vivo* in the tissues assessed.

16. The Committee concluded that 2,3-DCP can be regarded as having no significant genotoxic potential *in vivo*.

May 2004

COM/04/S1

References

1. Nakamura A. Tateno N, Kogima s, Kaniwa M-A and Kawamura T (1979). The mutagenicity of halogenated alkanols and their phosphoric acid esters for Salmonella typhimurium. Mutation Research, 66; 373-380.

2. Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K. (1988) Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ Molec Mutagen 1 (S12); l – 158.

3. von der Hude W. Scheutwinkel M, Gramlich U, Fissler B and Basler A (1987). Genotoxicity of three-carbon compounds evaluated in the SCE test *In-Vitro*. Environmental Mutagenesis, 9; 401-410.

4. Beevers C. (2003) 2,3-Dichloropropan-2-ol (1,3-DCP): Induction of micronuclei in the bone marrow of treated rats. Report no 2150/2-D6172 from Covance Laboratories Ltd, Harrogate, North Yorkshire, England. Available from the Food Standards Agency.

5. Beevers, C. (2003) 2,3-Dichloropropan-2-ol (1,3-DCP): Induction of unscheduled DNA synthesis in rat liver using an *in vivo/in vitro* procedure. Report no 2150/4-D6172 from Covance Laboratories Ltd, Harrogate, North Yorkshire, England. Available from the Food Standards Agency.

6. Hammond AH and Fry JR . (1999). Effect of cyanamide on toxicity and glutathione depletion in rat hepatocyte cultures: differences between two dichloropropanol isomer. Chemical Biological Interactions, 122, 107-115.

7. Koga M, Inoue N, Imazu K, Yamada N and Shinoki T (1992). Identification and quantitative analysis of urinary metabolites of dichloropropanols in rats. J UOEH (University Occupational and Environmental Health, 14, 13-22.

Statement on the mutagenicity of trivalent chromium and chromium picolinate

Introduction

Background to COM review

1. Chromium is a Group 6 metallic element which is ubiquitous in the environment where it generally occurs in the hexavalent or trivalent form. Chromium is an essential element, involved in carbohydrate metabolism. Chromium (III) picolinate is a food supplement which is widely available in the UK.

2. Chromium was one of the minerals recently reviewed by the Expert Group on Vitamins and Minerals (EVM) (http://www.foodstandards.gov.uk/multimedia/pdfs/vitmin2003.pdf). The EVM noted the reports of genotoxicity associated with chromium picolinate and excluded it from their recommendations for a Safe Upper Level for chromium. Following the publication of the EVM report, the Food Standards Agency advised that consumers should use other forms of trivalent chromium supplements.

Advice requested from COM

3. The Food Standards Agency has asked the COM to review the available information on the mutagenicity of trivalent chromium and specifically on chromium picolinate in order to support consumer advice and, if appropriate, to recommend what further studies would be required to draw definite conclusions. A US National Toxicology Program (NTP) carcinogenicity bioassay of chromium picolinate is underway but the final report is unlikely to be available for several years.

Public Health Issue

4. Hexavalent chromium compounds are established human carcinogens on the basis of both animal studies and epidemiological evidence of carcinogenicity (ie considered Group 1 carcinogens by the WHO International Agency for Research on Cancer (IARC) (http://monographs.iarc.fr/). Trivalent chromium compounds have lower toxicity, which is generally attributed to their lower solubility and to their very limited ability to cross cell membranes. Trivalent chromium compounds were considered by IARC to be not classifiable with regard to carcinogenicity in humans (i.e. Group 3).

5. Trivalent chromium may be the ultimate carcinogen responsible for the effect of hexavalent chromium since it is able to bind DNA directly. However, it is unclear whether the reduction of hexavalent to trivalent chromium and the subsequent oxidative damage, or the direct binding of trivalent chromium to DNA, or both is responsible.

Data considered by COM

Chromium picolinate and other chromium compounds

6. Chromium picolinate contains trivalent chromium bonded to three molecules of picolinic acid. The formation of additional chromium co-ordination complexes with each molecule of picolinic acid through the lone pair of electrons present on the nitrogen aids the stability of the molecule. Picolinic acid is an isomer of niacin (vitamin B_3) and a minor metabolite of tryptophan metabolism. Unlike other trivalent chromium compounds, chromium picolinate is soluble in water at neutral pH.

Absorption, distribution, metabolism and excretion

7. The data on the absorption, distribution, metabolism and excretion (ADME) of chromium picolinate are limited. The available data from human volunteer studies and from experimental studies in rats suggest that the gastrointestinal absorption of chromium picolinate is significantly greater than of other forms of trivalent chromium and is comparable to that of hexavalent chromium.

