Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

		Official use only
	187 REFERENCE	
1.1 Reference	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Burki, H.R. and Okita, G.T. (1969). Effect of oral copper sulfate on 7,12-dimethylbenz(**α**)anthracene carcinogenesis in mice. Br. J. Cancer Sep; **23(3):** 591-596 (published).	
1.2 Data protection	No *(indicate if data protection is claimed)*	
1.2.1 Data owner	*Give name of company* Public domain.	
1.2.2 Companies with letter of access	*Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)* Letter of access not required.	
1.2.3 Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* No data protection claimed.	
	188 GUIDELINES AND QUALITY ASSURANCE	
188.1 Guideline study	No. This was a non-regulatory study designed to investigate the effects of oral $CuSO_4$ on the incidence of 7,12-dimethylbenz(**α**)anthracene (DMBA) induced ovarian tumours, tumours of the breast and lymphomas in C57BL/6J mice and of tumours of the lung in strain A mice. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
188.2 GLP	No. This was a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
188.3 Deviations	Yes. Refer to section 5.3.6 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
	189 MATERIALS AND METHODS	
189.1 Test material	*In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.* Cu^{2+} as $CuSO_4$ *or give name used in study report*	

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

189.1.1 Lot/Batch number	*List lot/batch number if available*	
189.1.2 Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	
189.1.3 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Copper sulphate ($CuSO_4.5H_2O$).	
189.1.4 Purity	*Give purity in % active substance* [redacted]	X
189.1.5 Stability	*Describe stability of test material* Not stated.	
189.2 Test Animals	Non-entry field	
189.2.1 Species	Mouse	
189.2.2 Strain	C57BL/6J mice (59 intact virgins and 65 pseudopregnant females) were used to investigate the incidence of ovarian tumours, tumours of the breast and lymphomas. Strain A mice (50 animals bred by brother-sister mating) were used to investigate tumours of the lung.	
189.2.3 Source	The Jackson Laboratory, Bar Harbor, Maine.	
189.2.4 Sex	Female.	
189.2.5 Age/weight at study initiation	*Experiment A:* 4 – 6 months. *Experiment B:* 12 – 15 weeks. *Experiment C:* 12 – 16 weeks. *Experiment D:* Not stated.	
189.2.6 Number of animals per group	*Experiment A:* Five C57BL/6J virgins were injected i.v. with 0.75 mg DMBA. Five C57BL/6J virgins were injected i.v. with 0.75 mg DMBA and received $CuSO_4$ in drinking water. *Experiment B:* Eleven C57BL/6J virgins were injected i.v. with 0.75 mg DMBA. Eleven C57BL/6J virgins were injected i.v. with 0.75 mg DMBA and received $CuSO_4$ in drinking water. *Experiment C:* Ten strain A virgins were injected i.v. once with 0.75 mg DMBA and, 12 days later, with 0.5 mg DMBA i.p. Nine strain A virgins received 0.75 mg DMBA i.v. , 0.5 mg DMBA i.p. and $CuCO_4$ in their drinking water. *Experiment D:* Nineteen C57BL/6J pseudopregnant females received 6 skin paintings of 0.5 ml of a 0.5% DMBA solution in olive oil at biweekly intervals.	

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of**
IUCLID: 5.4/12 & 5.7/02	**copper**
	Eighteen C57BL/6J pseudopregnant females received 6 DMBA skin paintings and $CuSO_4$ in the drinking water.
189.2.6.1 at interim sacrifice	Not applicable.
189.2.6.2 at terminal sacrifice	Refer to section 3.2.6.
189.2.7 Control animals	Experiment A: Five C57BL/6J mice. Experiment B: Ten untreated C57BL/6J mice and 12 C57BL/6J mice fed $CuSO_4$. Experiment C: Nineteen untreated strain A mice and 12 strain A mice fed $CuSO_4$. Experiment D: Eleven untreated C57BL/6J mice and 17 $CuSO_4$-fed pseudopregnant mice.
189.3 Administration/ Exposure	DMBA: dermal, intraperitoneal and intravenous. $CuSO_4$: Oral (in drinking water). *Fill in respective route in the following, delete other routes*
189.3.1 Duration of treatment	Experiment A: Terminated 74 weeks after DMBA treatment. Experiment B: Terminated 44 weeks after DMBA treatment. Experiment C: Terminated 33 weeks after first DMBA application. Experiment D: Terminated 50 weeks after first skin painting with DMBA.
189.3.2 Interim sacrifice(s)	None.
189.3.3 Final sacrifice	Refer to section 3.3.1.
189.3.4 Frequency of exposure	*Experiment A:* A single DMBA injection was administered i.v. to test animals. Relevant groups were started on $CuSO_4$ treatment 2 weeks before administration of DMBA. Feeding of $CuSO_4$ continued throughout the entire experimental period. These animals had access to the $CuSO_4$ solution *ad libitum*. *Experiment B:* A single DMBA injection was administered i.v. to test animals. Relevant groups were started on $CuSO_4$ treatment 2 weeks before administration of DMBA. Feeding of $CuSO_4$ continued throughout the entire experimental period. These animals had access to the $CuSO_4$ solution *ad libitum*. *Experiment C:* DMBA was administered once i.v. and, 12 days later, once i.p. Relevant groups were started on $CuSO_4$ treatment 2 weeks before the first application of DMBA. Feeding of $CuSO_4$ continued throughout the entire experimental period. These animals had access to the $CuSO_4$ solution *ad libitum*. *Experiment D:* Six dermal paintings of DMBA were administered at biweekly intervals. Relevant groups were started on $CuSO_4$ treatment 2 weeks before the

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of**
IUCLID: 5.4/12 & 5.7/02	**copper**

	first application of DMBA. Feeding of $CuSO_4$ continued throughout the entire experimental period. These animals had access to the $CuSO_4$ solution *ad libitum*.		
189.3.5 Postexposure period	None.		
	Oral		
189.3.6 Type	$CuSO_4$ was administered in drinking water.		
189.3.7 Concentration	$CuSO_4$ was dissolved in water to a concentration of 198 mg/l (approximately 50 mg Cu^{2+}/l). Treatment water was supplied *ad libitum*.		
189.3.8 Vehicle	Tap water.		
189.3.9 Concentration in vehicle	$CuSO_4$ was dissolved in water to a concentration of 198 mg/l (approximately 50 mg Cu^{2+}/l).		
189.3.10 Total volume applied	Not stated.		
189.3.11 Controls	Vehicle (water).		
	Dermal		
189.3.12 Area covered	Not stated.		
189.3.13 Occlusion	Not stated.		
189.3.14 Vehicle	Olive oil.		
189.3.15 Concentration in vehicle	5 mg/ml		
189.3.16 Total volume applied	(0.5%). 0.5 ml.		
189.3.17 Duration of exposure	Not stated.		
189.3.18 Removal of test substance	Not stated. *give solvent, detergent*		
189.3.19 Controls	Untreated.		
	Intraperitoneal/Intravenous/Intratracheal instillation		
189.3.20 Vehicle	For parenteral administration, a fatty emulsion of DMBA was produced in 1.2% w/w lecithin, 0.3% w/v poloxalkol, 15% cottonseed oil and water.	X	
189.3.21 Concentration in vehicle	0.5% w/v DMBA.		X
189.3.22 Total volume applied	0.1 or 0.15 ml.		
189.3.23 Controls	Untreated.		

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

189.4 Examinations			
189.4.1 Body weight	No.		
189.4.2 Food consumption	No.		
189.4.3 Water consumption	No.		
189.4.4 Clinical signs	No.		
189.4.5 Macroscopic investigations	Not reported.		
189.4.6 Ophthalmoscopic examination	No.		
189.4.7 Haematology	No.		
189.4.8 Clinical Chemistry	No.		
189.4.9 Urinalysis	No.		
189.4.10 Pathology	No.		
189.4.10.1 Organ Weights	No.		
189.4.11 Histopathology	Yes.		
	from:	all dose groups	
	from:	all surviving animals and all animals that died during the study.	
	Organs:	Thymus, liver, kidneys, spleen, ovaries.	X
Other examinations	*E.g. enzyme induction, cell proliferation, reversibility of effects*		
	Vaginal smears for investigation of effects on the incidence of oestrus. Chi-square test and Wilcoxon ranking test were applied as appropriate.		
189.5 Statistics			
189.6 Further remarks	Pseudopregnant females refers to virgin mice housed together with vasectomised males. Vasectomy was performed under pentobarbital anaesthesia (70 mg/kg). Each group consisted of 3-4 virgins and 1-2 vasectomised males per cage.		

190 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

190.1 Results

No effects / describe significant effects referring to data in results table

Non-entry field.

190.1.1 Experiments A & B The results of Experiments A and B are shown in **Table A6.5(02) & A6.7(02)-1**.

Histopathology:

A single application of 0.75 mg DMBA caused a high incidence of ovarian tumours in C75BL/6J virgin mice. These tumours varied in size from 8 – 15 mm in diameter, and were classified histologically as granulosa cell tumours. Mice receiving the combination of DMBA and

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
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	$CuSO_4$ showed a lower incidence of ovarian tumours than those treated with DMBA alone. Histologically, the ovaries of all mice injected with DMBA showed similar precancerous changes, as evidenced by the destruction of oocytes and loss of follicular structure. However, addition of $CuSO_4$ to the diet appears to delay progression of precancerous lesions to frank ovarian tumours. Feeding of $CuSO_4$ to DMBA-treated females appeared to increase the incidence of lymphomas in Experiment A, but not in Experiment B. *Other examinations:* The incidence of oestrus, 20 – 22 weeks after DMBA application, was significantly elevated ($P<0.25$, chi-square test) to 60% oestrus in DMBA-treated females, compared to 51% for solvent controls and 50% for $CuSO_4$ controls.
190.1.2 Experiment C	The results of Experiment C are shown in **Table A6.5(02) & A6.7(02)-2** *Histopathology:* The feeding of $CuSO_4$ had no effect on the incidence of DMBAinduced adenomas of the lung. However, the total number of all tumours observed in the group treated with DMBA and $CuSO_4$ was only 8, compared to 16 in the group receiving DMBA only. *Other examinations:* $CuSO_4$ added to the diet appeared to prolong the survival of DMBA-treated mice ($P<0.025$).
190.1.3 Experiment D	The results of Experiment D are shown in **Table A6.5(02) & A6.7(02)-3** *Histopathology*: Animals that received both $CuSO_4$ and DMBA had a greater cumulative number of breast tumours than those receiving DMBA only. No effort was made to count skin tumours, as many non-malignant lesions were also observed after skin-painting with DMBA. *Other examinations*: When $CuSO_4$ was added to the diet of DMBA-treated mice, the mean survival time increased to 25 weeks in comparison with 21 weeks for animals treated only with DMBA ($P<0.05$, Wilcoxon ranking test).
190.2 Discussion	*No effects / describe significant effects referring to data in results table* This study was carried out to investigate the incidence of DMBAinduced tumours in mice kept on a diet supplemented with $CuSO_4$. It was shown in Experiments A and B that one injection of 0.75 mg DMBA induced ovarian tumours in nearly all C57BL/6J virgin females within 44 weeks. Conversely, $CuSO_4$ added to the diet of DMBAtreated females appeared to reduce the incidence of ovarian tumours and to prevent the increased incidence in oestrus observed in DMBAtreated females. However, all ovaries of mice treated with DMBA + $CuSO_4$ showed pre-cancerous changes, indicating that $CuSO_4$ had no

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
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effect on the initiation step of DMBA oncogenesis. Instead, it appeared that the greater availability of copper in the body delayed the full expression of the carcinogenic lesions induced by DMBA.

In Experiment A, it was observed that the incidence of lymphomas were greater in DMBA + $CuSO_4$-treated mice than in those receiving DMBA only. However, this finding could not be repeated in subsequent experiments (Experiment B), and it was concluded that $CuSO_4$ had no effect on the induction of lymphomas by DMBA.

$CuSO_4$ did not alter the incidence of adenomas of the lung in DMBA-treated strain A females (Experiment C).

The increased incidence of breast tumours observed in $CuSO_4$-fed pseudopregnant C57BL/6J mice receiving DMBA skin paintings (Experiment D) may have been related to the prolonged survival observed in this group, compared to animals treated only with DMBA skin paintings. The increased survival in strain A mice treated with DMBA + $CuSO_4$, compared to animals receiving DMBA only, is unexplained (Experiment C).

No toxic effects were observed in otherwise untreated mice fed $CuSO_4$ at the concentration used in these experiments.

190.3 Time to tumours *For dermal route and skin tumours: give mean time until appearance of tumour or time until appearance of first tumour or other measure*

The cumulative number of breast tumours occurring over time in pseudopregnant females treated with 6 skin paintings of DMBA are shown in **Table A6.5(02) & A6.7(02)-3**.

190.4 Other *Describe any other significant effects*

None.

191 APPLICANT'S SUMMARY AND CONCLUSION

191.1 Materials and methods *Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines*

A study was carried out to investigate the effects of oral $CuSO_4$ on the incidence of 7,12-dimethylbenz(**α**)anthracene (DMBA) induced ovarian tumours, tumours of the breast and lymphomas in C57BL/6J mice and of tumours of the lung in strain A mice. The study was divided into four separate experiments, designated A, B, C and D.

In all cases, $CuSO_4$ was dissolved in drinking water at a concentration of 198 mg/l (equivalent to approximately 50 mg Cu^{2+}/l). $CuSO_4$-treated animals had access to the solution *ad libitum* over the entire experimental period.

Experiment A: $CuSO_4$ was administered in the drinking water of 5 female mice (C57BL/6J) aged 4 – 6 months. Two weeks after commencement of copper treatment, the mice received an intravenous (i.v.) injection of 0.75 mg dimethylbenz(**α**)anthracene (DMBA), a known carcinogen. A second group of 5 mice received DMBA alone. Five untreated mice served as controls. The experiment was terminated

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

~~74 weeks after DMBA treatment.~~

Experiment B: $CuSO_4$ was administered in the drinking water of 11 female mice (C57BL/6J) aged 12 – 15 weeks. After commencement of copper treatment, the mice received an i.v. injection of 0.75 mg DMBA. A second group of 11 mice received DMBA alone. Ten untreated mice and 12 mice receiving $CuSO_4$ served as controls. The experiment was terminated 44 weeks after DMBA treatment.

Experiment C: $CuSO_4$ was administered in the drinking water of 9 female mice (strain A) aged 12 – 16 weeks. After commencement of the copper treatment, the mice received an i.v. injection of 0.75 mg DMBA and, 12 days later, an intraperitoneal (i.p.) injection of 0.5 mg DMBA. Ten other mice received 0.75 mg DMBA i.v., and 0.5 mg DMBA i.p only. Nineteen untreated mice and 12 mice receiving $CuSO_4$ served as controls. The experiment was terminated 33 weeks after the first DMBA treatment.

Experiment D: $CuSO_4$ was administered in the drinking water of eighteen pseudopregnant C57BL/6J female mice (i.e. virgins housed with vasectomised males), each of which also received 6 dermal applications of 0.5 ml of a 0.5% DMBA solution in olive oil at biweekly intervals. A separate group of 19 pseudopregnant females received dermal applications of DMBA, but did not receive $CuSO_4$ in their drinking water. Eleven untreated mice and 17 pseudopregnant mice receiving $CuSO_4$ served as controls. The experiment was terminated 50 weeks after the first DMBA treatment.

Animals in all experiments were observed daily. All mice found dead and those sacrificed were subject to post-mortem evaluation. Sections of the liver, lung, kidney, spleen, thymus, ovaries and all tumour-like structures were fixed in 10% formalin in phosphate buffer at pH 7.4. Specimens were embedded in wax, sectioned for light microscopy and stained by haematoxylin and eosin. Vaginal smears were also taken and stained with Wright's stain.

