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MUT/05/15

**COMMITTEE ON, MUTAGENICITY OF CHEMICALS IN FOOD,
CONSUMER PRODUCTS AND THE ENVIRONMENT. (COT/COC/COM)**

OVERVIEW OF NANOMATERIAL TOXICOLOGY (March 2005)

CONSIDERATION OF MUTAGENICITY DATA

Introduction.

1. The risk assessment of nanomaterials has been identified by COT/COC/COM as an area of interest during horizon scanning discussions for 2004. The Committee's interest in this area was prompted by the publication of the royal society review of nanotechnology. (<http://www.nanotec.org.uk/>) The appended documents have been drafted to provide baseline toxicology information for all three committees. These comprise; The objective is to collect initial views from the COC (21 April), COM (26 May) and COT (12 July) and draft a short statement.
2. The overview paper has been submitted to COM for information (MUT/05/15).
3. A short review of the retrieved mutagenicity data is given below.

Advice requested from COM

Nanoparticles

2. The COC discussed the overview at its meeting of 21 April 2005. There were no specific carcinogenicity data available and COC was therefore unable to reach any conclusions on carcinogenic hazard.
3. There is a limited amount of mutagenicity data available on ultrafine particulate titanium dioxide or zinc oxide compared to µmetre sized material. These data were briefly reviewed in the COM horizon scanning paper. A more detailed evaluation of the titanium dioxide data is tabulated at the end of this paper. Very little additional published information was retrieved on zinc oxide so that only a limited consideration of the mutagenicity data on this material is provided.

Titanium dioxide TiO₂

[Some additional data are appended as Annex 1]

4. The critical paper cited in the published literature for tests with ultrafine TiO₂ (Rahman Q et al Env Health Perspectives, 110, 797-800, 2002), reports the finding of mutagenicity and apoptosis induced *in-vitro* in SHE fibroblasts

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by ultrafine TiO₂ (≤ 20 nm) whilst no mutagenic effects was reported with fine (μ metre-sized) TiO₂ (>200 nm). No information on particle uptake by SHE cells was reported. A number of other published mutagenicity studies titanium dioxide have documented both negative and positive responses *in-vitro* in mammalian cells with μ metre-sized TiO₂. The results of such tests appear to be influenced significantly by the preparation of suspensions of TiO₂ with positive results documented using μ metre-sized TiO₂ in one study when DMSO was used to prepare the test material. A number of authors report precipitation when aqueous systems are used. The mechanism of TiO₂ induced mutagenic effects involve the generation of free radicals. It is difficult from the available to derive any conclusions as to whether nanoparticulate TiO₂ has any particular mutagenic activity in *in-vitro* mutagenicity test systems that cannot also be demonstrated with μ metre sized TiO₂ in appropriate studies

5. How would COM members recommend the mutagenicity of nanoparticulate and μ metre sized TiO₂ be further considered?

6. A weak positive mutagenic response was reported in an *in-vivo* bone marrow micronucleus assay in mice using the three-exposure protocol developed by Shelby et al in the early 1990s (Env Mol Mutagenesis, 21, 160-179, 1993) using test material obtained from the NTP chemical repository (specification not provided, see appended data from publication, Annex 1). From these data would COM members consider whether there was a need for appropriate *in-vivo* tests with nanoparticulate materials?

Zinc oxide

[Some relevant data appended as Annex 2]