8. In a recent *in-vivo* ADME study, rats were given a daily intravenous dose of radiolabelled chromium picolinate (^{51}chromium or ^{3}H- picolinate) for 14 days[1]. Retention of chromium was substantial, with daily urinary and faecal excretion of approximately 10% of the ^{51}Cr at the beginning of the experiment, increasing to approximately 20% by the end. The majority of the radiolabel was found in the urine and when subject to column chromatography co-eluted with chromodulin. ^{3}H-labelled material was largely excreted via the urine. At the end of the treatment period, both ^{51}Cr and ^{3}H labels were widely distributed in the tissues but were predominantly present in the liver, where the sub-cellular pattern of distribution differed to that in other tissues. Thus the absorption, distribution, metabolism and excretion of chromium picolinate is complex and includes some degree of dissociation.

Oxidative damage

9. Hexavalent chromium is readily reduced to trivalent chromium both *in vitro* and *in vivo*, resulting in oxidative and cytotoxic damage to cells. The evidence for oxidative damage *in vivo*, after treatment with chromium picolinate includes increased urinary excretion of 8-hydroxy-2' deoxyguanosine and increased lipid peroxidation in liver and kidney cells of rats[2]. Evidence of *in vitro* oxidative damage includes damage to mitochondria from CHO cells and lipid peroxidation in cultured macrophage J774A.1 cells[3,4]. However the only other *in-vivo* study available does not report any oxidative damage associated with chromium picolinate or with other forms of trivalent chromium[5]. Overall, it can be concluded that chromium picolinate induces oxidative damage *in vivo* to a lesser extent than hexavalent chromium compounds. However, *in-vivo* oxidative damage associated with chromium picolinate treatment has been reported by only one research group, where the test material was synthesised in the laboratory.

Interactions with DNA

10. Trivalent chromium compounds are able to bind DNA and RNA in cell free systems. While some studies with chromium picolinate suggest little direct interaction with DNA[2], Speetjens and colleagues[6], have shown a dose-dependent relaxation of supercoiled plasmid DNA to the circular nicked form by trivalent chromium picolinate in the presence of ascorbic acid and air. The authors noted that chromium picolinate was stable and thus could be incorporated into cells intact. They further speculated that ascorbate could reduce trivalent chromium to divalent chromium, which could then enter Fenton or Haber-Weiss reaction cycles to produce hydroxyl radicals leading to oxidative damage. Members considered that more direct evidence was required to confirm this suggestion.

11. Chromium picolinate induces DNA fragmentation in cultured J774A.1 murine macrophages[7].

In vitro mutagenicity

12. Trivalent chromium compounds are negative in a large number of *in-vitro* bacterial mutagenicity tests. In contrast, positive results have been documented for hexavalent chromium compounds. An adequate study using chromium picolinate was conducted in *Salmonella typhimurium* strains as part of the US NTP. This study reported negative results[8]. Other published bacterial tests with chromium picolinate have also yielded negative results[9,10].

13. Chromium picolinate has been reported to cause up to a 40 fold increase in mutation at the *hprt* locus in CHO AA8 cells in the absence of exogenous metabolic activation[11]. However the results were from a single, unusually long, treatment time of 48 hours. The chromium picolinate used had been synthesised by the testing laboratory and there was uncertainty regarding the nature and quantities of impurities in test material used.

14. In response to recommendations made by the COM in October 2003, the ability of chromium picolinate to cause *hgprt* mutations *in vitro* in CHO cells was tested using commercial grade material using a protocol adhering to International standards[12]. Chromium picolinate was negative in both S9 activated and non-activated assays. In order to fully replicate the work of Stearns and colleagues[11], an additional experiment was conducted using a 48 hour incubation in the absence of S9 activation[13]. This was also negative. Additional information on dosing solution analysis and historical control data were reported to the COM in October 2004. The Committee was satisfied regarding the identity of the test material and dosing solution analysis. Overall it was agreed that the data should be considered as indicating a negative result.

15. Positive results have been documented for both hexavalent and trivalent chromium compounds in *in-vitro* clastogenicity tests in mammalian cells. The significance of the results with trivalent compounds is uncertain as the evidence of mutagenicity was documented at high cytotoxic concentrations and under prolonged exposure conditions where it was considered that endocytic uptake of test material had occurred. In a study by Stearns and colleagues[14] chromium picolinate was tested in both a solubilised and a particulate form and resulted in a dose-related increase in chromosomal aberrations in CHO AA8 cells following incubation for 24 hours in the absence of exogenous metabolic activation. The magnitude of the response (3-18 times control) reported clearly suggested a clastogenic effect. The Committee noted that the test material used had been synthesised in the testing laboratory and commented that there was uncertainty regarding the nature and quantities of impurities in the test material used in this assay. The Committee also noted that chromium picolinate would have been expected to be poorly soluble in the solvent used (acetone). Overall it was agreed that there was a need for independent replication of the study undertaken by Stearns before any conclusions could be reached. In response to recommendations made by the COM in October 2003, the ability of chromium picolinate to cause chromosome aberrations in CHO cells *in vitro* was tested and reported to be negative[15]. The Committee considered that the submitted study had been adequately conducted according to internationally accepted guidelines but observed that there were limitations regarding this study (eg the wide historical positive control range for structural aberrations both in the absence and presence of exogenous metabolic activation). Additional information on dosing solution analysis and historical control data were reported to the COM in October 2004. The Committee was satisfied regarding the identity of the test material and dosing solution analysis. Overall it was agreed that although the committee noted some limitations in the study, the data should be considered as indicating a negative result.