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

191.2 Results and discussion	*Summarize relevant results; discuss dose-response relationship.* *Experiments A and B:* The incidences of ovarian tumours in Experiment A after 76 weeks were 0/5, 4/5, and 0/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu-treated mice, respectively. The incidences of these tumours in Experiment B after 46 weeks were 0/10, 0/12, 11/11 and 6/11 in the untreated controls, copper-treated mice, DMBA-treated mice and DMBA/copper treated mice respectively. The results of these two experiments suggested that $CuSO_4$ may inhibit DMBA-induced tumour development. The incidences of lymphomas in Experiment A were 0/5, 1/5, and 5/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu treated mice respectively. Although these results implied that incidence of lymphomas were greater in DMBA plus $CuSO_4$-treated mice than in those receiving DMBA only, this finding could not be repeated in Experiment B (incidences of lymphoma 1/10, 2/12, 3/11 and 3/11 in the untreated controls, Cu-treated mice, DMBA-treated mice and DMBA plus Cu-treated mice, respectively). It was therefore concluded that $CuSO_4$ had no effect on the induction of lymphomas by DMBA. *Experiment C:* Tumour incidence in the 12 mice given $CuSO_4$ alone (1 breast tumour, 2 lymphomas and no lung or ovarian tumours) was similar to that in the 19 untreated controls (2 lymphomas, no breast, lung or ovarian tumours). $CuSO_4$ had no effect on the incidence of DMBA-induced lung adenomas (incidence 4/9 in DMBA plus Cutreated mice and 4/10 in mice treated with DMBA only), although it appeared to prolong the survival of DMBA-treated mice (mean survival 28 weeks compared with 19 weeks in mice treated with DMBA only), and to slightly reduce the total number of tumours seen, as compared with mice given DMBA only. *Experiment D:* No information was given on the tumour incidence in mice given $CuSO_4$ alone. However, mice given DMBA plus $CuSO_4$ had a greater number of mammary tumours (9 tumours amongst an original group of 18) than those given DMBA alone (5 tumours amongst an original group of 19). This increase was attributed to the greater longevity of Cu-treated mice. No toxic effects were observed in otherwise untreated mice fed $CuSO_4$ at the concentration used in these four experiments.
191.3 Conclusion	DMBA was injected or administered by skin paintings to C57BL/6J and to strain A female mice kept on a diet supplemented with $CuSO_4$. It was found that $CuSO_4$ had no effect on the incidence of DMBAinduced adenomas of the lung, lymphomas and breast tumours. $CuSO_4$ did not prevent the induction of pre-cancerous lesions in the ovary, but may have delayed the development of granulosa cell tumours.
191.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4*

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	
191.3.2 Deficiencies	Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP. There were also a number of deficiencies in the methodology used, when compared with the requirements of currently accepted guidelines for the conduct of carcinogenicity studies (e.g. OECD 451), including the following:

- The test substance was not characterised in detail;
- The number of animals per test group was smaller than recommended;
- Only a single $CuSO_4$ test concentration was used;
- The range of tissues examined macroscopically was limited;
- The range of tissues examined microscopically was limited;
- Body and organ weights were not reported;
- No blood sampling was reported for adversely affected animals;
- Feed and water consumption were not reported;
- Study duration was shorter than recommended (33 – 74 weeks, rather than the recommended 2 years).

However, these deficiencies do not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the carcinogenicity of copper.

Overall, this is an adequately-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper carcinogenicity. A reliability indicator of 2 has been assigned on this basis.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	

Evaluation by Competent Authorities
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

Guidelines and quality assurance

Materials and Methods

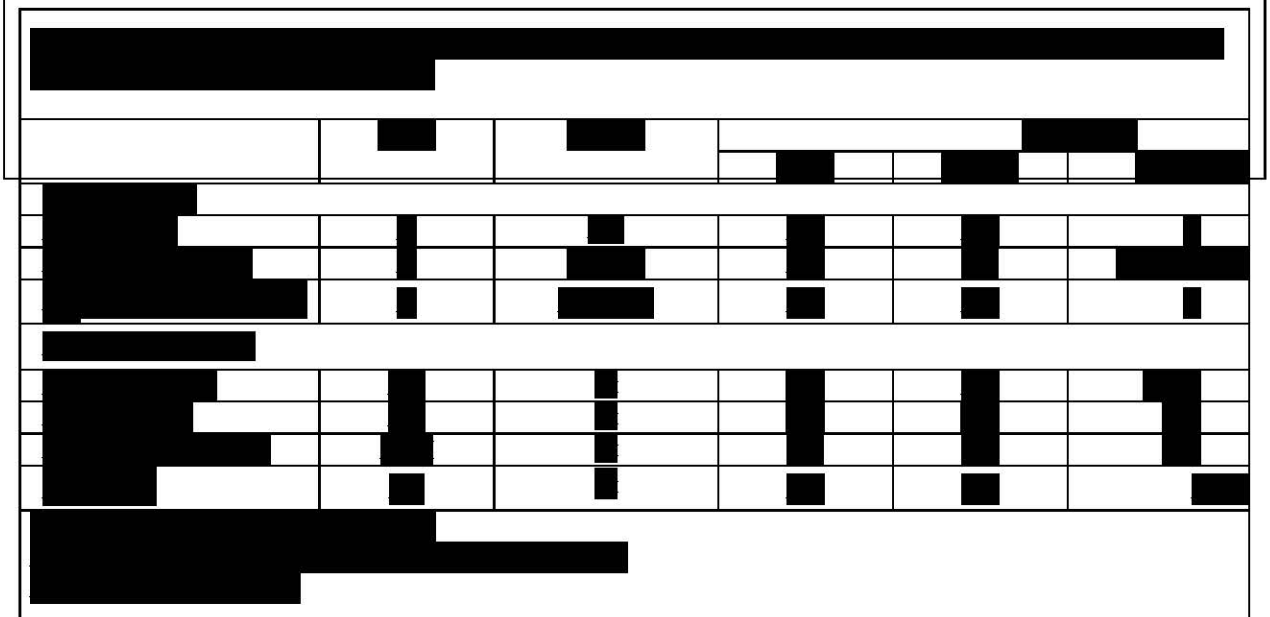
Results and discussion

Conclusion

Reliability

Acceptability

Remarks



COMMENTS FROM ...

Date *Give date of comments submitted*

Materials and Methods *Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Discuss if deviating from view of rapporteur member state

Results and discussion *Discuss if deviating from view of rapporteur member state*

Conclusion *Discuss if deviating from view of rapporteur member state*

Reliability *Discuss if deviating from view of rapporteur member state*

Table A6.5(02) & A6.7(02)-1 Effect of Oral Copper Sulphate on Incidence of DMBA-induced Tumours in C57BL/6J Female Mice.

	Number of Mice	Survival Weeks	Mice With Tumours		
			Ovary	Lymphomas	Other Tumours
Experiment A					
Controls	5	74*	0/5	0/5	--
DMBA i.v.#	5	47-74	4/5	1/5	1 papilloma (skin)
DMBA i.v.# + $CuSO_4$	5	52-67	0/5	5/5	--
Experiment B					
Controls	10	44*	0/10	1/10	--
$CuSO_4$ **	12	44*	0/12	2/12	--
DMBA i.v.#	11	44*	11/11	3/11	--
DMBA i.v.# + $CuSO_4$	11	44*	6/11	3/11	1 leukaemia

* Mice were killed
# 0.75 mg DMBA i.v.
** $CuSO_4$ in the drinking water (50 mg Cu^{2+}/litre)

Table A6.5(02) & A6.7(02)-2 Effect of Oral Copper Sulphate on Incidence of DMBA-induced Tumours in C57BL/6J Female Mice (Experiment C).

Groups	Number of Mice	Survival Weeks	Mice With Tumours		
			Lung	Ovary	Other Tumours
Controls	19	33*	0/19	0/19	2 lymphomas
$CuSO_4$ **	12	33*	0/12	0/12	2 lymphomas 1 breast tumour
DMBA i.v.#	10	19	4/10	5/10	2 lymphomas 2 breast tumours 1 hepatoma 2 papillomas (skin)
DMBA i.v.# + $CuSO_4$ **	9	28##	4/9	3/9	1 lymphoma

* Mice were killed
** $CuSO_4$ in the drinking water (50 mg Cu^{2+}/litre)
# 0.75 mg DMBA i.v.
$P < 0.025$ compared to group 3 (Wilcoxon ranking test)

Table A6.5(02) & A6.7(02)-3 Effect of Oral Copper Sulphate on Incidence of Breast Tumours in Pseudopregnant Females Treated with 6 Skin Painitings of DMBA (Experiment D).

Weeks After Treatment	Group 3 DMBA#		Group 4 DMBA i.v.# + $CuSO_4$*	
	Survivors	Cumulative number of breast tumours	Survivors	Cumulative number of breast tumours
0	19	0	18	0
16	17	2	18	2
20	11	2	17	6
25	8	4	9	6
30	4	4	7	6
40	0	5	2	9

Last skin painting with DMBA 10 weeks after start of experiment
* $CuSO_4$ in the drinking water (50 mg Cu_{2+}/litre)

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

			Official use only
		192 REFERENCE	
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Harrison, J.W.E., Levin, S.E. and Trabin, B., (1954). The Safety and Fate of Potassium Sodium Copper Chlorophyllin and Other Copper Compounds. Journal of the American Pharmaceutical Association, **43(12):** 722-737 (published).	
1.2	**Data protection**	No *(indicate if data protection is claimed)*	
1.2.1	Data owner	*Give name of company* Public domain.	
1.2.2	Companies with letter of access	*Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)* Letter of access not required.	
1.2.3	Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* No data protection claimed.	
		193 GUIDELINES AND QUALITY ASSURANCE	
193.1	**Guideline study**	No. This was a non-regulatory study designed to investigate the chronic toxicity on rats of potassium sodium copper chlorophyllin, copper sulphate (anhydrous) and copper gluconate. For the purposes of this summary, only information relevant to the chronic toxicity of copper sulphate is presented herein. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
193.2	**GLP**	No. This was a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed. *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
193.3	**Deviations**	Yes. Refer to section 5.3.6 for a general discussion of deviations and deficiencies. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
		194 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.	

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

194.1 Test material

Cu^{2+} as copper sulphate ($CuSO_4$).

or give name used in study report

194.1.1 Lot/Batch number

Not stated.

List lot/batch number if available

194.1.2 Specification

Deviating from specification given in section 2 as follows

(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

194.1.3 Description

If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)

Refer to section 2.1.

X

194.1.4 Purity

Give purity in % active substance

[redacted]

194.1.5 Stability

Describe stability of test material

Not stated.

Non-entry field

194.2 Test Animals

194.2.1 Species

Rat

194.2.2 Strain

Sprague-Dawley

194.2.3 Source

Not stated.

194.2.4 Sex

Male and female.

194.2.5 Age/weight at study initiation

Initial bodyweights of weanling rats were as follows:

Group	Males (grams)	Females (grams)
Controls	81 ± 2.3	73 ± 2.3
0.135% $CuSO_4$ in the diet (530 ppm Cu).	72 ± 3.4	67 ± 3.3
1.406% $CuSO_4$ in the diet (1600 ppm Cu).	71 ± 9.3	73 ± 2.2

Changes in bodyweight over the course of the study are shown in **Table A6.5(03) & A6.7(03)-1**.

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

194.2.6 Number of animals per group

Group	Males	Females
Controls	23	25
0.135% $CuSO_4$ in the diet (530 ppm Cu)	25	25
1.406% $CuSO_4$ in the diet (1600 ppm Cu)	23	25

194.2.6.1 at interim sacrifice: Refer to **Table A6.5(03) & A6.7(03)-2**.

194.2.6.2 at terminal sacrifice: Not stated.

194.2.7 Control animals: Control animals received the basal diet only.

194.3 Administration/ Exposure: Oral in the diet.

Fill in respective route in the following, delete other routes

194.3.1 Duration of treatment: All surviving animals of all groups were sacrificed at weeks forty to forty-four.

194.3.2 Interim sacrifice(s): None.

194.3.3 Final sacrifice: Refer to section 2.3.1.

194.3.4 Frequency of exposure: 7 days a week

194.3.5 Postexposure period: None.

Oral

194.3.6 Type: $CuSO_4$ was administered in the diet.

194.3.7 Concentration: Not applicable.

194.3.8 Vehicle: Basal diet.

194.3.9 Concentration in vehicle: Treatment group animals received diets containing Cu at one of the following concentrations:
1600 ppm Cu as $CuSO_4$ (1.406% $CuSO_4$).
530 ppm Cu as $CuSO_4$ (0.135% $CuSO_4$).

194.3.10 Total volume applied: Not stated.

194.3.11 Controls: Controls received basal diet only.

194.4 Examinations

194.4.1 Body weight: Yes.

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

194.4.2 Food consumption	Yes.			
194.4.3 Water consumption	No.			X
194.4.4 Clinical signs	Yes.			
194.4.5 Macroscopic investigations	Yes.			
194.4.6 Ophthalmoscopic examination	No.			
194.4.7 Haematology	Yes			
	Number of animals:	Not stated.		
	Time points:	Unspecified intervals.		
	Parameters:	Routine examinations.		
	Other:	Oxygen carrying capacity.		
194.4.8 Clinical Chemistry	No			
	Number of animals:	None.		
	Time points:	None.		
	Parameters:	None.		
	Other:	None.		
194.4.9 Urinalysis	Yes			
	Number of animals:	Not stated.		
	Time points:	Unspecified intervals.		
	Parameters:	Routine examinations.		
	Other:	None.		
194.4.10 Pathology	Yes.			
194.4.10.1 Organ Weights	Yes			
	from:	at interim sacrifice, at terminal sacrifice		
	Organs:	Liver, kidneys, testes, seminal vesicles, uterus, ovaries, spleen, brain, heart, lungs, stomach, brain.		
	Other:	None.		
194.4.10.2 Histopathology	Yes			
	from:	High dose group animals sacrificed at 30 – 35 weeks and also on the liver, kidneys and testes of animals receiving the lower level after 40 – 44 weeks.		
	Organs:	Spleen, adrenals, small intestine, large intestine, stomach, sciatic nerve, kidney, liver, testes, ovaries.		
	Other:	None.		

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

194.4.11 Other examinations *E.g. enzyme induction, cell proliferation, reversibility of effects*

Tissue-stored copper and iron were determined in the liver, kidneys and some spleens of animals from all groups.

194.5 Statistics

Simple statistical methods were applied, as appropriate.

194.6 Further remarks

In order to maintain a reasonably consistent ratio of copper sulphate intake per gram of animal weight over the duration of the study, a movable percentage in the diet was maintained. During the first 14 days on test when food intake is highest per gram of animal weight, 25% of the stated concentrations were fed; during the second 14 days, 50% of the stated concentrations were fed; thereafter for the balance of the study, 100% of the stated concentrations were fed.

195 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

195.1 Results

No effects / describe significant effects referring to data in results table

Non-entry field.

195.1.1 Body weight

The growth of animals on the high level of $CuSO_4$ was adversely affected by treatment (**Table A6.5(03) & A6.7(03)-1**). This retardation became readily discernible at the 26th week, when the male control animals and the animals receiving 530 ppm Cu weighed at least 50% more than those animals upon the 1600 ppm Cu intake.

195.1.2 Blood & urine examinations.

All factors examined were within normal expected ranges, except blood nonprotein (NPN) nitrogen levels. High NPN (83 mg. %) was noted in males receiving 1600 ppm Cu. Males receiving 530 ppm Cu and females from both treatment groups were just above the expected range of 60 – 70 mg. % of NPN.

Gasometric determinations of the oxygen-carrying capacity of the blood compared satisfactorily with the haemoglobin value determined by the iron and acid haematin methods.

195.1.3 Organ weights

Other than stomachs of female animals in the 1600 ppm group, the average weight of the various organs per 100 g bodyweight were within the expected ranges, when compared with controls of the same age (**Table A6.5(03) & A6.7(03)-2**).

195.1.4 Gross pathology

The following findings were common in animals in the 1600 ppm group: Bronzed kidneys exhibiting sharp demarcation between the cortex and the medulla; bronzed or yellowish livers; hypertrophied ridges between the cardiac and peptic portions of the stomach, occasional ulcers and some blood; bloody mucous in the intestinal tract.

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	
195.1.5 Histopathology	Histopathological studies were performed on the organs of test animals receiving the high level of $CuSO_4$ (sacrificed at 30 to 35 weeks), and also on the liver, kidney and testes of animals receiving the lower level of $CuSO_4$ (sacrificed at 40 to 44 weeks). The following organs were found to be normal in all the test and control animals: Spleen; adrenals; small intestine; large intestine; stomach; sciatic nerve. Kidney sections of animals receiving the high level of $CuSO_4$ showed minor changes which did not correlate well enough throughout the animals to draw a definite conclusion. Liver sections of animals receiving the high level of $CuSO_4$ showed well-defined abnormalities of a toxic nature in both males and females; their icteric pigmentation was increased and cytoplasmic staining properties were abnormal. Varying degrees of testicular degeneration were noted in both the high and low $CuSO_4$ treatment levels; the ovaries of the females were not noticeably affected to any degree. The kidneys, liver and testes of all the control animals were found to be normal.
195.1.6 Tissue-stored copper	The liver, kidneys and some spleens of animals from all groups were examined as to their total Cu and Fe content (**Table A6.5(03) & A6.7(03)-3**). Liver Cu averaged less than 2 mg/100 g of tissue in control animals. Animals receiving 530 ppm Cu as $CuSO_4$ for 40 weeks had Cu concentrations of 12.47 and 32.36 mg/100g liver in males and females, respectively. Those on the 1600 ppm diet for a similar duration had concentrations of 38.28 and 45.77 mg Cu/100g liver. Cu storage in the kidneys of animals receiving 530 ppm Cu was somewhat higher than that of control animals, while that in animals receiving 1600 ppm Cu was higher again.
195.2 Discussion	*No effects / describe significant effects referring to data in results table* This study confirmed that high doses of Cu as $CuSO_4$ cause metal toxicity in albino rats, increased storage of Cu (especially in the liver, kidney and spleen), damage to these organs, and high mortality.
195.3 Time to tumours	*For dermal route and skin tumours: give mean time until appearance of tumour or time until appearance of first tumour or other measure* No tumours were reported in any test animal.
195.4 Other	*Describe any other significant effects* None.

196 APPLICANT'S SUMMARY AND CONCLUSION

196.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to investigate the chronic toxicity to rats of potassium sodium copper chlorophyllin, copper sulphate (anhydrous) and copper gluconate. However, for the purposes of this summary, only information relevant to the chronic toxicity and carcinogenicity of copper sulphate is presented.