7. A number of positive *in-vitro* mutagenicity tests were reported with micronised ultrafine zinc oxide (reported to be practically insoluble in water but soluble in dilute mineral acids). It is notable that the limited specification cited in the SCCNFP opinion on zinc oxide reports the average particle size to be 200 nm or less with a narrow distribution. Under the definitions used for the Royal society review this specification is either too limited to define or just falls outside the definition used by the Royal Society for nanoparticulate material (i.e. one dimension <100 nm). Cytogenetic effects were reported in CHO and V-79 cells in tests where zinc oxide was prepared in aqueous systems (culture medium) or using an emulsion (10% emulsion in 35 Tego Care 450 (polyglyceryl-3 methylglucose disteareate)). Enhanced mutagenic effects were reported in photo mutagenicity assays in mammalian cell cytogenetic assays. A positive response was also reported in a COMET assay using v-79 cells (using deionised water to suspend zinc oxide). Very little additional information on the mutagenicity of zinc oxide was retrieved. In one recent study (Hikiba H J Pharmacol Sci, 97, 146-152, 2005) some limited evidence for a clastogenic effect was documented in SHE cells exposed to zinc oxide (dissolved in 0.1 N HCl (50 mM) at 0-180 μ M for 24 hours. A commercial source of zinc oxide was used and no information on particle size was given.

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It is possible that this latter test may reflect exposure to soluble zinc ions rather than particulate zinc oxide. In the absence of appropriate studies using a range of particle sizes of zinc oxide, it is not possible to derive conclusions on the influence of particle size on mutagenicity of zinc oxide.

8. What are members views on the available information on zinc oxide?

Fullerenes

[Some relevant data appended in Annex 3]

9. Fullerenes are molecules of carbon arranged in cage-like hollow structures. (The C₆₀ or Buckminsterfullerene is the most studied form). Environmental generation of a range of fullerenes has been reported during imperfect combustion of wood. A number of potential medical applications involve incorporating side chains onto the cage structure. The structural diversity of fullerenes has suggested there may be other applications e.g. potential uses as catalysts or starting materials for chemical synthesis. Fullerenes have been shown to have free radical generation properties. This leads to the suggestion that fullerenes may induce oxidative damage in cells. It is noted that a C₆₀ fullerene soot was not an initiator or promoter in a skin promotion assay in mice.

10. The only investigation of *in-vitro* mutagenicity was cited in the HSE review of nanomaterials (MUT/05/18, information paper (Annex 1 of MUT/05/15 page 24). C₆₀ fullerene (>99.9% pure dissolved in polyvinylpyrrolidone) was tested in plate incorporation assays in *S.typhimurium* TA102, TA 104 and YG3003 (repair deficient strain derived from TA 102) both in presence and absence of exogenous metabolic activation using Aroclor 1254 rat liver S-9 mix and with irradiation using visible light (100 W, 400-600 nm- 25 cm from plates for 20 mins) or without irradiation (plates kept in dark room). Dose related mutagenic responses were documented in TA102 in the presence of irradiation. The potency in TA102 using 300 µg/ml C₆₀ fullerene was lower than in comparison with the mutagenic response of methylene blue (30 µg/ml). Dose-related C₆₀ fullerene mutagenicity was also reported in the presence of irradiation and exogenous metabolising fraction in TA104 and YG3003. In addition some evidence for a mutagenic effect of C₆₀ fullerene was also documented in YG3003 in the presence of S-9 mix but without irradiation.

11. The *in-vitro* mutagenicity of C₆₀ fullerene in TA102 was enhanced by phospholipase A2 or by sonication and was reduced in the presence of β-carotene or parabromophenacyl bromide (an inhibitor of phospholipase). The authors demonstrated the C₆₀ fullerene generation of free radicals in phospholipids and 8-OHdG (in a rat liver microsomal system or using methyl linoleate incubated with 2 deoxyguanine), the oxidation of phospholipids, and generation of free radicals using calf thymus DNA or guanine. It was suggested that the mutagenicity of C₆₀ fullerene in the test strains of

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S.typhimurium used was due to generation of free radicals in phospholipids and subsequent oxidative damage of DNA.

12. No additional mutagenicity studies of fullerenes or modified fullerenes were retrieved. It is notable that the addition of side chains to the fullerene molecule appears to alter the solubility and toxicological profile compared to unmodified fullerene. (Annex 2 to MUT/05/18, page 14).

13. How would COM members consider the mutagenicity of fullerenes be further considered?

Discussion/ Questions for COM.