In vivo mutagenicity

16. Chromium picolinate but not trivalent chromic chloride was active in a multi-generation *Drosophila* study where it was observed to delay pupation and decrease pupal viability. Further analysis indicated that chromium picolinate increased lethal mutations and dominant female sterility[16]. It is not possible to extrapolate such data to *in-vivo* exposure in mammals.

17. Three *in-vivo* mutagenicity studies of chromium picolinate have been reported; two in rats and one in mice. No evidence of chromosomal damage was reported in a study in which rats were given an oral dose of up to 2,000 mg/kg bw. The data were published in abstract form[10,17] with further details being contained in the study report[18].

18. No increase in micronuclei was reported in peripheral blood samples from F344 rats given gavage doses of up to 2,500 mg/kg chromium picolinate for 3 days[19]. This study has been undertaken as part of the preliminary studies of the US NTP prior to commissioning carcinogenicity bioassays. The ratio of polychromatic to normochromatic erythrocytes was not altered in this study. However it is not possible to conclude that the bone-marrow has been exposed to chromium picolinate in this study since previous work[20] had suggested that trivalent chromium may be retained in the fatty tissue surrounding the bone-marrow. There are no comparable absorption data available for chromium picolinate. As part of the US NTP study, B6C3F1 mice were given diets containing up to 50,000 ppm chromium picolinate for 13 weeks and micronuclei in peripheral blood erythrocytes counted[21]. There was no clear evidence for a mutagenic effect, although a statistical test for a dose-related trend had reported a positive finding in female mice (P=0.005). Pair-wise comparisons with control suggested no mutagenic effect. The NTP data have not yet been published in a peer-reviewed form.

Picolinic acid

19. An increase in *hgprt* mutations and chromosomal aberrations in the absence of exogenous metabolic activation was also documented in CHO AA8 cells[11,12]. These tests were undertaken concurrently with the tests using chromium picolinate described previously. Chromium picolinate was reported to be more mutagenic in the *hgprt* system than the equivalent concentrations of either free picolinate or trivalent chromic chloride. Similarly, the clastogenic effects of chromium picolinate were apparent at lower equivalent concentrations than those associated with free picolinate.

20. Picolinic acid was also studied in a multigeneration *Drosophila* study[16]. Treatment with picolinic acid alone also increased the numbers of individuals arrested during pupation and reduced larval and adult viability. The significance of this with respect to the mutagenicity of picolinic acid is unclear. It is also not possible to extrapolate such data to *in-vivo* exposure in mammals.

Conclusions

21. The evaluation of the mutagenicity of chromium picolinate is complex and the available data are conflicting. Chromium picolinate has given positive results in some *in-vitro* mutagenicity tests. The mechanism by which this occurs is unclear. However, in these studies the test material had been synthesised in the laboratory concerned and an adequate specification was not available. Replication of the tests using commercial grade material in tests conducted to internationally accepted protocols gave negative results. The Committee expressed some reservations regarding the conduct of these studies (possible limitations in sensitivity) and in particular regarding the repeat in-vitro chromosome aberration study in CHO cells. However, overall it can be concluded that the balance of the data suggest that chromium picolinate should be regarded as not being mutagenic *in vitro*.

22. The available *in-vivo* tests in mammals with chromium picolinate are negative. In view of the negative *in-vitro* results with commercial grade chromium picolinate, there is no further requirement for *in-vivo* testing at the current time.

23. The ongoing US NTP carcinogenicity bioassays of chromium picolinate will provide important *in-vivo* data in the future. The in-life phase of the NTP bioassays is due to end in July 2004. These data should be considered when the full results of the bioassay are available.

December 2004

COM/04/S3

References

1. Hepburn D D D and Vincent J B (2002). *In Vivo* Distribution of Chromium from Chromium Picolinate in Rats and Implications for the Safety of the Dietary Supplement. Chemical Research in Toxicology, 15, 93-100.