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

Two groups of individually housed, weanling Sprague-Dawley rats received diets supplemented with anhydrous $CuSO_4$, giving dietary Cu concentrations of 530 ppm (0.135%) and 1600 ppm (1.147%). A third control group received the basal diet only. Each test group contained approximately 50 animals, equally divided between the sexes. In order to maintain a reasonably consistent ratio of copper sulphate intake per gram of animal weight over the duration of the study, a movable percentage in the diet was maintained. During the first 14 days on test when food intake was highest per gram of animal weight, 25% of the stated concentrations were fed; during the second 14 days, 50% of the stated concentrations were fed; thereafter for the balance of the study, 100% of the stated concentrations were fed. X

The maximum duration of the study was 44 weeks. The weight of each animal was determined weekly, as well as the amount of food and water consumed. Animals were individually inspected at least three times each week. An interim sacrifice was carried out at 33 weeks in which 4 animals from the control group and 4 animals from the group fed 1600 ppm Cu were sacrificed. The balance of the animals were continued in the study, and all surviving animals of all groups were sacrificed at 40 – 44 weeks.

Factors investigated in this study included growth (weight gain); blood and urine examinations; organ weights; gross pathology; histopathology and tissue storage of Cu. After dry-ashing at 525°C, determination of tissue Cu content was by the diethylthiocarbamate procedure.

196.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Bodyweight: The growth of animals on the high level of $CuSO_4$ was adversely affected by treatment. This was readily discernible at the 26th week, when male control animals and animals receiving 530 ppm Cu weighed at least 50% more than animals on the 1600 ppm Cu intake.

Blood and urine examinations: All factors examined were within normal expected ranges, except blood nonprotein (NPN) nitrogen levels, which were high (83 mg. %) in males receiving 1600 ppm Cu. Levels in males receiving 530 ppm Cu and females from both treatment groups were just above the expected range of 60 – 70 mg. % of NPN.

Organ weights: Other than consistently elevated weights for stomachs of female animals in the 1600 ppm group, the average weights of the various organs per 100 g bodyweight were within the expected ranges, when compared with controls of the same age. Other organs examined were heart, lungs, liver, spleen, kidneys, uterus, ovaries, seminal vesicles, testes and brain.

Gross pathology: The following findings were common in animals in the 1600 ppm group: Bronzed kidneys exhibiting sharp demarcation between the cortex and the medulla; bronzed or yellowish livers; hypertrophied ridges between the cardiac and peptic portions of the

stomach, occasional ulcers and some blood; bloody mucous in the intestinal tract. No treatment-related adverse findings were reported for animals in either the control or 530 ppm treatment group. No grossly

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

	obvious neoplasms were reported. *Histopathology:* Histopathology was performed on the organs of animals in the 1600 ppm group (sacrificed at 30 to 35 weeks), and also on the liver, kidney and testes of animals in the 530 ppm group (sacrificed at 40 to 44 weeks). The following organs were normal in all animals: Spleen; adrenals; small intestine; large intestine; stomach; sciatic nerve. The livers of animals in the 1600 ppm group showed well-defined abnormalities of a toxic nature in both males and females; icteric pigmentation was increased and cytoplasmic staining properties were abnormal. The kidneys of animals in the 1600 ppm group showed minor changes. Varying degrees of testicular degeneration were noted in both treatment groups; the ovaries of the females were not noticeably affected to any degree. The kidneys, liver and testes of all the control animals were found to be normal. No microscopic evidence of neoplasms was reported. *Tissue-stored copper:* Liver Cu averaged less than 2 mg/100 g of tissue in control animals. Animals in the 530 ppm group for 40 weeks had Cu concentrations of 12.47 and 32.36 mg/100g liver in males and females, respectively. Those in the 1600 ppm group for a similar duration had concentrations of 38.28 and 45.77 mg Cu/100g liver. Cu storage in the kidneys of animals receiving 530 ppm Cu was somewhat higher than control, while that in animals receiving 1600 ppm Cu was higher again.
196.3 Conclusion	The growth of rats receiving 1600 ppm Cu as $CuSO_4$ was adversely affected, although organ weights were apparently unaffected (other than markedly increased stomach weight in females). Well-defined abnormalities of a toxic nature were evident in rats of the 1600 ppm treatment group upon histological examination, and varying degrees of testicular degeneration was evident in animals from both the 530 ppm and the 1600 ppm groups. There were no reports of evidence of neoplasms in any treatment group.
196.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
196.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. There were also a number of deficiencies in the methodology used, when compared with the requirements of currently accepted guidelines for the conduct of carcinogenicity studies (e.g. OECD 451), including the following: • The test substance was inadequately characterised; • Environmental controls were not described in detail; • Only two $CuSO_4$ test concentration were used; • The range of tissues reported upon was limited;

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

- ~~Body and organ weights were not reported;~~
- The duration of the study was a maximum of 44 weeks;
- Microscopic investigations were carried out in a limited number of tissues in animals sacrificed at study termination.
- Experimental results were inadequately reported in some cases, e.g. haematology and urinalysis.

However, these deficiencies do not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the carcinogenicity of copper.

Overall, this is an adequately-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper carcinogenicity. A reliability indicator of 2 has been assigned on this basis.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Sections A6.5 & A6.7 Combined Chronic toxicity/Carcinogenicity

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(03) & A6.7(03), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/13 & 5.7/03	

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Guidelines and quality assurance [redacted]

Materials and Methods [redacted]

Results and discussion [redacted]

Conclusion [redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks [redacted]

Copper Oxide

Table A6.5(03) & A6.7(03)-1. Average Body Weights of Rats Receiving Copper Sulphate (grams)

Treatment Group	0 week	4th week	8th week	12th week	26th week	35th week
Females						
Controls	73 ± 2.3 g[a]	172 ± 3.2 g	204 ± 4.0 g	220 ± 3.9 g	261 ± 4.5 g	265 ± 4.3 g
Number of rats	25	24	24	24	24	24
530 ppm Cu	67 ± 3.3 g	154 ± 2.8 g	207 ± 3.5 g	232 ± 3.2 g	270 ± 3.5 g	260 ± 5.1 g
Number of rats	25	25	25	25	25	25
1600 ppm	**73 ± 2.2** g	**153 ± 3.4 g**	**198 ± 2.7 g**	**224 ± 3.1 g**	**220 ± 4.2 g**	**257 ± 3.6 g**
N	25	25	25	25	**24**	**20**
Males						
Controls	81 ± 2.3 g	218 ± 7.2 g	310 ± 6.2 g	382 ± 7.0 g	438 ± 17.3 g	459 ± 17.3 g
Number of rats	23	23	23	23	23	22
530 ppm Cu	72 ± 3.4 g	194 ± 6.5 g	279 ± 1.3 g	358 ± 5.8 g	425 ± 10.7 g	431 ± 3.7 g
Number of rats	25	25	25	25	24	23
1600 ppm	71 ± 9.3 g	174 ± 5.7 g	247 ± 6.3 g	280 ± 9.1 g	282 ± 10.6 g	335 ± 9.5 g
Number of rats	23	23	23	23	20	16

[a] Standard error.

[b] Depleted due to high mortality.

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6.5(03) & A6.7(03)-1. Average Body Weights of Rats Receiving Copper Sulphate (grams)

Treatment Group	0 week	4th week	8th week	12th week	26th week	35th week
Females						
Controls	73 ± 2.3 g[a]	172 ± 3.2 g	204 ± 4.0 g	220 ± 3.9 g	261 ± 4.5 g	265 ± 4.3 g
Number of rats	25	24	24	24	24	24
530 ppm Cu	67 ± 3.3 g	154 ± 2.8 g	207 ± 3.5 g	232 ± 3.2 g	270 ± 3.5 g	260 ± 5.1 g
Number of rats	25	25	25	25	25	25
1600 ppm	75 ± 2.5 g	170 ± 2.9 g	200 ± 3.1 g	235 ± 4.1 g	204 ± 3.8 g	182 ± 11.7 g
N	25	25	25	25	23	6[b]
Males						
Controls	81 ± 2.3 g	218 ± 7.2 g	310 ± 6.2 g	382 ± 7.0 g	438 ± 17.3 g	459 ± 17.3 g
Number of rats	23	23	23	23	23	22
530 ppm Cu	72 ± 3.4 g	194 ± 6.5 g	279 ± 1.3 g	358 ± 5.8 g	425 ± 10.7 g	431 ± 3.7 g
Number of rats	25	25	25	25	24	23
1600 ppm	71 ± 9.3 g	174 ± 5.7 g	247 ± 6.3 g	280 ± 9.1 g	282 ± 10.6 g	335 ± 9.5 g
Number of rats	23	23	23	23	20	16

[a] Standard error.

[b] Depleted due to high mortality.

Table A6.5(03) & A6.7(03)-2. Average Weight of Tissues, grams per 100 grams of Body Weight.

Group	N	Heart	Lungs	Liver	Spleen	Kidneys	Uterus (Seminal Vesicles)	Ovaries (Testes)	Stomach	Brain	Approx. Weeks on Test
Females											
Contols	9	0.317	0.500	3.214	0.203	0.717	0.274	0.038	0.615	0.656	42
530 ppm Cu	15	0.295	0.553	3.250	0.182	0.714	0.212	0.037	0.628	0.630	42
1600 ppm Cu	10	0.301	0.564	3.778	0.209	0.799	0.179	0.040	0.821	0.684	42
Males											
Contols	8	0.268	0.495	3.586	0.169	0.798	0.827	0.350	0.518	0.424	42
530 ppm Cu	12	0.282	0.487	3.674	0.189	0.792	0.666	0.357	0.585	0.423	42
1600 ppm Cu	6	0.301	0.488	4.072	0.198	0.889	0.839	0.405	0.686	0.505	42
Females											
Contols	4	0.336	0.770	3.524	0.188	0.753	0.230	0.039	0.645	0.668	33
1600 ppm Cu	4	0.333	0.569	3.767	0.185	0.670	0.135	0.024	0.795	0.669	33
Males											
Contols	4	0.301	0.713	3.556	0.173	0.777	0.923	0.359	0.531	0.479	33
1600 ppm Cu	4	0.297	0.518	3.492	0.176	0.720	0.700	0.255	1.061	0.572	33

Table A6.5(03) & A6.7(03)-3. Copper Content of Tissues of Rats Receiving Copper Sulphate, mg Cu/g tissue (wet basis).

Tissue	Control Diet		530 ppm Cu		1600 ppm Cu	
	Male	Female	Male	Female	Male	Female
Liver						
Av.	1.16	1.78	12.47	32.36	38.28	45.77
S.E.	0.31	0.39	2.52	14.6	13.85	5.18
N.	6	6	6	6	6	6
Kidney						
Av.	2.48	3.53	3.49	6.91	15.83	12.11
S.E.	0.20	0.33	0.54	0.48	6.21	4.80
N.	6	6	6	6	6	6
Spleen						
Av.	3.34	4.83	5.63	5.12	13.91	6.07
S.E.	0.63	0.33	1.5	1.3	7.50	1.72
N.	6	6	6	6	6	6

Official use only

197 REFERENCE

197.1 Reference

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).

[redacted] 1994. Study to Determine the Ability of Copper II Sulphate Pentahydrate to Induce Mutation in Five Histadine-Requiring Strains of Salmonella typhimurim. Hazleton Europe. Report No. 456/31 (unpublished).

197.2 Data protection

Yes

(indicate if data protection is claimed)

197.2.1 Data owner

Give name of company

Wood Preservative Copper Taskforce

197.2.2 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:

Data submitted to the MS after 13 May 2000 on existing [a.s. / b.p.] for the purpose of its [entry into Annex I/IA / authorisation]

198 GUIDELINES AND QUALITY ASSURANCE

198.1 Guideline study

Yes - The study was carried out according to the following test guidelines;
OECD Guidelines 471
EC Directive 2000/32 Annex V Test B14
UKEMS Guidelines

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

198.2 GLP

Yes

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

198.3 Deviations

No

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

199 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

199.1 Test material

Copper sulphate pentahydrate

or give name used in study report

199.1.1 Lot/Batch number *List lot/batch number if available*

A668269 350

Section A6.6.1	**Genotoxicity in vitro**
Annex Point IIA6.6.1	*Specify section no., heading, route and test system as appropriate*
IUCLID: 5.5/01	**A6.6.1(01), *In-vitro* Gene Mutation Study in Bacteria**

199.1.2 Specification		As given in section 2 *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
199.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* blue crystalline solid
199.1.2.2	Purity	*Give purity in % active substance* [redacted]
199.1.2.3	Stability	*Describe stability of test material* Stable at room temperature
199.2 Study Type		*Select / delete as appropriate:* Ames test
199.2.1 Organism/cell type		*Select / delete as appropriate:* Salmonella typhimurium Strains TA98, TA100, TA1535, TA1537, TA102
199.2.2 Deficiencies / Proficiencies		*Select / delete as appropriate:* With the exception of strain TA102, these strains require biotin as well as histidine for growth. In strain TA102 the critical mutation in the histidine gene is located on a multicopy plasmid pAQ1. This strain is particularly sensitive to the activities of oxidative and cross-linking mutagens. The pKM101 plasmid derivatives (TA98, TA100 and TA102) have increased sensitivity to certain mutagens as the pKM101 codes for an error-prone DNA repair system.
199.2.3 Metabolic activation system		Tests were carried out in both the presence and absence of metabolic activation - Aroclor 1254-induced rat liver (Sprague-Dawley male rat) post-mitochondrial fraction (S-9 mix). *state species, organ, induction y/n, induction substance used, give short description*
199.2.4 Positive control		*give name of substance* Details of the positive controls are in Table A6.6.1_1 Positive Controls.
199.2.5 Negative control		Yes, tests carried out with purified water in quintuplicate both with and without metabolic activation
199.3 Administration / Exposure; Application of test substance		Non-entry field
199.3.1 Concentrations		*give concentrations of test substance* Following a range finding study, two experiments were carried out with concentrations of 1.6, 8, 40, 200 and 1000 X

Section A6.6.1	**Genotoxicity in vitro**
Annex Point IIA6.6.1	*Specify section no., heading, route and test system as appropriate*
IUCLID: 5.5/01	**A6.6.1(01), *In-vitro* Gene Mutation Study in Bacteria**

µg/l in experiment one and 50, 100, 200, 400 and 800 µg/l in experiment two. The tests were carried out in triplicate. X

199.3.2 Way of application *describe how test substance was applied and state solvent, e.g. "dissolved in medium", "as impregnation on paper discs" or other*

The test article was dissolved in sterile purified water.

199.3.3 Pre-incubation time Only Experiment two included a pre-incubation step for the tests with metabolic activation. The test substance (or control substance), bacteria and S-9 mix were mixed together and incubated for 1 hour at 37 °C before the addition of 2.5 ml molten agar at 46 °C. Plating of these treatments then proceeded as for normal plate-incorporation procedure.

199.3.4 Other modifications *e. g. addition of catalase, peroxidase or other enzymes*

Not applicable

199.4 Examinations *see tables in appendix for examinations and results*

199.4.1 Number of cells evaluated *give number (i.e. for micronucleus test, chromosome aberrations)*

Colonies were counted electronically using a Seescan Colony Counter and the background lawn inspected for signs of toxicity.

199.5 Statistical analysis The m-statistic was calculated to check that all the data were Poisson-distributed, and the Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

4 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

4.1 Genotoxicity *Non-entry field*

4.1.1 without metabolic activation

No

There was no evidence of genotoxicity in either Experiment 1 or Experiment 2 in the absence of metabolic activation.

If yes, give concentrations with positive result

4.1.2 with metabolic activation

No

There was no evidence of genotoxicity was observed in either Experiment 1 or Experiment 2 in the presence of metabolic activation.

If yes, give concentrations with positive result

4.2 Cytotoxicity

Yes

Evidence of toxicity was observed in all Experiment 1 treatments of 1000 µg/plate. Some evidence of toxicity was also observed following strain TA102 treatments of 200 µg/plate in the presence of S-9 only.

Section A6.6.1	**Genotoxicity in vitro**
Annex Point IIA6.6.1	*Specify section no., heading, route and test system as appropriate*
IUCLID: 5.5/01	**A6.6.1(01), *In-vitro* Gene Mutation Study in Bacteria**

In Experiment 2, toxicity was observed following all treatments (with and without metabolic activation) of 800 µg/plate. Some treatments in the presence of S-9 at lower doses also produced evidence of toxicity.
The higher degree of toxicity observed with Experiment 2 treatments of S-9 was attributed to the use of a preincubation step, which allowed an enhanced exposure of the bacteria to the test article.

If yes, give concentrations with positive result

5. APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods *Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines*

Copper II sulphate pentahydrate was assayed for mutation in 5-histaidine requiring strains (TA98, TA100, TA1537 and TA102) of *Salmonella typhimurium*, both in the presence and absence of metabolic activation by Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9) in 2 separate experiments. Following a range finding study, two experiments were carried out with concentrations of 1.6, 8, 40, 200 and 1000 µg/l in experiment one and 50, 100, 200, 400 and 800 µg/l in experiment two. The tests were carried out in triplicate. Both positive and negative controls were included.

XX
X
X

The study complied with the following guidelines and was conducted in accordance with GLP;

OECD Guidelines 471
EC Directive 2000/32 Annex V Test B14 UKEMS Guidelines

5.2 Results and discussion *Summarize relevant results; discuss dose-response relationship.*

None of the dose concentrations in any of the test strains in either the absence or presence of S-9 resulted in an increase in revertant numbers that were statistically significant at the 1% level when analysed using a Dunnett's test. It was therefore concluded that copper II sulphate pentahydrate was unable to induce mutation in 5 strains of *S. typhimurium*, when tested at concentrations extending to the toxic range, in both the absence and presence of rat liver metabolic activation system.