14. The COT/COC/COM have been asked to provide a baseline review of the available information on nanomaterials. An overview review was drafted based on the HSE review of nanomaterials (MUT/05/18). Very little information was obtained regarding mutagenicity. These data have been reviewed in this cover paper. Some specific questions regarding mutagenicity testing strategy have been derived for the COM. These are reproduced below

i) How would COM members recommend the mutagenicity of nanoparticulate and μ metre sized TiO_2 be further considered? Is there a need for appropriate *in-vivo* tests with nanoparticulate sized TiO_2 .

ii) What are members views on the available mutagenicity information on zinc oxide?

iii) How would COM members consider the mutagenicity of fullerenes be further considered?

Secretariat May 2005

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ANNEX 1 TO MUT/05/15

MUTAGENICITY DATA ON TITANIUM DIOXIDE

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ANNEX 1 TO MUT/05/15: MUTAGENICITY REVIEW OF TITANIUM DIOXIDE

Nanomaterials developed for pharmaceutical uses have been excluded from this review.

Overview of mutagenicity studies using titanium dioxide

Introduction

1. Titanium dioxide occurs naturally in several crystalline forms of which rutile and anatase (octahedrite) are primarily used for industrial/commercial applications such as coating welding rods, and use as a white pigment in cosmetics, enamels, inks, dyes, and paints. Rutile has better surface coverage properties than anatase. Titanium dioxide is insoluble in water, dilute and concentrated acids. Titanium dioxide is considered an inert substance of low acute mammalian toxicity. (refs Merk Index, 12th edition, 1996, Soldana Pigments, Denmark).
2. Titanium dioxide is not carcinogenic in experimental animals in studies using oral (rat/mouse), subcutaneous (rat), intratracheal (hamster) or intraperitoneal (rat/mouse) routes of administration. An increase in the incidence of cystic keratinising lesions diagnosed as squamous cell carcinomas has been documented in rats in an inhalation bioassay. These lesions are typical of rat lung overload phenomena seen with inert dust materials such as carbon black. The COC considered the reported tumourigenic responses in rats exposed to inert particulates (such as carbon black and titanium dioxide represented an overload of normal lung clearance mechanisms in the lung and were specific to the rat) IARC considered there was limited evidence for carcinogenicity of titanium dioxide in the rat but overall inadequate evidence regarding carcinogenicity to humans (group 3, IARC, Monograph vol 47, 1989).

Mutagenicity data

3. *In-vitro* studies using cytogenetic end points and commercial samples of TiO₂ (i.e µmetre sized material) undertaken in 1980s, and up to mid 1990s reported predominantly negative results (see table below). A weak positive response in the mouse bone marrow micronucleus test was reported by Shelby in 1993 in the publication in Environmental Molecular Mutagenesis which presented an evaluation of the three-exposure protocol (data are appended from the tabulated information in Shelby's report). This report may have stimulated a number of other *in-vitro* tests in microbial and mammalian tests systems published in the late 1990s which have documented positive results *in-vitro* for titanium dioxide. It is possible that the choice of solvent and solubility of the test material in the medium used may be relevant to the differences in the reported results. The mechanism by which commercial samples of titanium dioxide could induce positive results in such mutagenicity tests is unclear. However essential elements in mutagenic responses could

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include adsorption/adherence to cells, intracellular uptake, oxidative damage and generation of free radicals leading to lipid which using the enamels

Summary of mutagenicity tests with titanium dioxide.