2. Hepburn D D D, Burney J M, Woski S A, Vincent J B (2003a). The Nutritional Supplement Chromium Picolinate Generates Oxidative DNA Damage and Peroxidised Lipids *In Vivo*. Polyhedron, 22, 455-463.

3. Manygoats K R, Yazzie M, Stearns D M (2002). Ultrastructural Damage in Chromium Picolinate-Treated Cells. Journal of Biology and Inorganic Chemistry, 7, 791-197.

4. Bagchi D, Bagchi M, Balmoori J, Ye X, Stohs S J (1997). Comparative Induction of Oxidative Stress in Cultures j774A.1 Macrophage Cells by Chromium Picolinate and Chromium Nicotinate. Research Communications in Molecular Pathology and Pharmacology, 97, 335-346.

5. Preuss H G, Grojec P L, Lieberman S, Anderson R A (1997). Effects of Different Chromium Compounds on Blood Pressure and Lipid Peroxidation in Spontaneously Hypertensive Rats. Clinical Nephrology, 47, 325-330.

6. Speetjens J K, Collins R A, Vincent J B, Woski S A (1999). The Nutritional Supplement Chromium (III) Tris (Picolinate) Cleaves DNA. Chemical Research in Toxicology, 12, 483-487.

7. Bagchi D, Stohs S J, Downs B W, Bagchi M, Preuss H G (2002). Cytotoxicity and Oxidative Mechanisms of Different forms of chromium. Mutation Research, 180, 5-22.

8. NTP, National Toxicology Program- Salmonella assay (unpublished results downloaded from NTP website).

9. Jutura V and Komorowski J R (2002). Antimutagenic activity of Chromium Picolinate in the Salmonella Assay, The Toxicologist, 44 (2) supp 1, Abstract 38.2, A59.

10. Esber H J, Moreno V, Loveday K S (1997). Evaluation of Chromium Picolinate in the Ames and the rat In Vivo Chromosomal Aberration. Mutation Research, 379, S89. [Abstract]

11. Stearns D M, Silveria S M, Wolf K K, Luke A M (2002). Chromium (III) trispicolinate is mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase locus in Chinese Hamster Ovary Cells, Mutation Research, 513, 135-142.

12. San R H C and Clarke J J (2004a). *In vitro* Mammalian Cell Gene Mutation (CHO/HGPRT) Test with an Independent Repeat Assay. Bioreliance Report no AA85MC.782001.BTL

13. San R H C and Clarke J J (2004b). *In vitro* Mammalian Cell Gene Mutation (CHO/HGPRT) Test with a 48-hour exposure. Bioreliance Report no AA85MC.782048.BTL

14. Stearns D M, Wise J P, Patierno S R, Wetterhan K E (1995). Chromium Picolinate Produces Chromosome Damage in Chinese Hamster Ovary Cells. FASEB J, 9, 1643-1645.

15. Gudi R and Rao M (2004a). *In vitro* Mammalian Chromosome Aberration Test. Bioreliance Report no AA85MC.331.BTL

16. Hepburn D D D, Xiao J, Bindom S, Vincent J B, O'Donell J (2003). Nutritional Supplement Chromium Picolinate Causes Sterility and Lethal Mutations in *Drosophila melangoster*. PNAS, 100, 3766-3771.

17. Greenberg D, Komorowski J R, Loveday K (1999). Rat Chromosomes are Unharmed by Orally Administered Chromium Picolinate. Journal of the American College of Nutrition, 18, 27.

18. Kiopes A L (1999). Evaluation of Chromium Picolinate in the Rat In Vivo Chromosomal Aberration Assay. Study Report no 30102. Published by Primedica Corporation Inc.

19. NTPb National Toxicology Program- Micronucleus assays (unpublished results downloaded from NTP website).

20. Kraintz L and Talmage R V (1952). Distribution of Radioactivity Following Intravenous Administration of Trivalent Chromium 51 in the Rat and Rabbit. Proceedings of the Society of Experimental Biology and Medicine, 81, 490-492.

21. NTPc National Toxicology Program- 13 week study (unpublished results downloaded from NTP website).

Joint COM and COC statement on the mutagenicity and carcinogenicity of malachite green (MG) and leucomalachite green (LMG)

Introduction

1. Malachite green (MG) is a cationic triphenylmethane dyestuff used in a number of industries including fish farming. Its use in fish for human consumption was banned in the EU in June 2002 but residues continue to be found in fish.

2. In 1999 the COM provided advice to the COT on the mutagenicity of malachite green and its lipophilic metabolite leucomalachite green (LMG). The COT had asked for advice in particular on the results from ^{32}P post-labelling studies investigating DNA adduct formation in the liver of rats and mice from 28 day repeated dose toxicity studies. These studies were part of the US National Toxicology programme, and were the range-finding studies prior to initiating carcinogenicity bioassays on MG and LMG.