5.3 Conclusion *Non entry field*

5.3.1 Reliability *Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4*

1

Section A6.6.1	**Genotoxicity in vitro**
Annex Point IIA6.6.1	*Specify section no., heading, route and test system as appropriate*
IUCLID: 5.5/01	**A6.6.1(01), *In-vitro* Gene Mutation Study in Bacteria**
5.3.2 Deficiencies	No *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 6.6.1_1 POSITIVE CONTROLS USED IN BACTERIAL REVERSE MUTATION ASSAY

CHEMICAL	STOCK* CONCENTRATION (µg/ml)	FINAL CONCENTRATION (µg/plate)	USE	
			STRAINS	S-9
2-nitrofluorene	500	50	TA98	-
Sodium azide	20	2	TA100 TA1535	-
9-aminoacridine	500	50	TA1537	-
Glutaraldehyde	250	25	TA102	-
2-aminoanthracene	50	5	At least one strain	+

* With the exception of sodium azide and glutaraldehyde, which were prepared in water, all stock solutions were prepared in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) and stored in aliquots at 1-10 °C in the dark

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/01**	**Genotoxicity in vivo – Mouse Micronucleus Test** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.4.4(01), In-vivo Mutagenicity Study**	
	200 REFERENCE	**Official use only**
200.1 Reference	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* [redacted] (1994). Copper II Sulphate Pentahydrate: Induction of Micronuclei in the Bone Marrow of Treated Mice. Hazleton Europe. Report No. 456/33 (unpublished).	
200.2 Data protection	Yes *(indicate if data protection is claimed)*	
200.2.1 Data owner	Wood Preservative Copper Taskforce	
200.2.2 Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* Data submitted to the MS after 13 May 2000 on existing [a.s. / b.p.] for the purpose of its [entry into Annex I/IA / authorisation]	
	201 GUIDELINES AND QUALITY ASSURANCE	
201.1 Guideline study	Yes – the study following the following guidelines: EEC Annex V test B12. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	X
201.2 GLP	Yes *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
201.3 Deviations	Yes Following test termination, test animals were sacrificed and the bone marrow extracted from both femurs of each test animal. However with one test animal (2338) only one femur was aspirated. This was not considered to have affected the outcome of the study. *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
	202 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
202.1 Test material	Copper sulphate *or give name used in study report*	X
202.1.1 Lot/Batch number	*List lot/batch number if available* A668269 350	
202.1.2 Specification	As given in section 2	

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/01**	**Genotoxicity in vivo – Mouse Micronucleus Test** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.4.4(01), In-vivo Mutagenicity Study**
	(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):
202.1.2.1 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* blue crystalline substance
202.1.2.2 Purity	*Give purity in % active substance* [redacted]
202.1.2.3 Stability	*Describe stability of test material* Stable at room temperature
202.1.2.4 Maximum tolerable dose	*usually the dose applied in single dose application* 338 mg/kg
202.2 Test Animals	Non-entry field
202.2.1 Species	Mouse
202.2.2 Strain	Out-bred CD-1
202.2.3 Source	Charles River, UK Ltd, Margate, UK
202.2.4 Sex	Male and female
202.2.5 Age/weight at study initiation	Ages ranged from 35-42 days for both males and females. Bodyweight ranged from 24-30 g and 21-26 g for males and females respectively.
202.2.6 Number of animals per group	15 males and 15 females were treated with the test substance (this includes and additional 5 mice per sex to be used in the event of deaths among similarly dosed animals), 10 males and 10 females were treated with the negative control and 5 males and 5 females were treated with the positive control.
202.2.7 Control animals	Yes
202.3 Administration/ Exposure	Oral *Fill in respective route in the following, delete other routes*
202.3.1 Number of applications	2
202.3.2 Interval between applications	24 h
202.3.3 Postexposure period	Test animals were sacrificed at either 24 or 48 hours following the second dose administration.
	Oral
202.3.4 Type	By gavage
202.3.5 Concentration	Following a range finding study the test concentration was 447 mg/kg
202.3.6 Vehicle	Purified water

Section A6.6.4	**Genotoxicity in vivo – Mouse Micronucleus Test**
Annex Point IIA6.6.4	*Specify section no., heading, route and species as appropriate Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)*
IUCLID: 5.6/01	**A6.4.4(01), In-vivo Mutagenicity Study**
202.3.7 Total volume applied	20 ml/kg
202.3.8 Controls	Cyclophosphamide (CPA) was dissolved in purified water at 4 mg/ml to serve as a positive control, and administered at 80 mg/kg with a dose volume of 20 ml/kg. The positive control was administered as a single dose. The negative control was purified water administered twice at the same sampling points as the test substance.
202.4 Examinations	Non entry field
202.4.1 Clinical signs	No
202.4.2 Tissue	Bone marrow
202.4.3 Number of animals and time points	Test substance and vehicle treated mice were sacrificed in groups of 5 male and 5 female after 24 or 48 hours; CPA mice were sacrificed after 24 hours.
202.4.4 Number of cells	Initially the relative proportions of polychromatic erthrocytes and normochromatic erythrocytes were determined until a total of at least 1000 cells had been analysed. Counting continued until at least 2000 polychromatic erythrocytes had been observed.
202.4.5 Type of cells	Erythrocytes in bone marrow
202.4.6 Parameters	Polychromatic/normochromatic erythrocytes ratio
	4 RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
4.0 Clinical signs	*No effects / describe significant effects referring to data in results table* Not reported

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/01**	**Genotoxicity in vivo – Mouse Micronucleus Test** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.4.4(01), In-vivo Mutagenicity Study**
4.1 Haematology / Tissue examination	*No effects / describe significant effects referring to data in results table* Mice treated with copper II sulphate pentahydrate exhibited polychromatic/normochromatic erythrocytes (PCE/NCE) ratios which were decreased compared to concurrent vehicle controls at 24 hour sampling point. This is indicative of cellular toxicity and evidence of the test substance penetration into the bone marrow. Mice sampled at 48 hours after being treated with copper II sulphate pentahydrate exhibited ratios which were similar to those in the vehicle controls. The number of micronucleated PCE seen at both sampling times were similar to those seen in the controls and were not significantly different by x2 analysis. The positive control induced a statistically significant increase in the frequency of micronucleated polychromatic erythrocytes See Table A6.6.4_1 Summary of Group Mean Data.
4.2 Genotoxicity	No
If genotoxic give effect dose	

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/01**		**Genotoxicity in vivo – Mouse Micronucleus Test** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.4.4(01), In-vivo Mutagenicity Study**
		5.0 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and methods**	*Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines* Copper II sulphate pentahydrate was assayed *in vivo* in a mouse bone marrow micronucleus test at a single dose level of 447 mg/kg (113.76 mg Cu/kg) for two consecutive days to groups of 5 male and 5 female mice sacrificed 24 or 48 hours after the second administration. Both negative (purified water) and positive controls (cyclophosphamide) were included in the study. The study was conducted in according to EEC Annex V test B12 guidelines and in compliance with GLP.
5.2	**Results and discussion**	*Summarize relevant results; discuss dose-response relationship.* Slides from all dose and control groups sacrificed after 24 and 48 hours were analysed. Negative control mice exhibited normal ratios of PCE to NCE (normochromatic erythrocytes) and normal frequencies of micronucleated PCE within historical negative control ranges. Mice treated with copper sulphate exhibited ratios of PCE to NCE that were decreased compared to concurrent vehicle controls when sampled after 24 hours, which was taken as evidence of copper sulphate absorption into the bone marrow. The PCE/NCE ratios seen in animals sampled at 48 hours were similar to those seen in the vehicle controls. Mice treated with copper sulphate exhibited frequencies of micronucleated PCE which were similar to vehicle controls at all sampling times. There were no instances of statistically significant increases in micronucleus frequency for any group receiving the test chemical at either sampling point. It was concluded that copper sulphate did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of mice treated with 447 mg/kg/day.
5.3	**Conclusion**	*Non entry field*
5.3.1	Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 1
5.3.2	Deficiencies	No *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

X

Section A6.6.4
Annex Point IIA6.6.4
IUCLID: 5.6/01

Genotoxicity in vivo – Mouse Micronucleus Test

Specify section no., heading, route and species as appropriate
Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)

A6.4.4(01), In-vivo Mutagenicity Study

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Guidelines and quality assurance	• [redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/01**	**Genotoxicity in vivo – Mouse Micronucleus Test** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.4.4(01), In-vivo Mutagenicity Study**

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 6.6.4_1 Summary Of Group Mean Data

TREATMENT GROUP (mg/kg X2)	SAMPLING POINT (hours)	*SEX*	MEAN RATIO PCE/NCE	GROUP MEAN FREQUENCY OF MICRONUCLEATED PCE (per 1000)	
				PER SEX	PER TREATMENT GROUP
Vehicle control (purified water)	24	Male	1.07	0.40	0.35
		Female	1.20	0.30	
	48	Male	1.44	0.38	0.33
		Female	0.83	0.30	
447 (copper sulphate pentahydrate)	24	Male	0.70	0.60	0.50
		Female	0.84	0.40	
	48	Male	1.12	0.50	0.45
		Female	1.32	0.40	
Positive control (CPA – single dose only)	24	Male	0.52	26.87	28.07
		Female	0.48	29.27	

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/02**	**Genotoxicity in vivo – Unscheduled DNA Synthesis** *Specify section no., heading, route and species as appropriate Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.6.4(02), *In-vivo* Mutagenicity Study**

		Official use only
	203 REFERENCE	
203.1 Reference	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* [redacted], (1994). Copper II Sulphate Pentahydrate: Measurement of Unscheduled DNA Synthesis in Rat Liver Using an in vivo/in vitro Procedure. Hazleton Europe. Report No. 456/32 GLP, Unpublished.	
203.2 Data protection	Yes *(indicate if data protection is claimed)*	
203.2.1 Data owner	*Give name of company* Wood Preservative Copper Taskforce	
203.2.2 Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* Data submitted to the MS after 13 May 2000 on existing [a.s. / b.p.] for the purpose of its [entry into Annex I/IA / authorisation]	
	204 GUIDELINES AND QUALITY ASSURANCE	
204.1 Guideline study	No – The study was not conducted in accordance with any internationally recognised guidelines. However, the methods employed in the study are comparable to OECD Guidelines 486 "Genetic Toxicology: DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells *in vivo*". *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
204.2 GLP	Yes *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
204.3 Deviations	No *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	
	205 MATERIALS AND METHODS *In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*	
Test material	Copper sulphate *or give name used in study report*	X
3.1.0 Lot/Batch number	*List lot/batch number if available*	

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/02**		**Genotoxicity in vivo – Unscheduled DNA Synthesis** *Specify section no., heading, route and species as appropriate Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.6.4(02), *In-vivo* Mutagenicity Study**
		A668269 350
205.1.1 Specification		As given in section 2 *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
205.1.1.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Blue crystalline solid
205.1.1.2	Purity	*Give purity in % active substance* [redacted]
205.1.1.3	Stability	*Describe stability of test material* Stable at room temperature
205.1.1.4	Maximum tolerable dose	*usually the dose applied in single dose application* <2000 mg/kg
205.2 Test Animals		Non-entry field
205.2.1 Species		Rat
205.2.2 Strain		Wistar
205.2.3 Source		Charles River UK Ltd, Margate, UK
205.2.4 Sex		Male
205.2.5 Age/weight at study initiation		Test animals were 41-51 days old with a bodyweight range of 189-254 g.
205.2.6 Number of animals per group		6 animals
205.2.7 Control animals		Yes
205.3 Administration/ Exposure		Oral *Fill in respective route in the following, delete other routes*
205.3.1 Number of applications		1 *give reasons for more than one application*
205.3.2 Interval between applications		Not applicable
205.3.3 Postexposure period		12-14 hours for Experiment 1, 2-4 hours for Experiment 2
		Oral
205.3.4 Type		Gavage
205.3.5 Concentration		Following a range finding study dose concentrations were set at 632.5 mg/kg and 2000 mg/kg (equivalent to 161 or 509 mg Cu/kg) See Table A6.6.4_1 for further information.
205.3.6 Vehicle		Purified water

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/02**	**Genotoxicity in vivo – Unscheduled DNA Synthesis** *Specify section no., heading, route and species as appropriate* *Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.6.4(02), *In-vivo* Mutagenicity Study**
205.3.7 Concentration in vehicle	Not reported
205.3.8 Total volume applied	10 ml/kg
205.3.9 Controls	Purified water was used as the negative control. 7.5 mg/ml 2-Acetamidofluorene (2-AAF) suspended in corn oil was the positive control for the 12-14 hour experiment. 1.0 mg/ml dimethylnitrosamine (DMN) dissolved in purified water was used as the positive control for the 2-4 hour experiment. Both positive controls were dosed at 10 ml/kg giving achieved doses of 75 mg/kg and 10 mg/kg for the 12-14 and 2-4 hour experiments respectively. See Table A6.6.4_1 for further information.
205.4 Examinations	Non entry field
205.4.1 Clinical signs	No
205.4.2 Tissue	Liver
205.4.3 Number of animals	Cultures from 5 animals were taken
205.4.4 Number of cells	150,000 viable cells/ml
205.4.5 Time points	12-14 hours in Experiment 1, 2-4 hours in Experiment 2
205.4.6 Type of cells	hepatocytes from the liver
205.4.7 Parameters	After approximately 12-14 hours (experiment 1) or 2-4 hours (Experiment 2) after dose administration the animals were sacrificed and the livers perfused with collagenase to provide a primary culture of hepatocytes. Cultures were made from 5 animals in each dose group and were treated with [^{3}H] thymidine. Six slides were prepared with fixed hepatocytes and of these, 3 were dipped in photographic emulsion to prepare autoradiograms. Slides were examined microscopically after development of the emulsion and staining, and the net grain count (NG) and the number of grains present in the nucleus minus the mean number of grains in 3 equivalent areas of cytoplasm was determined for each of the at least 2 of the three slides from each animal in each dose group. **4 RESULTS AND DISCUSSION** *Describe findings. If appropriate, include table. Sample tables are given below.*

Section A6.6.4	**Genotoxicity in vivo – Unscheduled DNA Synthesis**
Annex Point IIA6.6.4	*Specify section no., heading, route and species as appropriate Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)*
IUCLID: 5.6/02	**A6.6.4(02), *In-vivo* Mutagenicity Study**
4.1 Clinical signs	*No effects / describe significant effects referring to data in results table* Not reported
4.2 Haematology / Tissue examination	*No effects / describe significant effects referring to data in results table* Treatment with copper II sulphate pentahydrate at doses up to 2000 mg/kg yielded group mean net grain counts of less than 0, producing group mean net grain counts over the 2 experiments in the range of -1.0 to -3.2, well below the value of 5 net grain counts required for a positive response. No more than 1.0% of the cells were seen in repair at any dose of test substance. The data obtained indicate that oral treatment of male rats with 632.5 or 2000 mg/kg copper II sulphate pentahydrate did not result in increased unscheduled DNA synthesis in hepatocytes isolated approximately 12-14 or 2-4 hours after dosing. The positive control chemicals (2-AAF and DMN) induced increases in the group mean net grain count of 5 or more (12.7 and 17.2 respectively), and 50% or more of the cells (90% and 99.6% respectively) had net grain counts of 5 or more. This result showed that the test system was sensitive to 2 known DNA damaging agents requiring metabolism for their action and that the experiment was valid. The group mean net grain count for the vehicle-treated animals was less than 0 (-1.3 and -2.2 or Experiments 1 and 2 respectively). For further information on results, please refer to Table A6.6.4_2.
4.3 Genotoxicity	No *If genotoxic give effect dose*
4.4 Other	*Describe any other significant effects* Not applicable

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods *Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines*

Copper II sulphate pentahydrate was tested for its ability to induced unscheduled DNA systhesis (UDS) in the livers of orally dosed male rats using an *in vivo/in vivo* procedure. Groups of 6 male rats were treated once with copper sulphate at 632.5 or 2000 mg/kg by oral gavage at a dose volume of 10 ml/kg. For the negative control, a further 6 male rats received purified water as a negative control at the same dose volume. Positive control animals for the12-14 hour experiment, 6 male rats were dosed orally with 75

Section A6.6.4 **Annex Point IIA6.6.4** **IUCLID: 5.6/02**	**Genotoxicity in vivo – Unscheduled DNA Synthesis** *Specify section no., heading, route and species as appropriate Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)* **A6.6.4(02), *In-vivo* Mutagenicity Study**

mg/kg 2-acetamidofluorene, suspended in corn oil. Dimethylmitrosamine, dissolved in purified water, was the positive control for the 2-4 hour experiment.

Approximately 12-14 hours (experiment 1) or 2-4 hours (Experiment 2) after dose administration the animals were sacrificed and the livers perfused with collagenase to provide a primary culture of hepatocytes. The net grain count, number of grains present in the nucleus minus the mean number of grains in 3 equivalent areas of cytoplasm were determined.

5.2 Results and discussion *Summarize relevant results; discuss dose-response relationship.*

Negative control animals gave a group mean net grain of less than 0 with no cells in repair. Group mean net grain values were increased by both positive controls to more than 5 with more than 50% of cells found to be in repair. This was consistent with historical control data.