Test system	Exposure details	Result	Comments/Conclusions
<i>In-vitro</i>			
CA and SCEs in CHO cells Commercial TiO ₂ (American Cyanamide 98.5% pure) . Culture medium used as vehicle. Ivett JL et al, Env Mol Mut, 14, 165-187, 1989.	For SCEs 25 h exposure (or 2h + S-9). Dose level limited by solubility, with three highest evaluated. BrdU added after 2 h of incubation. Colcemid added for last 2-2.5 h of incubation. For CA, 7 dose levels, with highest based on solubility. Three highest evaluated for CAs. Cells exposed for 8 h and colcemid added for 2-2.5 h (or 2h + S-9 followed by 8 h before colcemid added. 100-200 cells scored for CAs from the three highest analysable concentrations (0, 15, 20, 25 µg/ml). (Gaps/endoreduplication assessed but not included in analyses or data presented).	No increase in SCEs reported. A weak positive reported at mid dose level in presence of S-9 in first trail, but not repeated in a second trail. Overall reported to be negative. Positive controls MMC (-S-9) and CPA (+ S-9) gave adequate results.	It is likely that TiO ₂ would have been insoluble at the dose levels tested. No information on precipitate formation given. Study was part of validation exercise. Positive results were reported with malaoxon, phenol and sulfisoxazole.
MN in CHO-K5 cells. TiO ₂ from Sigma chemicals (purity not stated). DMSO used as vehicle, up to 1% as final concentration in medium Miller BE et al Env Mol Mut, 26, 240-247, 1995.	Approximately 9000 cells culture for 24 h prior to test material addition. Cells exposed for 48 h (10 concentrations up to 1 µg/ml) (or for 3 h + S-9, 6 concentrations up to 10 µg/ml). Cells washed, fixed, and stained with Giemsa. MN assessed in 1000 cells/culture (minimum 2 cultrures/trial). MN identified as clearly surrounded by nuclear membrane, area less than one third of nucleus, nonrefractility, not linked to main nucleus, location in cytoplasm. Mononucleated cells with fewer than 5 MN and no nuclear fragmentation were scored. Independent experiment undertaken.	No evidence for an increase in MN reported in either presence or absence of exogenous metabolising fraction (Phenobarbital/β-naphthoflavone-induced rats liverS-9). Visible precipitate noted at 0.5 µg/ml (-S-9) and at 1.0 µg/ml (+S-9) Positive control substances (bleomycin, CPA gave adequate results, although it is notable that CPA (5µg/ml gave a similar magnitude of response (12.% and 11.7% cells with MN) to 2mg/ml phenol + S-9).	
CAs and SCEs in CHO-K1 cells. TiO ₂ from Sigma chemicals.(purity not stated). DMSO used as vehicle up to 0.5% in medium. Lu PJ et al Mut Res, 414, 15-20, 1998.	For SCEs, 5x10 ⁵ cells exposed to TiO ₂ (0-5µM) and BrdU (20 µM) for 24h. Colcemid (0.2 µg/ml) added for last 2 h. Metaphase cells collected for SCE evaluation. Cell cycle kinetics evaluated by differential chromatid labelling. For MN logarithmically growing CH-K1 cells exposed to TiO ₂ (0-20 µM) for 18 h. Cells fixed, stained with Giemsa and MN/1000 interphase cells scored. Cytokinesis Block (CB) MN cells exposed to exposed to TiO ₂ (0-20 µM) for 24 h along with 1 µg/ml cytocholasin B. MN/1000 binucleate cells scored. Experiments repeated 3 times. Uptake of TiO ₂ in CHO cells measured through atomic absorption analysis for titanium.	A dose-dependent uptake of Ti was demonstrated which was evident at all concentrations used (i.e. just below 2 µM was lowest concentration used) Authors report that concentrations chosen for SCE/MN analysis were intended to be within the non-cytotoxic range. A statistically significant dose-related increase in SCE and MN was reported in all experiments. An enhancement in the number of MN was reported in the CB MN assay. (see appended figure 2 from publication)	Increase in SCEs reported to be 1.59 fold at 5 µM. Increase in MN in CB assay was 2.5-3 fold at 5-20 µM. Authors considered that the negative study reported by Ivett et al 1989 was probably due to different methods of test sample preparation (i.e. insolubility in the Ivett study). Authors considered that the negative results reported by Miller may have resulted from long exposure time and possibility of MN loss during cell division. Authors speculate that mutagenicity of TiO ₂ observed in-vitro is likely to be due to generation of free radicals.