Mutagenicity

COM Advice 1999

3. In 1999 the COM agreed the following conclusions regarding the mutagenicity of MG and LMG, which were incorporated in the 1999 COT statement on MG and LMG in farmed fish.[1]

Malachite Green

i) There is one report of a Salmonella assay done to an acceptable protocol; malachite green oxalate (>90% pure) was shown to induce mutations in TA98 in the presence of an exogenous metabolic activation system.[2] There is also some evidence of clastogenicity in Chinese hamster lung cells.[3] MG should thus be regarded as having mutagenic potential.

ii) Negative results were obtained in a poorly reported bone marrow micronucleus test in mice using a single oral dose of malachite green oxalate (>90% pure) at the MTD (75% of the LD50).[2] Members agreed that the high dose level of 37.5 mg/kg was adequate, but it was difficult to assess the value of the negative results in the absence of appropriate information on bone marrow toxicity or data to show that MG and/or metabolites reached the bone marrow.

iii) Recent ^{32}P-post-labelling studies using a 28 day dietary exposure and carried out with NTP range – finding studies (used in designing carcinogenicity bioassays) indicate that MG induces DNA adducts in the liver of rats and mice.[4,5]

iv) The Committee concluded that although a limited negative *in-vivo* micronucleus test was available, the results of the recently conducted ^{32}P- post-labelling studies indicated that it would be prudent to assume that MG may be a potential in-vivo mutagen.

Leucomalachite green

i) The Committee were concerned that only limited information was available on LMG. The lack of information on LMG prevents an adequate mutagenicity assessment for this compound.

ii) The Committee recommended that *in-vitro* studies in bacteria for gene mutations, in mammalian cells for clastogenicity and a mammalian cell assay for gene mutation (preferably the mouse lymphoma assay), conducted according to current OECD guidelines, should be undertaken as an important step to evaluating the mutagenic potential of LMG.

iii) The Committee, however, concluded that the results of the recently conducted post labelling studies[4,5] indicated that it would be prudent to assume that LMG may also be a potential *in-vivo* mutagen.

Updated advice from COM 2004

4. The results of the NTP carcinogenicity bioassays on MG and LMG were published in 2004[6]. The NTP report in addition included the results of a number of new studies to investigate the mutagenicity of these 2 compounds, most of which had also been published separately. The COM reviewed these new data in 2004 with a view to updating their conclusions on the mutagenicity of these two compounds.

5. New data were now available from *in-vitro* studies to investigate the ability of MG and LMG to induce mutations in the Salmonella assay, the CHO/*hprt* assay, and an *in-vitro* comet assay[6,7]. Negative results were obtained except for the comet assay with MG which was positive.

6. The ^{32}P post-labelling studies considered by the COM in 1999 have now been published in full. Male F344 rats and female B6C3F_1 mice were fed 9, 100 and 600 ppm MG or 0, 96 or 580 ppm LMG in the diet for 28 days[8]. ^{32}P post-labelling analysis of liver DNA indicated a single adduct, or co-eluting adducts, with both compounds, with the level of adducts increasing significantly as a function of dose. In rats the level of adducts seen was similar with MG and LMG. However in the mice MG gave a clear dose-related increase in binding which was slightly lower than that seen in the rats, whereas LMG produced only very low levels of adducts, of doubtful significance. In later studies with female Big Blue rats and mice LMG again produced evidence of DNA adduct formation in the rat but not the mouse.[9]

7. The ability of MG or LMG to induce micronuclei in peripheral blood or in *hprt* mutations in the spleen was also investigated in the above studies; negative results were obtained[6,9]. The Committee noted that these studies, which used repeated exposure over 4-32 weeks, did not optimise the chances of detecting a mutagenic response.

8. Studies to investigate induction of *lacI* mutations by LMG in the liver of female Big Blue rats gave equivocal results[10]. Samples were obtained at 3 time periods (4, 16, 32 weeks) at 5 dose levels. An increase in *lacI* mutations was seen at only a single time point (16 weeks) and only at the top dose level. Since *lacI* mutations were not expected to decline with continued exposure to compounds the nature of the DNA sequence alterations was investigated in DNA from the isolated positive result. When corrected for clonality there was no significant difference between the LMG treated and the control frequency. The authors considered that this isolated positive was an artifact due to the disproportionate expansion of spontaneous *lacI* mutations.

9. Studies to investigate *cII* mutations induced by LMG in the liver of Big Blue B6C3F$_1$ mice did, however, give a positive result[6]. At a dietary level of 408 ppm LMG produced a statistically significant ($p < 0.05$) increase in mutations. Further analysis showed that these contained a spectrum of mutations that was distinct from that in the control animals. In contrast MG did not produce any significant increase in such mutations in the mice. In similar studies in Big Blue rats, LMG did not produce any increase in *cII* mutation frequency in liver DNA.