Treatment with 632.5 or 2000 mg/kg copper sulphate pentahydrate (equivalent to 161 or 509 mg Cu/kg) did not produce a group mean net grain value greater than -1.0 nor were any more than 1.0% cells found in repair at either dose.

It was concluded that copper II sulphate pentahydrate has no genotoxic activity detectable in this test system under the experimental conditions employed.

5.3 Conclusion *Non entry field*

5.3.1 Reliability *Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4*

1

5.3.2 Deficiencies No

The study was not conducted according to an internationally recognised guideline although it is GLP compliant.
When compared with generally accepted principles to be applied in OECD Guidelines 486 Genetic Toxicology: DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian Cells *in vivo*, it is apparent that the study follows these guidelines and there are no apparent deficiencies.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Section A6.6.4
Annex Point IIA6.6.4
IUCLID: 5.6/02

Genotoxicity in vivo – Unscheduled DNA Synthesis

Specify section no., heading, route and species as appropriate
Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)

A6.6.4(02), *In-vivo* Mutagenicity Study

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

Section A6.6.4

Annex Point IIA6.6.4

IUCLID: 5.6/02

Genotoxicity in vivo – Unscheduled DNA Synthesis

Specify section no., heading, route and species as appropriate
Specify type of test (micronucleus test, cytogenetic in-vivo-test [chromosomal analysis], UDS in vivo or other special investigation)

A6.6.4(02), *In-vivo* Mutagenicity Study

EXPERIMENT: 2-4 HOUR SACRIFICE TIME

DOSE (mg/kg)	NET NUCLEAR GRAIN COUNT		NET GRAIN COUNT OF CELLS IN REPAIR		PERCENT OF CELLS IN REPAIR (Net Grain Count ≥5)	
	Mean	SD	Mean	SD	Mean	SD
0 water	-2.2	0.3	0	-	-	-
632.5 copper II sulphate pentahydrate	-2.2	0.2	0	-	-	-
2000 copper II sulphate pentahydrate	-3.2	0.5	0	-	-	-
10 DMN	17.2	2.8	17.3	2.7	99.6	0.9

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

TABLE A6.6.4_1 SUMMARY OF ADMINISTERED DOSES

TREATMENT	DOSE (mg/kg)	DOSE VOLUME (mg/kg)	NUMBER OF ANIMALS DOSED	
			EXPERIMENT 1 (12-14 HOURS)	EXPERIMENT 2 (2-4 HOURS)
Purified water	0	10	3+3	3+3
Copper II sulphate pentahydrate	632.5	10	3+3	3+3
Copper II sulphate pentahydrate	2000	10	3+3	3+3
2-AAF	75	10	3+3	-
DMN	10	10	-	3+3
Copper II sulphate pentahydrate	2000	10	2+1	0+3

A6.6.4_2 SUMMARY OF RESULTS

EXPERIMENT 1: 12-14 HOUR SACRIFICE TIME

DOSE (mg/kg)	NET NUCLEAR GRAIN COUNT		NET GRAIN COUNT OF CELLS IN REPAIR		PERCENT OF CELLS IN REPAIR (Net Grain Count ≥5)	
	Mean	SD	Mean	SD	Mean	SD
0 water	-1.3	0.6	0	-	-	-
632.5 copper II sulphate pentahydrate	-1.3	0.3	10.2	6.4	0.6	0.9
2000 copper II sulphate pentahydrate	-1.0	0.3	5.5	0.9	1.0	1.0
75 2-AAF	12.7	0.9	13.7	0.8	90.0	4.0

EXPERIMENT: 2-4 HOUR SACRIFICE TIME

DOSE (mg/kg)	NET NUCLEAR GRAIN COUNT		NET GRAIN COUNT OF CELLS IN REPAIR		PERCENT OF CELLS IN REPAIR (Net Grain Count ≥5)	
	Mean	SD	Mean	SD	Mean	SD
0 water	-2.2	0.3	0	-	-	-
632.5 copper II sulphate pentahydrate	-2.2	0.2	0	-	-	-
2000 copper II sulphate pentahydrate	-3.2	0.5	0	-	-	-
10 DMN	17.2	2.8	17.3	2.9	99.6	0.9

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

Official use only

206 REFERENCE

206.1 Reference

Lecyk, M. (1980). Toxicity of CuSO4 in mice embryonic development. Zoologica Poloniae, 28(2): 101-105 (published).

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).

206.2 Data protection

No.
(indicate if data protection is claimed)

206.2.1 Data owner

Give name of company

Public domain.

206.2.2 Companies with letter of access

Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)

Letter of access not required.

206.2.3 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation in support of AnnexVI) and delete the others:

No data protection claimed.

207 GUIDELINES AND QUALITY ASSURANCE

207.1 Guideline study

No. This was a non-regulatory study carried out to determine the effect of $CuSO_4$, added to the food of pregnant female mice, on the development of their offspring.

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

207.2 GLP

No. This was a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed.

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

207.3 Deviations

Yes. Refer to section 5.3.6 for a general discussion of deviations and deficiencies.

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

208 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

208.1 Test material

Cu^{2+} as $CuSO_4$

or give name used in study report

208.1.1 Lot/Batch number *List lot/batch number if available*

Not stated.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

208.1.2 Specification		Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
208.1.2.1	Descriptio n	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* $CuSO_4$ was added to the diet as an aqueous solution.
208.1.2.2	Purity	*Give purity in % active substance* [redacted]
208.1.2.3	Stability	*Describe stability of test material.* Not stated.

Non-entry field

208.2 Test Animals

208.2.1 Species	Mouse
208.2.2 Strain	C_{57}BL and DBA
208.2.3 Source	Institute of Immunology and Experimental Therapy, Polish Academy of Sciences.
208.2.4 Sex	Male and female
208.2.5 Age/weight at study initiation	Experimental animals were sexually mature at study initiation.

208.2.6 Number of animals per group

Group	*Number of females treated*	
	Strain C_{57}BL	**Strain DBA**
Control	21	17
1	10	10
2	18	10
3	7	14
4	10	10
5	22	18
6	18	20

Give number, specify, if there are differences for example for treatment and recovery groups

208.2.7 Control animals	Yes
208.2.8 Mating period	Not stated

208.3 Administration/ Exposure

Oral

Fill in respective route in the following, delete other routes

208.3.1 Duration of exposure

Female test animals were continuously exposed to the test substance in their diet from one month prior to mating until they were sacrificed on the 19th day of pregnancy. Males were also fed the appropriate test diet prior to mating.

rat/mouse: day 6-15 post mating

X

Section A6.8.1 **Teratogenicity Study**

Annex Point IIA6.8.1 *Specify section no., heading, route and species as appropriate*

IUCLID: 5.8.2/01 **A6.8.1(01), Teratogenicity of copper**

	Hamster:	day 6-14	post mating
	rabbit:	day 6-18	post mating
	or other		

208.3.2 Postexposure period: None. Females were killed on day 19 of pregnancy.

Oral

208.3.3 Type: In food

208.3.4 Concentration: food consumption per day ..... ad libitum.

Group	*g $CuSO_4$/ kg food*	
	Strain $C_{57}BL$	**Strain DBA**
Control	0	0
1	0.5	0.5
2	1.0	1.0
3	1.5	1.5
4	2.0	2.0
5	3.0	3.0
6	4.0	4.0

208.3.5 Vehicle: Aqueous solution

208.3.6 Concentration in vehicle: Not stated.

208.3.7 Total volume applied: Not stated.

208.3.8 Controls: Plain diet.

No entry field

208.4 Examinations

208.4.1 Body weight: Not stated.

208.4.2 Food consumption: Not stated.

208.4.3 Clinical signs: Not stated.

208.4.4 Examination of uterine content: Gravid uterine weight not stated.
Number of corpora lutea not stated
Number of implantations not stated..

208.4.5 Examination of foetuses: No entry field

208.4.5.1 General: Litter size, Nr. of living foetuses, Nr. of dead foetuses, foetal weight, Nr. of abnormal foetuses.

208.4.5.2 Skelet: Yes

208.4.5.3 Soft tissue: Yes

208.5 Further remarks None.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

209 RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

209.1 Maternal toxic Effects

No effects / describe significant effects referring to data in results table; give concentrations of test substance resulting in toxic effects if any

Maternal toxic effects were not reported.

209.2 Teratogenic / embryotoxic effects

No effects / describe significant effects referring to data in results table

$CuSO_4$ doses in the range 0.5 to 1.0 g per kg feed had no harmful effects on the embryonic growth of mice, and may even have stimulated growth to some extent. This is indicated by the absence of foetal abnormality and slightly higher weights of foetuses than those of the controls (**Tables A6.8.1(01)-2a** and **A6.8.1(01)-2b** for C57BL and DBA strains, respectively). Adverse effects on the foetus were recorded only in the foetuses of females fed a diet containing 3 or 4 g $CuSO_4$/kg food, where greater mortality rates and decreased litter weights were observed (**Tables A6.8.1(01)-2a** and **A6.8.1(01)-2b** for C57BL and DBA strains, respectively).

Developmental malformations were seen in a number of foetuses from the top two dose groups. In $_{C57BL}$ mice from the 4 g $CuSO_4$/kg food group, a thoracic wall hernia was found in one foetus, hydrocephalis in another, and coalescence of two adjacent thoracic vertebrae and ribs in a third. The last lumbar vertebra was included in the sacral region of a single $_{C57BL}$ foetus from the 3 g $CuSO_4$/kg food group (**Table A6.8.1(01)-3a**). In the foetuses of DBA mice from the 4 g $CuSO_4$/kg food group, two had encephaloceles, and another two showed inclusion of a half of the last lumbar vertebra in the sacral region. Two DBA strain foetuses from the 3g $CuSO_4$/kg food group had unilateral coalescence of adjacent ribs (**Table A6.8.1(01)-3b**).

209.3 Other effects *Describe any other significant effects*

None reported.

210 APPLICANT'S SUMMARY AND CONCLUSION

210.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to investigate the effects of $CuSO_4$ added to the diet of pregnant mice on the development of their offspring. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Sexually mature male and female mice of the $_{C57BL}$ and DBA strains were divided into six experimental groups and one control group. Animals in the experimental groups were then fed diets containing, 0.5, 1.0, 1.5, 2.0, 3.0 or 4.0 g of $CuSO_4$ per kg of feed *ad libitum* for one month. The diet was prepared by crushing the standard "Murigran" diet and mixing it with aqueous solutions of $CuSO_4$. Control animals received the standard diet only. Male and female animals of each group were held in separate cages during this period.

Section A6.8.1 **Teratogenicity Study**

Annex Point IIA6.8.1 *Specify section no., heading, route and species as appropriate*

IUCLID: 5.8.2/01 **A6.8.1(01), Teratogenicity of copper**

After one month, the males and females of each group were paired, and the females continued to receive the appropriate diet through the first 19 days of the resulting pregnancy. The number of pregnant females in each treatment group were as follows:

Group	*Number of females treated*	
	Strain C57BL	**Strain DBA**
Control	21	17
1	10	10
2	18	10
3	7	14
4	10	10
5	22	18
6	18	20

On the 19th day of pregnancy, females were killed and living and dead foetuses were removed, counted and weighed. Half the foetuses of each group were examined by Wilson's method. The other half were stained with alizarin red S in 1% KOH and cleared in glycerin.

210.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

$CuSO_4$ in the diet at concentrations of 0.5 and 1.0 g per kg feed had no apparent adverse effects on mouse embryonic growth. Indeed, the slightly higher weights of foetuses in groups receiving up to 2.0 g $CuSO_4$/kg food (when compared to controls) may indicate that supplementation of the diet with $CuSO_4$ stimulated growth to some extent (**Tables A6.8.1(01)-2a** and **A6.8.1(01)-2b** for C57BL and DBA strains, respectively).

Adverse effects were recorded in the highest dose groups. Foetuses of females fed a diet containing 3 or 4 g $CuSO_4$/kg food, appeared to have markedly higher mortality rates and decreased litter weights, when compared to controls (**Tables A6.8.1(01)-2a** and **A6.8.1(01)-2b** for C57BL and DBA strains, respectively).

Developmental malformations were seen in a number of foetuses from females fed diets containing 3 or 4 g $CuSO_4$/kg food. In $_{C57BL}$ mice from the 4g $CuSO_4$/kg food group, a thoracic wall hernia was found in one foetus; a hydrocephalis in a second, and coalescence of two adjacent thoracic vertebrae and ribs in a third. The last lumbar vertebra was included in the sacral region of a single $_{C57BL}$ foetus from the 3 g $CuSO_4$/kg food group (**Table A6.8.1(01)-3a**).

In foetuses of DBA mice from the 4 g $CuSO_4$/kg food group, two had encephaloceles, and another two had inclusion of a half of the last lumbar vertebra in the sacral region. Two DBA foetuses from the group fed 3 g $CuSO_4$/kg had unilateral coalescence of adjacent ribs (**Table A6.8.1(01)-3b**).

210.3 Conclusion No entry field

Section A6.8.1	**Teratogenicity Study**	
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*	
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**	
210.3.1 LO(A)EL maternal toxic effects	*Give critical effect and dose/concentration* Not reported	
210.3.2 NO(A)EL maternal toxic effects	*Give dose/concentration, if necessary separately for males and females* Not reported	
210.3.3 LO(A)EL embryotoxic / teratogenic effects	*Give critical effect and dose/concentration* 3 g $CuSO_4$/kg diet	
210.3.4 NO(A)EL embryotoxic / teratogenic effects	*Give dose/concentration* 2 g $CuSO_4$/kg diet	
210.3.5 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2	X
210.3.6 Deficiencies	Yes This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles to be applied to teratogenicity studies, as set out in OECD guideline 414, it is also apparent that experimental details/results were poorly reported in places, including: • Housing and feeding conditions of test animals; • Information on the age and weight of test animals; • In several dose groups, the number of pregnant animals was smaller than recommended by the guideline (16 animals). • In the absence of information on the weight of test animals and the weight of treated diet consumed, it was not possible to accurately determine the dose received on a mg/kg bodyweight basis. • No information on maternal toxicity was presented in the report. • No post-mortem information was presented in the report for dams. • No information was presented on: the weight of gravid uteri; the number of corpora lutea; degrees of resorption of dead foetuses. • The sex ratio of live foetuses was not reported. These deficiencies do not, however, necessarily compromise the validity of the data reported, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes and that the information that did appear in the report was clearly presented. Furthermore, this research (including its methodology) was published in a peer-reviewed publication, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of the embryotoxic/teratogenic potential of copper. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of the embryotoxic / teratogenic potential of copper. A reliability indicator of 2 has been assigned on this basis.	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ...
Date	*Give date of comments submitted*

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/01	**A6.8.1(01), Teratogenicity of copper**

Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6.8.1(01)-1. Table for Teratogenic effects (separate data for all dosage groups) Maternal effects

Modify if necessary and give historical data if available

Parameter	**control data**		**low dose**	**medium dose**	**high dose**	**dose-response + / -**
	historical	study				
Number of dams examined						
Clinical findings during application of test substance						
Mortality of dams *state %*						
Abortions						
Body weight gain *day 0-x, day 0-y, day x-y, day 0-end of test,*						
Food consumption						
Water consumption *if test substance is applied with drinking water*						
Pregnancies *pregnancy rate or*						
Necropsy findings in dams dead before end of test						

Table A6.8.1(01)-2a. Table for Teratogenic effects (separate data for all dosage groups) Litter response (Caesarean section data) for $_{C57BL}$ stock Modify if necessary and give historical data if available

Parameter	Concentration of $CuSO_4$ in food (g/kg)							dose-response + / -
	0 (control)	0.5	1.0	1.5	2.0	3.0	4.0	
Corpora lutea state total/number of dams								
Implantations state total/number of dams								
Resorptions state total/number of dams								
total number of fetuses								
pre-implantation loss state %								
post-implantation loss state %								
total number of litters								
Mean fetuses / litter (± SD)	3.09 (±0.83)	4.60 (±1.64)	4.50 (±1.15)	4.42 (±1.13)	4.20 (±1.39)	2.50 (±1.01)	1.94 (±0.80)	
No. of live fetuses / dose group (%)	65 (83.1)	46 (89.2)	81 (86.5)	31 (87.1)	42 (78.6)	55 (72.8)	35 (71.5)	
No. of dead fetuses / dose group (%)	11 (16.9)	5 (10.8)	11 (13.5)	4 (12.9)	9 (21.4)	15 (27.2)	10 (28.5)	
Mean fetus weight in g (± SD)	1.10 (±0.15)	1.35 (±0.18)	1.22 (±0.13)	1.14 (±0.12)	1.25 (±0.24)	1.00 (±0.14)	0.99 (±0.10)	
placenta weight (mean) [g]								
crown-rump length (mean) [mm]								
Fetal sex ratio [state ratio m/f]								

Table A6.8.1(01)-2b. Table for Teratogenic effects (separate data for all dosage groups) Litter response (Caesarean section data) for DBA stock Modify if necessary and give historical data if available