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<p>Microbial mutagenicity in <i>Salmonella typhimurium</i>. Single cell gel DNA damage and mutation assay with L5178Y cells and CA with Chinese hamster CHL/IU cells.</p> <p>Range of TiO₂ commercial samples used</p> <p>p-25 nanometre material (21 nm), µmetre sized WA and WR samples (255 nm) and TP-3 (420 nm).</p> <p>Test materials suspended in Earle's balanced salt solution (EBSS).</p> <p>Experiments undertaken using either presence or absence of illumination using Uv/visual light designed to give spectra identical to natural light. A range of different energy levels were used.</p> <p>Nakagawa Y et al Mut Res, 394, 125-132, 1997.</p>	<p>Mutation assays were undertaken in <i>Salmonella typhimurium</i> TA100, TA 98, TA 102 by plate incorporation using TiO₂ (p-25 nanometre sample) suspended in distilled water. Plates were irradiated for 10 or 50 mins to give 1 J/cm² or 5 J/cm²</p> <p>L5178Y SCG assay was undertaken using all types of TiO₂ samples. 1x 10⁶ cells (0.5 ml) were incubated with 0. 5 ml of TiO₂ (0- 800 µg/ml for P-25. 0- 3200 µg/ml for other samples) in microwell plates. Following 1 h incubation (37⁰C, 5% CO₂) in the dark. Plates were irradiated with 0.61 J/cm² up to 5 J/cm² for 50 mins. Duplicate trials were kept in dark conditions. Each trial comprised of a single culture at each dose level. After irradiation, the cells were resuspended in pre cooled medium incubated for a further 20 h and processed for SCG DNA damage (lysing solution for 2h, alkaline treatment for 20 mins, electrophoresis 25 V for 25 min visualisation using ethidium bromide. Tail length from 50 nuclei/treatment measured).</p> <p>For mutation assay in 5 X 10⁶ LY5178Y cells were incubated with 0-2mg/ml P-25 TiO₂ for 1 h in the dark followed by 50 mins under varying intensities of irradiation. An expression time of 11-13 days was used with triflourothymidine as the selective agent. A concurrent study without irradiation was not undertaken.</p> <p>For CA, 2x10⁴ CHL/IU cells were incubated with p-25 TiO₂ (0-50 µg/ml (with irradiation, 0-800 µg/ml in absence of irradiation) for 1 h in the dark followed by irradiation for 50 mins (1.25 J/cm² up to 5 J/cm²). After irradiation, the cells were rinsed with EBSS and incubated for 20 h (last 2 h with demecolin 0.1 µg/ml) 100 metaphase spreads (with 22-26 chromosomes were examined for aberrations.</p>	<p>Results for non-irradiated studies are presented first as the remit of the COM review is to look at particles rather than photogenotoxicity.</p> <p>There was no evidence of a mutagenic response in the <i>S. typhimurium</i> assays.</p> <p>There was no evidence of DNA damage in the SCG assays except for sample WA (µmetre sized) where evidence of a dose response and a clear effect at 800 µg/ml were documented.</p> <p>No mammalian cell mutation assay was undertaken using non-irradiated conditions.</p> <p>I</p> <p>There was no evidence for a clastogenic effect in the CA assay using p-25 (nanometre sized).</p> <p><u>Irradiation studies.</u></p> <p>There was no mutagenic effect with p-25 in presence of irradiation in the <i>S. typhimurium</i> assays</p> <p>There was a clear dose related increase in DNA damage as assessed in the SCG assays with all types of TiO₂ samples (i.e. both nanometre and µmetre sized) both with respect to dose of test material and dose level of light energy administered.</p> <p>A dose related increase in CAs was reported in the p-25 study in the presence of irradiation CAs comprised predominantly of chromatid breaks and exchanges. (Ofloxacin was used as a positive control for photogenotoxicity and gave an appropriate response). Trials to evaluate the effect of varying light intensity revealed a statistically significant effect (at 50 ug/ml TiO₂ p-25) only in the presence of 5 J/cm².</p>	<p>The studies undertaken under non-irradiated conditions are interesting in that information for a range of TiO₂ particle sizes is available. However the protocols used could be considered limited both in terms of the methods used to suspend the test material and the test protocols used (in particular limited duration of incubation). Nevertheless there was some evidence of DNA damage with one µmetre sized TiO₂ sample.</p> <p>The authors demonstrated through a number of test systems that irradiated leads to photoexcitation of both nanometre and µmetre sized samples of TiO₂ with result DNA damaging and mutagenic effects.</p>