10. Members considered the lack of concordance between the results of studies investigation DNA adducts and mutations in transgenic mice and the observation of an apparently specific mutation in the *cII* transgene in mice represented an unusual data set and hence there was uncertainty in deriving conclusions from these studies.

11. The COM agreed to revise their 1999 conclusions regarding the mutagenicity of MG and LMG as follows:

Malachite green

i) There is one report of a *Salmonella* assay done to an acceptable protocol; MG oxalate (>90% pure) was shown to induce mutations in TA98 in the presence of an exogenous metabolic activation system[2]. There is also some evidence of clastogenicity in Chinese Hamster Lung cells[3]. MG has also been shown to produce DNA damage in CHO cells using the Comet assay[6]. MG should thus be regarded as having mutagenic potential.

ii) Negative results were obtained in a poorly reported bone marrow micronucleus test in mice using a single oral dose of malachite green oxalate (>90% pure) at the MTD (75% of the LD50)[2]. Members agreed that the high dose level of 37.5 mg/kg was adequate, but it was difficult to assess the value of the negative results in the absence of appropriate information on bone marrow toxicity or data to show that malachite green and/or metabolites reached the bone marrow.

iii) ^{32}P post-labelling studies using a 28 day dietary exposure have indicated that MG induces DNA adduct(s) in the liver of both F344 rats and B6C3F$_1$ mice[7]. This has been confirmed in a separate study using 16 weeks dietary exposure[1]. MG did not induce micronuclei in peripheral lymphocytes nor *hprt* mutations in splenic lymphocytes in the 28 day study; the experimental design of these studies did not, however, optimise the chances of obtaining a positive response and no definite conclusions can be drawn.

iv) In view of the demonstration of DNA adduct formation in samples from both rats and mice, MG should be regarded as an *in-vivo* mutagen.

Leucomalachite green

i) Data are now available on the *in-vitro* mutagenicity of LMG from limited Salmonella and CHO/HGPRT assays, and also from a Comet assay in CHO cells[7]. Negative results were obtained. However in view of the limitations of these studies, and the fact that no data are available on clastogenicity, it is not possible to make an adequate assessment of the mutagenic potential of LMG from these data.

ii) ^{32}P Post-labelling studies using a 28 day dietary exposure have indicated that LMG induces a low level of DNA adduct(s) in the liver of F344 rats; only a marginal response was however seen in $B6C3F_1$ mice, and no conclusions can be drawn from these data[8]. This has been confirmed in a separate unpublished study in mice using 16 weeks dietary exposure; there was no evidence for DNA adduct formation in the liver[6]. LMG did not induce micronuclei in peripheral lymphocytes nor *hprt* mutations in splenic lymphocytes in the 28 day study[9], but the experimental design of these studies did not optimise the chances of obtaining a positive response and no definite conclusions can be drawn.

iii) Gene mutation studies have indicated that LMG can induce mutations *in vivo* in liver DNA. Studies in Big Blue F344 rats gave equivocal results. LMG produced an increase in *lacI* mutations only at the highest dose tested (543 ppm in diet) and at a single time point (16 weeks). No increase was seen after 32 weeks. Evidence was provided to suggest that this isolated positive may have been due to disproportionate expansion of spontaneous *lacI* mutations[9,10]. However studies in female Big Blue $B6C3F_1$ mice indicated that LMG produced an increase in *cII* mutant frequency in liver DNA of mice treated with 408 ppm LMG in the diet[6]. Furthermore it was shown that the spectrum of mutation seen in the DNA was distinct from that of the control mice. These data provide evidence of *in-vivo* mutagenicity at the target site in the carcinogenicity bioassay.

iv) In view of the demonstration of the induction of mutations in liver DNA of female $B6C3F_1$ mice, LMG should be regarded as an *in-vivo* mutagen.

Carcinogenicity

COC Advice 2004

12. The COC considered the results of the NTP carcinogenicity studies on MG and LMG in June 2004. They noted that prior to the publication of the NTP bioassay data on MG and LMG there had been no data available to make any meaningful assessment of the carcinogenicity of these compounds.

13. The carcinogenicity of MG was investigated in female F344 rats and in female B6C3F$_1$ mice, compound being given in the diet for 104 weeks. It was noted that the rationale for testing only in females was that this had been shown in range finding studies to be the most sensitive gender. In the study in rats groups of 48 female rats were given diets containing 0, 100, 300 and 600 ppm MG (equivalent to average daily doses of approximately 0, 1, 21 and 43 mg/kg bw/day). Survival in all groups was comparable, but mean body weight gain was slightly lower at 300 and 600 ppm (approximately 10% reduction compared to controls). At autopsy it was noted that the relative liver weights were increased at the top dose. In the study in mice groups of 48 animals were fed diets containing 0, 100, 225 and 450 ppm MG (equivalent to average daily doses of approximately 0, 15, 33 and 67 mg/kg bw/day). Survival was again comparable in all groups but there was a slight reduction in body weight gain (5-10%) at the top dose. Effects on relative kidney weight were noted at autopsy.