Parameter	Concentration of $CuSO_4$ in food (g/kg)							dose-response + / -
	0 (control)	0.5	1.0	1.5	2.0	3.0	4.0	
Corpora lutea state total/number of dams								
Implantations state total/number of dams								
Resorptions state total/number of dams								
total number of fetuses								
pre-implantation loss state %								
post-implantation loss state %								
total number of litters								
Mean fetuses / litter (± SD)	4.47 (±1.46)	5.40 (±1.17)	5.10 (±1.19)	4.14 (±1.93)	4.10 (±0.87)	3.11 (±1.27)	2.70 (±1.26)	
No. of live fetuses / dose group (%)	76 (84.3)	54 (90.8)	51 (88.3)	58 (82.8)	41 (83.0)	56 (75.0)	45 (70.4)	
No. of dead fetuses / dose group (%)	12 (15.7)	5 (9.2)	6 (11.7)	10 (17.2)	7 (17.0)	14 (25.0)	16 (29.6)	
Mean fetus weight in g (± SD)	0.96 (±0.16)	1.24 (±0.12)	1.19 (±0.18)	1.17 (±0.20)	1.13 (±0.11)	1.11 (±0.10)	1.09 (±0.18)	
placenta weight (mean) [g]								
crown-rump length (mean) [mm]								
Fetal sex ratio [state ratio m/f]								

Table A6.8.1(01)-3a. Table for Teratogenic effects (separate data for all dosage groups) Examination of the fetuses ($C_{57}BL$ stock) Modify if necessary and give historical data if available

Parameter	control data	0.5	1.0	1.5	2.0	3.0	4.0	dose-response +/-
Number of external malformations* (%)	0	0	0	0	0	0	1 (2.8)	+
Number of external anomalies* (%)	0	0	0	0	0	0	0	-
Number of skeletal malformations* (%)	0	0	0	0	0	1 (1.8)	1 (2.8)	+
Number of skeletal anomalies* (%)	0	0	0	0	0	0	0	-
Number of skeletal variants* (%)	0	0	0	0	0	0	0	-
Number of visceral malformations* (%)	0	0	0	0	0	0	1 (2.8)	+
Number of visceral anomalies* (%)	0	0	0	0	0	0		-
Number of variants visceral* (%)	0	0	0	0	0	0	0	-

Table A6.8.1(01)-3b. Table for Teratogenic effects (separate data for all dosage groups) Examination of the fetuses (DBA stock) Modify if necessary and give historical data if available

Parameter	control data	0.5	1.0	1.5	2.0	3.0	4.0	dose-response +/-
Number of external malformations* (%)	0	0	0	0	0	0	2 (3.7)	+
Number of external anomalies* (%)	0	0	0	0	0	0	0	-
Number of skeletal malformations* (%)	0	0	0	0	0	2 (3.7)	2 (3.7)	+
Number of skeletal anomalies* (%)	0	0	0	0	0	0	0	-

Number of skeletal variants* (%)	0	0	0	0	0	0	0	-
Number of visceral malformations* (%)	0	0	0	0	0	0	0	-
Number of visceral anomalies* (%)	0	0	0	0	0	0	0	-
Number of variants visceral* (%)	0	0	0	0	0	0	0	-

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

Official use only

211 REFERENCE

211.1 Reference

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)
If necessary, copy field and enter other reference(s).

Barlow, S.M., Knight, A.F. and House, I. (1981). Intrauterine exposure to copper IUDs and prenatal development in the rat. J. Rep. Fert. 123 – 130 (published).

211.2 Data protection

No.

(indicate if data protection is claimed)

211.2.1 Data owner

Give name of company

Public domain.

211.2.2 Companies with letter of access

Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)

Letter of access not required.

211.2.3 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation in support of AnnexVI) and delete the others:

No data protection claimed.

212 GUIDELINES AND QUALITY ASSURANCE

212.1 Guideline study

No. This was a non-regulatory study carried out to investigate the effects of intrauterine exposure to copper IUDs and prenatal development in the rat. No guideline is available specifically to address this endpoint.

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

212.2 GLP

No. This is a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed.

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

212.3 Deviations

Yes. Refer to section 4.3.6 for a general discussion of deviations and deficiencies.

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

213 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

213.1 Test material

Cu^{2+} as copper wire.

or give name used in study report

213.1.1 Lot/Batch number *List lot/batch number if available*

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

Not available.

213.1.2 Specification — Deviating from specification given in section 2 as follows

(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

213.1.2.1 Description — *If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)*

Copper wire, 0.1 mm diameter.

213.1.2.2 Purity — *Give purity in % active substance*

[redacted]

213.1.2.3 Stability — *Describe stability of test material*

Not applicable to inorganic substances.

213.2 Test Animals — Non-entry field

213.2.1 Species — Rat.

213.2.2 Strain — Wistar strain.

213.2.3 Source — Charles river, Kent, UK.

213.2.4 Sex — Female.

213.2.5 Age/weight at study initiation — Age: approximately 12 weeks.
Weight: 200 – 250 g.

213.2.6 Number of animals per group

Experiment	Group	Number of animals
1	1 (Copper IUD)	9
	2 (Sham-operated)	10
	3 (No operation)	10
2	4 (Copper IUD)	13
	5 (Steel IUD)	14
	6 (No operation)	7
3	7 (Copper IUD)	2
	8 (Steel IUD)	2
	9 (Control)	1

Give number, specify, if there are differences for example for treatment and recovery groups

213.2.7 Control animals — Yes. See section 5.4.7.

213.2.8 Mating period — Not specified. Females were housed in groups of 3 and a proven male of the same strain was introduced into each cage in the morning. Males were removed and vaginal smears taken in the evening; the day of finding spermatozoa in the smear was designated day 1 of pregnancy.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**
213.3 Administration/ Exposure	Intrauterine exposure. **Experiment 1:** Animals were allocated to 3 groups representing treatment with copper IUDs (Group 1), sham-operated controls (Group 2) and untreated controls (Group 3). IUDs were made of 2 cm lengths of wire coiled into spirals approximately 2 mm in length and 1 mm in diameter. A coil was surgically inserted between each implantation site in either the right or left uterine horn, leaving the other horn as an unoperated control. Gravimetric analysis of the copper IUDs before insertion on Day 9 and after removal on Day 21 of pregnancy showed a mean ± s.e.m. copper loss of 48 ± 3 µg, i.e. about 4 µg/coil/day. **Experiment 2:** Animals in Groups 4, 5 and 6 were fitted as described for Experiment 1 with copper IUDs, steel IUDs or left as untreated controls, respectively. The coils were made of 4 cm lengths of copper or steel wire, and the mean ± s.e.m. copper loss/coil was 74 ± 4 µg, i.e. about 6 µg/coil/day. No significant reduction in weight of the steel coils between insertion and removal was found. **Experiment 3:** To determine whether copper released from the IUDs penetrated into the foetuses, pregnant rats were treated as follows. On Day 9 of pregnancy, copper IUDs were inserted between each embryo in both uterine horns of 2 rats (Group 7). In another 2 rats, steel IUDs were similarly inserted in both horns (Group 8). One rat was left as an unoperated control. *Fill in respective route in the following, delete other routes*
213.3.1 Duration of exposure	Developing foetuses were exposed to intrauterine copper from days 9 to 21 of pregnancy.
213.3.2 Postexposure period	None. Females were sacrificed on day 21 of exposure.
213.4 Examinations	No entry field
213.4.1 Body weight	Yes
213.4.2 Food consumption	No
213.4.3 Clinical signs	Yes
213.4.4 Examination of uterine content	Number of corpora lutea Number of implantations
213.4.5 Maternal plasma copper exstimation	Yes
213.4.6 Maternal tissue copper levels	Yes
213.4.7 Examination of foetuses	No entry field

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

213.4.7.1 General — Number and position of live and full-term dead foetuses (no signs of maceration), late resorptions (maceration, death occurring at the foetal stage), and early resorptions (death occurring at the embryonic stage); Foetal Weight; Foetal tissue copper levels.

213.4.7.2 Skeleton Yes

213.4.7.3 Soft tissue Yes

213.5 Further remarks None.

RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

213.6 Maternal toxic Effects

No effects / describe significant effects referring to data in results table; give concentrations of test substance resulting in toxic effects if any

Experiment 1: Of a total of 63 coils inserted, 59 were recovered at autopsy. The majority were superficially embedded in the fibrous ring around the edge of the placentae and the remainder were free in the uterine lumen between the amniotic sacs. Maternal plasma copper levels (mean ± s.e.m.) on Day 21 of pregnancy were 203 ± 5 (n = 9), 208 ± 12 (n = 10) and 200 ± 5 (n = 10) µg/100 ml in Groups 1, 2 and 3 respectively. The differences between the groups were not significant. Two rats had unilateral pregnancies, the remainder were bilateral. The only significant differences in comparisons of the 5 sub-groups of uterine horns (**Table A6.8.1(02)-1**) were between resorptions in Group 1A and Group 2A or 2B ($P < 0.015$) and between Group 1A and Group 3 ($P = 0.03$).

Experiment 2: Mean ± s.e.m. copper levels in maternal plasma on Day 21 of pregnancy were 207 ± 6 (*n* = 13), 194 ± 9 (*n* = 12) and 208 ± 14 (*n* = 7) µg/100 ml in Groups 4, 5 and 6, respectively. The differences are not significant. The outcome of the pregnancies is shown in **Table A6.8.1(02)-3**. There was a significant increase in the incidence of resorptions in Groups 4A and 5A in comparison with Groups 4B and 5B ($P < 0.005$). There was no significant difference between Groups 4A and 5A.

213.7 Teratogenic / embryotoxic effects

No effects / describe significant effects referring to data in results table

Experiment 1: There were no significant differences between the 5 sub-groups in either the overall incidence of abnormal foetuses or in specific abnormalities and anomalies seen (**Table A6.68.1(02)-2**).

Experiment 2: There were no significant differences in the overall incidence of abnormalities (**Table A6.8.1(02)-4**). The only significant difference in the incidence of specific soft tissue abnormalities was an excess of tracheobronchomegaly in Group 4A compared with Group 4B ($P < 0.02$). However, the difference between Group 4A and Group 6 was not significant. The only significant difference in the incidence of skeletal anomalies was a slight excess of extra 14th rib in foetuses from Group 4B in comparison with Group 6 ($P < 0.05$).

213.8 Other effects *Describe any other significant effects?*

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

Experiment 3: Rats were killed on Day 22 of pregnancy and samples of maternal liver and uterus, all foetal brains, foetal livers and placentae were removed for copper analysis.

Foetal brain, foetal liver and placental copper levels were elevated in Group 7, compared with tissues from animals in Group 8 or the unoperated control (**Table A6.8.1(02)-5**). In Group 7, variance in foetal tissue copper levels was low, suggesting uniform exposure of embryos and foetuses. Maternal liver levels of copper were not elevated in Group 7 (5.0 and 6.8 µg/g) compared with Group 8 (4.9 and 5.2 µg/g) or the unoperated control (4.5 µg/g). Uterine copper levels were considerably elevated in Group 7 (33.1 and 21.3 µg/g), compared with values in Group 8 (2.0 and 2.1 µg/g) and the control animal (1.8 µg/g).

214 APPLICANT'S SUMMARY AND CONCLUSION

214.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to investigate the potential for intrauterine copper IUDs to affect prenatal development in the rat. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Animals: Female Wistar rats aged about 12 weeks and weighing 200-250 g were used in this study. For 2 weeks before mating and throughout the experiment they were held at 21-24°C under reversed lighting conditions (12 h red light, 12 h white light). Food and water were fed *ad libitum*. At the beginning of the experimental period, female rats were housed in groups of 3 and a male was introduced into each cage in the morning. Males were removed in the evening and vaginal smears taken. The day on which spermatozoa were found in the smear was designated Day 1 of pregnancy. Rats were weighed daily from Days 1 to 21 of pregnancy.

Insertion of IUDs: On Day 9 of pregnancy, rats were assigned randomly to treatment groups. Animals receiving IUDs were anaesthetized and one uterine horn exposed through an incision in the flank. IUDs were made from 2 or 4 cm lengths of 0.1 mm diameter copper wire (99.9% pure), coiled into spirals 2 mm in length and 1 mm in diameter. A coil was inserted between each implantation site by making an incision in the uterus with an intravenous cannula with cutting needle. When the incision had been made, the needle was withdrawn leaving the cannula in place. The IUD was then pushed down the cannula into the uterus and the cannula removed. The other horn was left unoperated as a control. To control for the physical presence of devices in the uterus, some animals had similar-sized coils of stainless-steel wire inserted into one horn, leaving the other unoperated. All coils were weighed before insertion and their position noted. To control for the stress of the operation and other factors such as loss of uterine fluid, other animals were sham-operated, with no IUDs inserted. Animals in another group were left unoperated. Rats were returned to the animal room until sacrifice on Day 21.

Section A6.8.1 **Teratogenicity Study**

Annex Point IIA6.8.1 *Specify section no., heading, route and species as appropriate*

IUCLID: 5.8.2/02 **A6.8.1(02), Teratogenicity of Copper**

Examination of foetuses: Rats were anaesthetised on Day 21 of pregnancy and a maternal blood sample taken for copper analysis. After sacrifice, the uterus was exposed and opened up. In IUDbearing animals, copper or steel coils were removed, washed and weighed. The number and position of live and full-term dead foetuses (no signs of maceration), late resorptions (maceration, death occurring at the foetal stage), and early resorptions (death occurring at the embryonic stage) were noted. Numbers of corpora lutea in each ovary were also noted. Foetuses were weighed and examined for gross external abnormalities. They were then either fixed in Bouin's fluid for examination of soft tissues by the slicing technique of Wilson or in alcohol and stained with Alizarin red S for skeletal examination.

Maternal plasma copper estimation: Blood samples were centrifuged and the plasma stored at 4°C until assayed by the colorimetric bathocuprein method with deproteinisation, using duplicate 1 ml aliquots from each sample.

Tissue copper levels: Samples were wet-ashed with 1 ml of a mixture of nitric, perchloric and sulphuric acids (20:10:1) and made up to 0.5 ml in deionised water. Copper was measured by atomic absorption spectrometry. Brains were assayed by flameless atomic absorption analysis using a graphite furnace and the remaining tissues by flame aspiration after addition of dilute hydrochloric acid.

Statistical Analysis: Differences between group means ± s.e.m. were compared by Student's t test, two-tailed. All other comparisons were examined by the Fisher exact probability test, two-tailed.

Study design:

The results of this study were reported in terms of three separate experiments. The details of Experiments 1 and 2 are shown in the following table:

Experiment	Group	No. of animals	Uterine horn*
1	1 (Copper IUD)	9	A operated (9) B unoperated (8)
	2 (Sham-operated)	10	A operated (10) B unoperated (9)
	3 (No operation)	10	Unoperated (20)
2	4 (Copper IUD)	13	A operated (13) B unoperated (13)
	5 (Steel IUD)	14	A operated (14) B unoperated (13)
	6 (No operation)	7	Unoperated (14)

* Figures in parentheses indicate number of horns containing implantation sites.

Experiment 3 was carried out to determine whether copper released from IUDs penetrated into foetuses. Pregnant rats were treated as

Section A6.8.1	**Teratogenicity Study**
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IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

follows: on Day 9 of pregnancy, copper IUDs were inserted between each embryo in both uterine horns of 2 rats (Group 7). In another 2 rats, steel IUDs were inserted in both horns (Group 8). One rat was left as an unoperated control. Test animals were killed on Day 22 of pregnancy, and samples of maternal liver and uterus, all foetal brains, foetal livers and placentae were removed for copper analysis.

214.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Experiment 1 results: Gravimetric analysis of the IUDs before insertion on Day 9 and after removal on Day 21 of pregnancy showed a mean ± s.e.m copper loss of 48 ± 3 µg (about 4 µg/coil/day). Of a total of 63 coils inserted, 59 were recovered at autopsy. Maternal plasma copper levels (mean ± s.e.m.) on Day 21 of pregnancy were 203 ± 5 (n = 9), 208 ± 12 (n = 10) and 200 ± 5 (n = 10) µg/100 ml in Groups 1, 2 and 3 respectively. Differences between the groups were not significant. Two rats had unilateral pregnancies, the remainder were bilateral. The only significant differences in comparisons of the 5 subgroups of uterine horns were between resorptions in Group 1A and Group 2A or 2B ($P < 0.015$) and between Group 1A and Group 3 ($P = 0.03$). There were no significant differences between the sub-groups in either overall incidence of abnormal foetuses or specific abnormalities and anomalies.

Experiment 2 results: Gravimetric analysis of the IUDs before insertion on Day 9 and after removal on Day 21 of pregnancy showed a mean ± s.e.m copper loss/coil of 74 ± 4 µg, i.e. about 6 µg/coil/day. No significant reduction in weight of the steel coils was found between insertion and removal. Mean ± s.e.m. copper levels in maternal plasma on Day 21 of pregnancy were 207 ± 6 (n = 13), 194 ± 9 (n = 12) and 208 ± 14 (n = 7) µg/100 ml in Groups 4, 5 and 6, respectively. The differences are not significant. There was a significant increase in the incidence of resorptions in Groups 4A and 5A in comparison with Groups 4B and 5B ($P < 0.005$). There was no significant difference between Groups 4A and 5A. There were no significant differences in the overall incidence of foetal abnormalities. The only significant difference in the incidence of specific soft tissue abnormality was an excess of tracheobronchomegaly in Group 4A compared with Group 4B ($P < 0.02$). However, the difference between Group 4A and Group 6 was not significant. The only significant difference in the incidence of skeletal anomalies was a slight excess of extra 14th rib in foetuses from Group 4B in comparison with Group 6 ($P < 0.05$).