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<p>SHE Fibroblasts Ultrafine TiO₂ ≤ 20 nm Fine TiO₂ >200 nm</p> <p>Particles suspended in Phos[phate buffered saline 1µg/ml.</p> <p>Rahman Q et al, Env Health perspect, 110, 797-800, 2002</p>	<p>SHE fibroblasts obtained from 13-day-old embryos and cultures at 37°C, 12% CO₂ in modified Dulbecco's Eagle's reinforced medium.</p> <p>Cells were treated with 0.5, 1.0, 5.0, or 10.0 µg/ml for 12, 24, 48, 66 or 72 h.</p> <p>For MN assay, cells were fixed at end of incubation and stored at -20°C for at least 30 minutes. DNA was stained with bisbenzimidazole (1µg/ml for 4 mins). MNs (< than one third of diameter of nucleus. 200 nuclei from each of 3 separate experiments evaluated.</p> <p>For kinetochore staining, fixed cell preparations were stained with CREST at 37°C for 1h and incubated with FITC conjugated goat anti-human antibodies before applying bisbenzimidazole. 100 MN/dose level examined for kinetochore staining.</p> <p>TEM was undertaken for cells exposed for 48 and 66 h to 5 or 10 µg/ml or 100 µM cisplatin. DNA fragmentation during apoptosis was also measured</p>	<p>A statistically significant increase in MN was documented with ultrafine TiO₂ after 24 and 48 h exposure at 1.0 and 5.0 µg/ml. A statistically significant increase in Mn was also reported at 1.0 µg/ml at 66 and 72 h exposure (non-statistically significant increase at 5.0 µg/ml).</p> <p>Authors reported cytotoxicity at 10 µg/ml.</p> <p>No increase in MN reported in studies using Fine TiO₂ (but data were not presented)</p> <p>Authors report that there was no significant increase in kinetochore positive staining MN.</p> <p>Bisbenzimidazole staining of cells revealed evidence for the induction of apoptosis. TEM evaluations showed compaction and marginalisation of chromatin</p>	<p>These data provide evidence that ultrafine TiO₂ was mutagenic in-vitro whilst a µmetre sized TiO₂ sample was not. The secretariat have attempted to obtain further details of the results of the studies with µmetre sized TiO₂ from the authors. No information precipitation of test materials was provided.</p> <p>(Previous publication presented validation results for test system. Out of 48 carcinogens, 41 (85%) were positive and all 17 non carcinogens tested yielded negative results. Fritzenschaf H et al, Mut Res, 319, 47-53, 1993.)</p>
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- Size information for many of the chemicals supplied to laboratories were not presented in the published papers. Specifications obtained from the Sigma-Aldrich internet site indicate that a research material with 99.9% below 5µm is available. It is reasonable to assume that unless specified very little material used would have been <100 nm A nanopsecification product is also available. Size information was produced for Nagawa Y et al paper which included both nano- and µmetre sized test materials.

In-vivo mutagenicity data

3. In this study TiO₂ (NTP repository sample, size specification not given) was administered in corn oil by i.p injection on three successive days and bone-marrow samples and peripheral blood obtained 24 hours after the last dose. The initial test undertaken by Shelby was positive by trend analysis to 1000 mg/kg bw with the high dose group significantly elevated (1000 mg/kg bw). A repeat study using the a high dose of 1500 mg/kg bw was trend negative as were results from scoring blood samples from the dose-determination study. However because of elevated MN-PCE value in the peripheral blood samples at 1000 mg/kg and increasing frequencies of MN-PCE in the low and middle (1000 ,g/kg) dose groups of the repeat test, overall results were concluded to be positive.