14. The carcinogenicity of LMG was investigated in both male and female F344 rats and in female mice, the compound being given in the diet for 104 weeks. Groups of 48 male and female rats were fed diets containing 0, 91, 272 or 543 ppm LMG (equivalent to daily doses of 0, 5, 15 and 30 mg/kg bw/day in the males and 0, 6, 17 and 35 mg LMG/kg bw/day in the females). Survival was comparable in all groups (except for an increase in males given 272 ppm) but there was a reduction in body weight gain at the top dose (25% in females, 10-15% in males). At autopsy liver weight were noted to be increased in the males at the two higher doses, as were relative liver weights in the females.

15. In the study in female mice groups of 48 were given diets containing 0, 91, 204 or 408 ppm LMG (equivalent to average daily doses of 0, 13, 31 and 63 mg LMG/kg bw/day). Survival and body weight gain were comparable in all groups. At autopsy a decrease in relative kidney weight was noted.

16. Regarding the evidence for carcinogenicity in these bioassays the Committee agreed with the NTP conclusions, as modified by their peer review panel. They agreed the following conclusions regarding the carcinogenicity of MG and LMG.

Conclusions regarding carcinogenicity of malachite green

i) There was equivocal evidence of carcinogenicity in female F344 rats based on an increase in thyroid gland tumours (adenoma or carcinoma combined) hepatocellular adenomas and mammary gland carcinomas.

ii) There was no evidence of carcinogenic activity in female B6C3F$_1$ mice.

iii) Overall there was no convincing evidence for any carcinogenic effect with malachite green in these studies.

Carcinogenicity of leucomalachite green

i) There was evidence of carcinogenic activity in female B6C3F$_1$ mice based on an increase in hepatocellular adenoma or carcinoma combined.

ii) There was equivocal evidence of carcinogenic activity in male F344 rats based on an increase in interstitial cell adenoma of the testes and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined).

iii) There was equivocal evidence of carcinogenic activity in female F344 rats based on an increased incidence of hepatocellular adenoma or carcinoma (combined).

Overall conclusions regarding mutagenicity and carcinogenicity of MG and LMG

16. The COM concluded that both MG and LMG should be regarded as *in vivo* mutagens.

17. The COC concluded that the only convincing evidence for any carcinogenic effect of MG or LMG in the NTP bioassays was for LMG in female mice, based on an increase in hepatocellular adenoma or carcinoma combined.

18. The COC considered the possible mechanisms by which LMG induced tumours in the liver of the female mice. It was noted that the overall tumour profile was not that which would be expected of a genotoxic carcinogen, with activity being limited to effects in the liver of the female mouse; furthermore this was mainly due to an increase in adenomas. However it was also noted that there was no evidence from the NTP studies to support any non-genotoxic mechanism. In view of this, and taking into account the views of the COM, the Committee agreed that it was not possible to discount a genotoxic mechanism for the induction of the liver tumours in female mice and it would therefore be prudent to regard LMG as a genotoxic carcinogen.

December 2004

COM/04/S4 & COC/04/S7

References

1. COT Malachite Green and Leucomalachite Green in farmed fish. Annual Report of COT/COC/COM PP23-27(1999).

2. Clemmenson S, Jensen J C, Jensen N J *et al*. Toxicological studies on malachite green. Arch. Toxicol. 56, 43-5 (1984).

3. Ishidite M. Application of chromosome aberration tests *in-vitro*. Tests Courts Cancer. Quo Vades Symp. 57-79 (1981).

4. NTP (USA). Notification of approval protocol. 2 year bioassay in rats administered MG and LMG in the diet. Project. 212801. July 1998.

5. Culp S J, Blankenship L R, Kusewitt D F *et al*. Metabolic changes occurring in mice and rats fed LMG. Proc. 88th Ann. Meeting of Am. Soc. For Cancer Research, San Diego. P. 121 (1997).

6. NTP Technical Report TR-527 Toxicology and Carcinogenesis Studies of Malachite Green and Leucomalachite Green in F344 rats and B6C3F1 mice (feed studies) NTP US Dept Health and Human Services (2004).

7. Fessard V, Godard T, Huet S *et al*. Mutagenicity of MG and LMG in *in-vitro* tests. J. Appl. Toxicol 19, 421-30 (1999).