Experiment 3 results: Foetal brain and liver and placental copper levels were significantly elevated in Group 7 animals, compared with those from Group 8 or the unoperated control. Variance in foetal copper levels in Group 7 was low, suggesting relatively uniform exposure of embryos and foetuses. Maternal liver levels of copper were not elevated in Group 7 (5.0 and 6.8 µg/g) compared with Group 8 (4.9 and 5.2 µg/g) or the unoperated control (4.5 µg/g). Uterine copper levels were considerably elevated in Group 7 (33.1 and 21.3 µg/g) compared with values in Group 8 (2.0 and 2.1 µg/g) and the control animal (1.8 µg/g).

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

	Discussion: This study demonstrated that it is possible to achieve uniform exposure of embryos and foetuses to copper by inserting small coils of wire between implantation sites, and that the majority of offspring survive this procedure. Coils remain in the uterus throughout pregnancy and do not puncture the amniotic sacs. Examination of the offspring for structural abnormalities confirmed that copper had no significant teratogenic or growth-retarding effect in the rat. The incidence of major malformations was low in all groups and the minor disturbances that were seen in all groups are known to be common spontaneous malformations in the strain of rat used. The copper ions released from intrauterine wire were insufficient to elevate maternal plasma copper levels. Copper levels in the rat maternal liver were not elevated, but the copper released from the IUDs did penetrate the foetus. Foetal brain copper levels were increased by 65% and foetal liver levels by more than 100% in copper-exposed offspring compared with those from mothers with steel IUDs or no IUDs. The lack of teratogenicity of copper released from IUDs cannot therefore be attributed to lack of exposure of the conceptuses. Moreover, the embryos were exposed to copper throughout organogenesis. The IUDs were inserted on the morning of Day 9 of pregnancy, which corresponds to the primitive-streak stage marking the onset of organogenesis, and is well before the time of neural tube closure on Days 10-11. Intrauterine mortality rates of 19 and 24% in copper IUD horns were significantly higher than in sham-operated (4%) or untreated controls (0 and 8%), but were no higher than in horns carrying inert steel IUDs (25%). These results suggest that the deaths were probably due to trauma from the insertion and the physical presence of devices in the uterus, rather than to any specific effect of copper.	
214.3 Conclusion	There was no significant increase in the incidence of congenital malformations or growth retardation in foetuses from uterine horns containing copper coils, when compared with those from unoperated horns, sham-operated horns, or horns containing stainless-steel coils.	X
214.3.1 LO(A)EL maternal toxic effects	*Give critical effect and dose/concentration* No maternal toxic effects were observed.	
214.3.2 NO(A)EL maternal toxic effects	*Give dose/concentration, if necessary separately for males and females* No maternal toxic effects were observed.	
214.3.3 LO(A)EL embryotoxic / teratogenic effects	*Give critical effect and dose/concentration* No embryotoxic/teratogenic effects were observed.	
214.3.4 NO(A)EL embryotoxic / teratogenic effects	*Give dose/concentration* No embryotoxic/teratogenic effects were observed.	
214.3.5 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**
214.3.6 Deficiencies	Yes. This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles applied to teratogenicity studies, as set out in OECD 414 a number of deviations/ deficiencies are apparent, as follows: • IUDs were implanted on Day 9, and not prior to implantation (this was necessary in order to avoid the known ability of copper to prevent implantation as a result of embryotoxicity). • Group sizes are smaller than recommended by the guideline (20 females animals per group with implantation sites). • The number of dose levels is fewer than recommended (3). • Levels of food consumption are not reported. • Foetal sex is not reported. These deficiencies do not, however, compromise the validity of the data reported, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes and that the information that appears in the report is clearly presented. Furthermore, this research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition, this report has been included in a number of expert reviews of the toxicity of copper to reproduction. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of the embryotoxic / teratogenic potential of copper. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/02	**A6.8.1(02), Teratogenicity of Copper**

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	[redacted]
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and	*Discuss if deviating from view of rapporteur member state*
discussion Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Tables A6.8.1(02)-1 and A6.8.1(02)-2

Table 1. Outcome of pregnancy in rats carrying 2-cm coiled copper IUDs from Days 9 to 21 of pregnancy (Exp. 1)

Group	No. of rats	Uterine horn	No. of implantation sites	Fetuses		Resorptions		Mean ± s.e.m. fetal wt (g)
				Live	Dead	Early	Late	
1 (copper IUD)	9	A Operated (9)	63	51	0	9	3	2·95 ± 0·12
		B Unoperated (8)	42	39	0	2	1	3·02 ± 0·10
2 (sham-operated)	10	A Operated (10)	57	55	0	1	1	2·97 ± 0·10
		B Unoperated (9)	47	47	0	0	0	2·85 ± 0·07
3 (no operation)	10	Unoperated (20)	126	117	0	9	0	3·12 ± 0·05

Figures in parentheses indicate number of horns containing implantation sites.

Table 2. Results of morphological examinations of fetuses from rats in Exp. 1 carrying 2-cm coiled copper IUDs from Days 9 to 21 of pregnancy

	Group 1 (copper IUD)		Group 2 (sham-operation)		
	A Operated horn	B Unoperated horn	A Operated horn	B Unoperated horn	Group 3 (no operation)
Gross external examination					
Fetuses examined	51	39	55	47	117
Fetuses abnormal	2	0	0	0	0
Types of abnormality*					
Omphalocoele	1	0	0	0	0
Club foot	1	0	0	0	0
Soft tissue examination					
Fetuses examined	24	21	29	22	58
Fetuses abnormal	3	5	5	5	12
Types of abnormality*					
Hydronephrosis	1	3	3	1	2
Hydrocephaly	0	1	2	0	1
Tracheobronchomegaly	0	1	0	2	1
Hypertrophied tracheal/ oesophageal wall	0	1	0	1	3
Partly descended testis	0	0	0	1	3
Haemorrhage	2	0	1	0	2
Skeletal examination					
Fetuses examined	22	18	26	25	59
Fetuses anomalous	11	7	6	8	17
Types of anomaly*					
Split centra	4	4	3	6	5
Kinked ribs	6	3	3	4	12
Extra 14th rib(s)	2	1	0	1	1
Short rib(s)	0	0	0	0	1
Abnormal fusion of sternebrae	1	1	1	0	0

* Some fetuses had more than one abnormality or anomaly.

Tables A6.8.1(02)-3 and A6.8.1(02)-4

Table 3. Outcome of pregnancy in rats carrying 4-cm coiled copper IUDs from Days 9 to 21 of pregnancy (Exp. 2)

Group	No. of animals	Uterine horn	No. of implantation sites	Fetuses		Resorptions		Mean ± s.e.m. fetal wt (g)
				Live	Dead	Early	Late	
4 (copper IUD)	13	A Operated (13)	75	57	0	16	2	2·96 ± 0·08
		B Unoperated (13)	95	91	0	4	0	3·04 ± 0·07
5 (steel IUD)	14	A Operated (14)	98	75	0	13	10	2·83 ± 0·08
		B Unoperated (13)	110	108	0	2	0	2·86 ± 0·07
6 (no operation)	7	Unoperated (14)	102	102	0	0	0	2·79 ± 0·11

Figures in parentheses indicate number of horns containing implantation sites.

Table 4. Results of morphological examinations of fetuses from rats carrying 4-cm coiled copper IUDs from Days 9 to 21 of pregnancy (Exp. 2)

	Group 4 (copper IUD)		Group 5 (steel IUD)		Group 6 (no operation)
	A Operated horn	B Unoperated horn	A Operated horn	B Unoperated horn	
Gross external examination					
Fetuses examined	57	91	75	108	102
Fetuses abnormal	0	2	0	0	2
Types of abnormality*					
Omphalocoele	0	0	0	0	2
Club foot	0	1	0	0	0
Spina bifida	0	1	0	0	0
Soft tissue examination					
Fetuses examined	30	44	37	54	51
Fetuses abnormal	10	7	6	9	7
Types of abnormality*					
Hydronephrosis	1	2	2	4	2
Tracheobronchomegaly	5	0	2	0	2
Large unfolded oesophagus	3	2	1	1	2
Diaphragmatic hernia	1	0	0	0	1
Partly descended testis	0	0	1	1	0
Haemorrhage	0	3	1	3	1
Skeletal examination					
Fetuses examined	27	47	38	54	51
Fetuses anomalous	7	13	11	7	10
Types of anomaly*					
Split centra	0	3	2	2	3
Kinked ribs	4	5	9	4	9
Extra 14th rib(s)	3	5	0	0	0
Short rib(s)	0	1	2	1	1
Fused ribs	1	0	0	0	0

* Some fetuses had more than one abnormality or anomaly.

Table A6.8.1(02)-5

Table 5. Mean ± s.e.m. (range in parentheses) tissue copper levels (μg/g) after insertion of copper IUDs from Days 9 to 22 of pregnancy (Exp. 3)

Group	No. of samples	Fetal brain	Fetal liver	Placenta
7 (copper IUD)	14	2·0 ± 0·2† (1·3–3·9)	26·8 ± 1·5* (19·4–39·1)	5·8 ± 0·8* (3·1–14·0)
8 (steel IUD)	22	1·3 ± 0·1 (0·9–2·3)	12·9 ± 0·3 (10·4–15·7)	2·4 ± 0·1 (1·8–3·3)
9 (control)	12	1·3 ± 0·1 (0·9–2·1)	9·2 ± 0·3 (8·0–10·6)	2·6 ± 0·1 (2·1–3·0)

* $P < 0·001$ compared with steel IUD or control groups.
† $P < 0·001$ compared with steel IUD group and <0·05 compared with controls.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/03	**A6.8.1(03), Teratogenicity of copper**

Official use only

215 REFERENCE

215.1 Reference

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)
If necessary, copy field and enter other reference(s).

Barash, A., Shoham (Schwartz), Z., Borenstein, R. and Nebel, L. (1990). Development of Human Embryos in the Presence of a Copper Intrauterine Device. Gynecol. Obstet. Invest., **29:**203-206 (published).

215.2 Data protection

No.
(indicate if data protection is claimed)

215.2.1 Data owner

Give name of company

Public domain.

215.2.2 Companies with letter of access

Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)

Letter of access not required.

215.2.3 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation in support of AnnexVI) and delete the others:

No data protection claimed.

216 GUIDELINES AND QUALITY ASSURANCE

216.1 Guideline study

No. This was a non-regulatory study carried out to investigate the teratogenic potential of copper-releasing intrauterine contraceptive devices on the developing embryo.

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

216.2 GLP

No. This was a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed.

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

216.3 Deviations

Yes. Refer to section 5.3.6 for a general discussion of deviations and deficiencies

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

217 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

217.1 Test material

Cu^{2+} derived from a copper intrauterine device.

or give name used in study report

217.1.1 Lot/Batch number *List lot/batch number if available*

Not available.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/03	**A6.8.1(03), Teratogenicity of copper**

217.1.2 Specification		Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	
217.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Cu^{2+} was derived from either a Nova-T or a Multiload copper-bearing IUD *in situ.*	
217.1.2.2	Purity	*Give purity in % active substance* [redacted]	X
217.1.2.3	Stability	*Describe stability of test material* Not applicable to inorganic substances. Non-entry field	
217.2 Test Animals			
217.2.1 Species		Human subjects.	
217.2.2 Strain		Not applicable.	
217.2.3 Source		Not applicable.	
217.2.4 Sex		Female.	
217.2.5 Age/weight at study initiation		Age range 20 – 40 years	
217.2.6 Number of subjects per group		The study included 18 women, 11 of whom had conceived while using a copper-bearing IUD; 7 of whom (control group) had conceived spontaneously with no previous history of using an IUD. *Give number, specify, if there are differences for example for treatment and recovery groups*	
217.2.7 Control subjects		Yes	
217.2.8 Mating period		Not applicable	
217.3 Administration/ Exposure		Intrauterine exposure of developing foetuses *Fill in respective route in the following, delete other routes*	
217.3.1 Duration of exposure		Between 7 and 12 weeks of gestation, an artificial abortion was induced in all 18 women. rat/mouse: day 6-15 post mating hamster: day 6-14 post mating rabbit: day 6-18 post mating or other	
217.3.2 Postexposure period		None.	
		Intrauterine	
Vehicle		None; the copper was derived from an IUD *in-situ.*	
Concentration in vehicle		Not applicable.	

Section A6.8.1	**Teratogenicity Study**
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IUCLID: 5.8.2/03	**A6.8.1(03), Teratogenicity of copper**

Total volume applied	Not applicable.
Controls	Seven women who had conceived spontaneously with no previous history of using an IUD.
217.4 Examinations	No entry field
217.4.1 Body weight	No
217.4.2 Food consumption	No
217.4.3 Clinical signs	No
217.4.4 Examination of uterine content	Gravid uterine weight: Not reported.
	Number of corpora lutea: Not reported.
	Number of implantations: Not reported.
	Or other
217.4.5 Examination of foetuses	No entry field
217.4.5.1 General	All embryos were examined for gross malformation. The following organs were examined histologically: brain; eyes; inner ear; heart and lungs; liver; pancreas; mesonephron; kidneys; gonads; vertebrae and limbs.
217.4.5.2 Skeleton	Yes.
217.4.5.3 Soft tissue	Yes.

217.5 Further remarks Embryos were fixed in formalin, embedded in paraffin wax and sectioned sagitally at a thickness of 7 µm. The sections were stained with haematoxylin and eosin. Fixation, embedding and staining of the placentae were similarly carried out. All sections were stained for copper in accordance with Uzman's procedure for the protection of granular precipitates.

Maternal blood samples were taken on the day of the abortion and examined for plasma levels of copper and ceruloplasmin. Copper levels were determined by atomic absorption spectrophotometry. Ceruloplasmin was assessed using Richterich's method. The student t test was used to evaluate significant differences in means. Results are presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

Describe findings. If appropriate, include table. Sample tables are given below.

Maternal toxic Effects *No effects / describe significant effects referring to data in results table; give concentrations of test substance resulting in toxic effects if any*

None reported.

Teratogenic / embryotoxic effects *No effects / describe significant effects referring to data in results table*

No organic malformations were found in the 11 embryos of either the study group or in the 7 controls. Alternating serial sections were examined histologically. The embryonic tissue was free of copper

Section A6.8.1	**Teratogenicity Study**
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deposits, whether fine or coarse granular.

The histodifferentiation of the organs demonstrated no abnormal findings in relation to embryonic age.

The placentae of embryos in both groups were examined histologically. There was no structural impairment and no trace of copper deposits in the placentae.

Other effects

Describe any other significant effects

The maternal copper and ceruloplasmin plasma levels were within normal range, with no statistically significant difference between the study and control groups (163.4 ± 41.4 vs. 137.8 ± 37.9 µg % copper and 70.4 ± 14.9 vs. 69 ± 19.9 mg % ceruloplasmin).

APPLICANT'S SUMMARY AND CONCLUSION

Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study carried was carried out to investigate the teratogenic potential of copper-releasing intrauterine contraceptive devices on developing embryos. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

The study involved 18 healthy, fertile women, 11 of whom had conceived while using a copper-bearing IUD, and 7 of whom had conceived spontaneously with no previous history of using an IUD (these women constituted a control group). Between 7 and 12 weeks of gestation, an artificial abortion was induced in all 18 women. The reasons for this procedure were of a non-medical nature. All 18 embryos were removed without injury.

Embryos were fixed in neutral formalin and examined for gross malformation. They were then embedded in paraffin wax and sectioned sagitally at a thickness of 7 µm, allowing examination of the brain, eyes, inner ear, heart and lungs, liver, pancreas, mesonephron, kidneys, gonads, vertebrae and limbs. The sections were stained with haematoxylin and eosin. Fixation, embedding and staining of the placentae were similarly carried out. All sections were stained for copper in accordance with Uzman's procedure for the detection of granular precipitates.

To confirm the accuracy of this staining method, two mice were injected intraperitoneally and another was injected intravenously with 0.5 ml 2% $CuSO_4$. The internal organs were stained in accordance with Uzman's procedure. Copper aggregates were obtained in this control study by reduction of $CuSO_4$ with iron sulphate, whereby free aggregates remain in the solution. Injection of the diluted aggregates produced deposits of copper in the organs similar to those found after intraperitoneal injection of copper sulphate. This method facilitates the detection of copper deposits in embryonic tissues and placentae.