8. Culp S J, Blankenship L R, Kusewitt D F *et al*. Toxicity and metabolism of MG and LMG during short term feeding to Fischer 344 rats and B6C3F1 mice. Chem. Biol. Interactions. 122, 153-70 (1999).

9. Culp S J, Beland F A, Heflich R H *et al*. Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed LMG. Mut Res 506/7, 55-63 (2002).

10. Majanatha M G, Shelton S D, Bishop M *et al*. Analysis of mutations and bone marrow micronuclei in Big Blue rats fed LMG. Mut. Res. 547, 5-18 (2004).

2004 membership of the Committee on Mutagenicity of Chemicals in Food, Consumer products and the Environment

CHAIRMAN

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

MEMBERS

Dr Carolyn Allen BSc MSc PhD
Non specialist member

Dr Brian Burlinson CBiol MIBiol PhD
Director of Cellular and Molecular Toxicology, Huntingdon Life Sciences

Dr Gillian Clare BSc PhD
Study Director, Covance

Dr Julie Clements BSc PhD
Head of Genetic and Molecular Toxicology, Covance

Dr David Gatehouse BSc PhD CIBiol FIBiol FRCPath
Consultant in Genetic Toxicology, Covance

Mrs Rosie Glazebrook MA
Non specialist member

Dr Nigel J Gooderham BSc PhD CChem FRSC
Reader and Head of Molecular Toxicology, Division of Biomedical Sciences,
Imperial College London (South Kensington Campus)

Dr Ian Mitchell BA PhD
Consultant in Genetic and Molecular Toxicology,
Kelvin Associates and Chilfrome Enterprises Ltd

Dr Elizabeth M Parry BSc DPhil
Senior Research Fellow, School of Biological Sciences,
University of Wales Swansea

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

SECRETARIAT

Dr R Fielder BSc PhD Dip RCPath Scientific Secretary (until August 2004)
Dr D Benford BSc PhD Scientific Secretary – Food Standards Agency
Mr K Mistry Administrative Secretary
Mr J Battershill BSc MSc

Declaration of interests during the period of this report

Member	Personal Interest		Non-Personal Interest	
	Company	Interest	Company	Interest
Prof P B Farmer (Chairman)	Abbey National	Share Holder	American Chemistry Council	Research Support
	Bradford & Bingley	Share Holder		
	Celltech	Share Holder	CEFIC	Research Support
	Foreign & Colonial Friends Provident	Share Holder		
	Health Effects Institute	Share Holder Research Committee Member		
	Torotrak	Share Holder		
Dr C Allen	NONE	NONE	NONE	NONE
Dr B Burlinson	Huntingdon Life Sciences	Salary Employee Share Option Holder	NONE	NONE
Dr G Clare	Covance	Salary	NONE	NONE
	Allied Domecq	Share Holder		
	AstraZeneca	Share Holder		
	Diagco	Share Holder		
	HBOS	Share Holder		
	Marks & Spencer	Share Holder		
Dr J Clements	Covance	Salary Share Option Share Holder	NONE	NONE
Dr D Gatehouse	Covance	Salary Consultant	NONE	NONE
	Friends Provident	Share Holder		
	GlaxoSmithKline	Pension Share Option Holder Share Holder		
Mrs R Glazebrook	Dr Foster Ltd	Salary	NONE	NONE
	BT Group	Share Holder		
	Lloyds TSB	Share Holder		
	National Grid	Share Holder		

Member	Personal Interest		Non-Personal Interest	
	Company	Interest	Company	Interest
Dr N Gooderham	Abbey National	Share Holder	GlaxoSmithKline	BBSRC Collaborative Studentship
	Friends Provident	Share Holder		
	Game	Share Holder		
	ML Laboratories	Share Holder		
	Northern Rock	Share Holder		
	Proctor & Gamble	Consultant		
	Protherics	Share Holder		
	Sunderland AFC	Share Holder		
Dr I Mitchell	Kelvin Associates	Associate Consultant	NONE	NONE
	IM Enterprises	Director/Creditor		
	GlaxoSmithKline	Pensioner		
		Option and Share Holder		
		Consultant		
	Bass	Share Holder		
	Cable & Wireless	Share Holder		
	Cadbury Schweppes	Share Holder		
	Renishaw	Share Holder		
	Pfizer	Share Holder		
	RTZ	Share Holder		
	Shell	Share Holder		
	Unilever	Share Holder		
	Vodafone	Share Holder		
	Whitbread	Share Holder		
	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	Share Holder	NONE	NONE
	Banco Santander	Share Holder		
	BG Group	Share Holder		
	Bradford & Bingley	Share Holder		
	Centrica	Share Holder		
	Lattice Group	Share Holder		
	National Grid	Share Holder		
	Takeda	Consultant		