Maternal blood samples were taken on the day of the abortions and examined for plasma levels of copper and ceruloplasmin. Copper levels

Section A6.8.1	Teratogenicity Study
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	were determined by atomic absorption spectrophotometry. Ceruloplasmin was assessed using Richterich's method. The student t test was used to evaluate significant differences in means. Results are presented as the mean ± standard deviation.
Results and discussion	*Summarize relevant results; discuss dose-response relationship.* The maternal copper and ceruloplasmin plasma levels were within normal range, with no statistically significant difference between the study and control groups (163.4 ± 41.4 vs. 137.8 ± 37.9 µg % copper and 70.4 ± 14.9 vs. 69 ± 19.9 mg % ceruloplasmin). No organic malformations were found in the 11 embryos of either the study group or in the 7 controls. Alternating serial sections were examined histologically. The embryonic tissue was free of copper deposits, whether fine or coarse granular. The histodifferentiation of the organs demonstrated no abnormal findings in relation to embryonic age. The placentae of embryos in both groups were examined histologically. There was no structural impairment and no trace of copper deposits in the placentae. Copper deposits were, however, detected in the organs of mice injected with $CuSO_4$. Fine and coarse copper granulation was observed in the spleen, kidney, liver and peritoneum of these animals.
Conclusion	No foetal malformation was observed and there were no copper aggregates in the various organs and placentae. These findings suggest that copper-releasing intrauterine devices has no deleterious effect on foetal development.
LO(A)EL maternal toxic effects	*Give critical effect and dose/concentration* No maternal toxic effects were observed.
NO(A)EL maternal toxic effects	*Give dose/concentration, if necessary separately for males and females* No maternal toxic effects were observed.
LO(A)EL embryotoxic / teratogenic effects	*Give critical effect and dose/concentration* No embryotoxic / teratogenic effects were observed.
NO(A)EL embryotoxic / teratogenic effects	*Give dose/concentration* No embryotoxic / teratogenic effects were observed.
Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
Deficiencies	Yes. This study was not conducted and/or reported in compliance with GLP. When compared with generally accepted principles to be applied to embryotoxicity / teratogenicity studies, as set out in OECD guideline 414, it is also apparent that there were a number of deficiencies, including: • A relatively small number of subjects (the minimum number of

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/03	**A6.8.1(03), Teratogenicity of copper**

pregnant subjects recommended by the guideline is 16).

- No information is available on the absolute amounts of copper to which the women or their foetuses were exposed.

It should be noted, however, that the OECD guideline is written specifically for work in animals and that the factors highlighted above do not, therefore, necessarily compromise the validity of the data reported, or the author's interpretation of that data. Furthermore, this research was published in a peer-reviewed publication, and has been subject to the prior scrutiny of experts in the field. It has also been included in a number of expert reviews of the embryotoxic / teratogenic potential of copper.

Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of the embryotoxic / teratogenic potential of copper. A reliability indicator of 2 has been assigned on this basis.

(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/03	**A6.8.1(03), Teratogenicity of copper**

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date	[redacted]
Materials and Methods	[redacted]
Results and discussion Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	[redacted]

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**

Official use only

218 REFERENCE

218.1 Reference

Chang, C.C. And Tatum, H.J. (1973). Absence of teratogenicity of intrauterine copper wire in rats, hamsters and rabbits. Contraception, **7(5):** 413 - 434 (published).

Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).

218.2 Data protection

No.

(indicate if data protection is claimed)

218.2.1 Data owner

Give name of company

Public domain.

218.2.2 Companies with letter of access

Give name of company/companies which have the right to use these data on behalf of the data owner (see TNsG in support of AnnexVI)

Letter of access not required.

218.2.3 Criteria for data protection

Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:

No data protection claimed.

219 GUIDELINES AND QUALITY ASSURANCE

219.1 Guideline study

No. This was a non-regulatory study carried out to determine whether copper wire, placed within the uterus after implantation and kept *in situ* throughout pregnancy, produced any teratogenic effects on the embryo, or alters in any way development and subsequent growth of the offspring.

(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

219.2 GLP

No. This was a non-regulatory study. Furthermore, GLP was not compulsory at the time the study was performed.

(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

219.3 Deviations

Yes. Refer to section 4.3.4 for a general discussion of deviations and deficiencies.

(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

220 MATERIALS AND METHODS

In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.

220.1 Test material

Cu^{2+} as copper wire

Or give name used in study report

220.1.1 Lot/Batch number *List lot/batch number if available*

Not available.

220.1.2 Specification

Deviating from specification given in section 2 as follows

(describe specification under separate subheadings, such as the

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**

following; additional subheadings may be appropriate):

220.1.2.1 Description — *If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)*

Copper wire, 0.1 mm in diameter.

220.1.2.2 Purity — *Give purity in % active substance*

[redacted]

220.1.2.3 Stability — *Describe stability of test material*

Not applicable to inorganic substances.

220.2 Test Animals — Non-entry field

220.2.1 Species — Rat, hamster and rabbit.
if other, state reason for non standard species

220.2.2 Strain — **Rat:** Holtzman strain.
Hamster: Not stated.
Rabbit: New Zealand White.

220.2.3 Source — **Rat:** Not stated.
Hamster: Lakeview Hamster Colony, Newfield, New Jersey. **Rabbit:** Not stated.

220.2.4 Sex — Male and female.

220.2.5 Age/weight at study initiation — **Rat:** Weight 180 – 220 g; age not stated.
Hamster: Weight 100 – 120 g; age not stated.
Rabbit: Weight not stated; age 9 - 91/2 months.

220.2.6 Number of animals per group — *Give number*

Treatment Group (Parent Generation)	Species		
	Rat	Hamster	Rabbit
Copper	12	11	9
Control	7	6	5

should be enough to yield 20 pregnant females per group

220.2.7 Mating — The mating procedure and the symbols designated to descendants of either copper wire treated parent or control parent are shown in **Figure A6.8.1(04)-3**.

Rat P generation: 19 females were mated with untreated males. Of these females, copper wire was inserted into the endometrial cavities of both uterine horns of 12 animals. The remaining 7 animals were considered to be untreated controls.

Rat F_1 generation: When F_1 females reached the age of 90 days, each was cohabited with one fertile male for 10 days. When F_1 males reached the age of 120 days, each was cohabited with 2 virgin cycling females for 10 days.

Section A6.8.1	**Teratogenicity Study**	
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*	
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**	
	Rat F_2 generation: F_2 animals were tested in the same manner as F_1 animals, giving rise to an F_3 generation. **Hamster P generation:** 17 females were mated with untreated males. Of these females, copper wire was inserted into the endometrial cavities of both uterine horns of 11 animals. The remaining 6 animals were considered to be untreated controls. **Hamster F_1 generation:** When F_1 females reached the age of 90 days, each was cohabited with one fertile male for 10 days. When F_1 males reached the age of 120 days, each was cohabited with 2 virgin cycling female for 10 days. **Hamster F_2 generation:** F_2 animals were tested in the same manner as F_1 animals, giving rise to an F_3 generation. **Rabbit P generation:** 14 females were mated with untreated males. Of these females, copper wire was inserted into the endometrial cavities of both uterine horns of 9 animals. The remaining 5 animals were considered to be untreated controls. **Rabbit F_1 generation:** When F_1 animals reached the age of 71/2 - 8 months, each was cohabited with normal animals. **Rabbit F_2 generation:** No F_2 generation was bred in the rabbit.	
220.2.8 Duration of mating	*2 weeks or other* 10 days for mating of F_1 and F_2 animals.	
220.2.9 Deviations from standard protocol	*i.e. second mating of parent or F1 generations, standardisation of litter size.* Refer to section 6.5.5.	X
220.2.10 Control animals	Yes	
220.3 Administration/ Exposure	Intrauterine *Fill in respective route in the following, delete other routes*	
220.3.1 Animal assignment to dosage groups	*See table below* Not applicable.	
220.3.2 Duration of exposure before mating	None *10 weeks or other (mice at least 56 days, rats 70 days)*	
220.3.3 Duration of exposure in general females only). P, F1, F2 males, females	**Rat:** From day 6 of pregnancy until sacrifice of parent (P generation **Hamster:** From day 6 of pregnancy until sacrifice of parent (P generation females only). **Rabbit:** From day 7 of pregnancy until sacrifice of parent (P generation females only). F_1 and F_2 generation animals were not exposed to copper wire.	
220.3.4 Method of exposure	**Intrauterine** **Rat and Hamster:** Copper wire was inserted in the endometrial cavities of both uterine horns of rats and hamsters on Day 6 of pregnancy. The wire was inserted by means of a half-circle suture needle through the antimesometrial surface about 5 mm below the uterotubal junction and led through the uterine lumen and brought out 2 – 3 mm below the original entry. The two ends were tied together, thus	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**
	making a ring 5 – 7 mm in diameter. The surface area of copper wire within the uterine cavity was approximately 3 mm^2. **Rabbit:** Copper wire was inserted into the uterine horns on day 7 of pregnancy at a position approx. 2 cm below the utero-tubal junction and a ring of 1 – 1.5 cm in diameter was made. This provided a surface area of the copper wire within the uterus of approximately 6 mm^2.
220.3.5 Vehicle	None.
220.3.6 Copper dose received by test animals.	**Rat and Hamster:** It was estimated that the rate of dissolution of the wire was approximately 2.75 µg per day. **Rabbit:** It was estimated that the rate of dissolution of the wire was approximately 5.50 µg per day.
220.3.7 Controls	Untreated animals (no copper wire introduced).
220.4 Examinations	Non-entry field.
220.4.1 Clinical signs	Yes
220.4.2 Body weight	Yes
220.4.3 Food/water consumption	No
220.4.4 Oestrus cycle	No
220.4.5 Sperm parameters testis weight	
220.4.6 Offspring	number and sex of pups presence of gross anomalies weight gain physical or behavioural abnormalities Survival rate at time of weaning.
220.4.7 P, F_1 and F_2 organ weights	uterus ovaries testis ventral prostate (rat and hamster only) Epididymi (rabbit only) seminal vesicles adrenal glands
220.4.8 Histopathology P (females only), F_1 and F_2 (males and females).	uterus ovaries adrenal glands testis ventral prostate (rat and hamster only) Epididymi (rabbit only) seminal vesicle

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**
220.4.9 Histopathology P (females only), F_1 and F_2 (males and females) not selected for mating.	uterus ovaries adrenal glands testis ventral prostate (rat and hamster only) Epididymi (rabbit only) seminal vesicle
220.5 Further remarks Gestation period	
	RESULTS AND DISCUSSION *Describe findings. If appropriate, include table. Sample tables are given below.*
220.6 Effects	**Table A6.8.1(04)** has been amended to take account of available data. Copies of tables taken from the published report are appended, as follows: **Table A6.8.1(04)-1**: Effect of Copper Wire on Pregnancy and Parturition in Rats; **Table A6.8.1(04)-2**: Survival of F_1 Generation Rats at the Time of Weaning (25 days old); **Table A6.8.1(04)-3**: Organ Weights of F_1 Generation Rats; **Table A6.8.1(04)-4**: Organ Weights of F_2 Generation Rats; **Table A6.8.1(04)-5**: Effect of Copper Wire on Pregnancy and Parturition in Hamsters; **Table A6.8.1(04)-6**: Organ Weights of F_1 Generation Hamsters; **Table A6.8.1(04)-7**: Organ Weights of F_2 Generation Hamsters; **Table A6.8.1(04)-8**: Effect of Copper Wire on Pregnancy and Parturition in Rabbits; **Table A6.8.1(04)-9**: Organ Weights of F_1 Generation Rabbits. Growth rate data presented in graphical form are appended as follows: **Figure A6.8.1(04)-1**: Growth rates of F_1 Generation Female Rats; **Figure A6.8.1(04)-2**: Growth rates of F_1 Generation Male Rats.
220.6.1 Parent males	*No effects / describe significant effects referring to data in results table* **Rat:** Not applicable. Parental males were not treated. **Hamster:** Not applicable. Parental males were not treated. **Rabbit:** Not applicable. Parental males were not treated.
220.6.2 Parent females	*No effects / describe significant effects referring to data in results table* **Rat:** There was no difference in gestation periods between the mothers bearing the wire in the uterus and the controls (23 and 22.5 days,

respectively). All copper wire-treated and control mothers delivered normally. However, a comparison of the average number of pups delivered from treated mothers to those from untreated rats showed that

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**

~~the copper wire-treated females delivered 6.5 ± 0.7 pups, a number~~ significantly lower than that of the untreated controls (8.6 ± 0.6) at the 5% confidence level. Since the rat blastocysts are spaced along the longitudinal axis of the endometrial wall and may implant in the immediate vicinity of the utero-tubal junction, it seems likely that the incidence of fewer pups in the treated group was due to manipulation of the uterus and damage to the embryos at or near the site when the copper wire was inserted.

At autopsy, there were no gross anatomical deformities noted in female parents. Histological examination of the ovaries, uteri and adrenals did not show deviation from normal.

There was no evidence of teratogenic effects in pups of either sex. No abnormalities were observed at birth, at weaning or at fertility testing. There was no effect on survival rates of the F_1 generation animals at the time of weaning. Survival rates of the descendants of treated and untreated mothers indicate that lactation was not interrupted by the wire. Pups grew normally, as evidenced by the fact that the increase in body weight measured at 5-day intervals from 5 days up to 60 days of age in treated animals was similar to that in untreated animals.

Hamster: Copper wire had little effect on gestation or parturition when the wire was inserted into the uterine lumen after implantation. There was no difference in the average number of pups born between the group bearing copper wire and the control group (6.9 vs. 6.7). The gestation period for treated animals was not different from the controls (17 vs. 16.5 days). Lactation in treated mothers was considered to be normal, using as the criteria the average body weight of the pups and the percentage of loss of pups at weaning (25 days of age).

No teratogenic effects were observed in the F1 generation animals at birth and at weaning. Histological examination of the ovaries, uteri and adrenals of mothers with copper wire showed no deviation from normal.

Rabbit: At the time of insertion of the copper wire (Day 7 of pregnancy) there was no difference in the average number of implantation sites between the animals which were to be exposed to copper wire and the controls. However, at laparotomy on Day 15 of pregnancy (laparotomy was done only in animals bearing copper wire), the number of implantation sites was significantly less than that observed on Day 7 of pregnancy. The number of pups subsequently delivered from these animals was reduced as compared to that in the control animals (32.4% vs. 44.2%). This difference is thought to be due to manipulation of the uterus at the time of insertion of the copper wire.

There were no gross anatomical deformities noted in the F_1 generation at birth, at weaning or at autopsy.

The Parent females were autopsied after weaning. Histological examination of the ovaries, uteri and adrenals showed no deviations from normal.

Section A6.8.1 **Teratogenicity Study**

Annex Point IIA6.8.1 *Specify section no., heading, route and species as appropriate*

IUCLID: 5.8.2/04 **A6.8.1(04), Teratogenicity of copper**

Rat: The fertility of the F1 males was tested when the animals reached the age of 120 days. Each male was cohabited with two virgin cycling females for 10 days. Fourteen of 15 males (A') born to Parent mother bearing copper wire and 6 of 7 males (B') born to Parent untreated controls mated. The average number of pups born to group A' males was 9.3 ± 0.6 as compared to the average number of 8.8 ± 0.7 for group B' males.

The weights of the reproductive organs of the F1 generation animals are shown in **Table A6.8.1(04)**. There were no significant differences in organ weights of male offspring of copper wire-treated and untreated mothers. Since 93% of the animals in group A' have normal fertility, it is not surprising that the weights of their reproductive organs are comparable to those of the animals in group B'.

No gross anatomical deformities were noted at autopsy in F1 males. Histological examination of tissues showed no deviation from normal.

Hamster: There was no apparent effect on fertility of offspring of treated and untreated mothers in males of the F1 generation. Seven of 10 males in group A' and 6 of 10 males in group B' were mated with normal females. The average number of pups was 8.0 ± 0.8 in group A' and 6.4 ± 1.2 in group B'. These differences are not significant.

F1 generation males were autopsied at the age of 155-160 days. Weights of the reproductive organs are shown in **Table A6.8.1(04)**. There were no significant differences in organ weights of male offspring of copper wire treated and untreated mothers. Although s slight decrease was noted in the weight of the testes in these animals, fertility appeared not to be affected (although only 10 randomly selected animals from the total 32 were used). In addition, the weights of accessory sex organs, such as seminal vesicles and ventral prostates, increased in this group indicating that from a hormonal viewpoint, the function of the testes was not subnormal.

At autopsy, there were no demonstrable macroscopic anatomical deformities in F1 males. Histological examination of the tissues of F1 males showed no abnormalities.

Rabbit: The fertility of F1 generation males was tested when the animals reached the age of 7V2 - 8 months by mating with normal animals. The average number of implantation sites in females mated with A' males was 7.2 (range 1 - 10). This represents a normal degree of fertility.

Some of the F1 generation animals were autopsied at either the age of 3 or 6 months. No teratogenic effects were observed in these animals and their growth rate was normal. The reproductive organs were fixed for histological examination. The remaining animals were autopsied at the age of 8V2 months. There were no significant differences in body weight and organ weights between the male F1 generation animals from copper

treated and untreated mothers. Histological examination of the male reproductive tissues of F1 generation animals showed no deviations from normal.

Copper Oxide

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**
220.6.4 F1 females	*No effects / describe significant effects referring to data in results table*

Rat: The fertility of F1 females was tested when the animals reached the age of 90 days. Each female was housed with one fertile male for 10 days. Thirty-three of 38 females (A) born to Parent mother bearing copper wire, and 11 of 16 females (B) born to Parent untreated controls were mated successfully. The average number of pups was 10.1 ± 0.5 in group A as compared to the average of 8.5 ± 0.9 pups in group B. It was apparent that there were no significant differences in fertility of offspring of copper treated and untreated mothers of either sex in the F1 generation.

The weights of the reproductive organs of the F1 generation animals are shown in **Table A6.8.1(04)**. The females were autopsied after weaning their pups (F2 generation) at 125-130 days of age. There were no significant differences in organ weights of female offspring of copper wire treated and untreated mothers. Since 86% of the animals in group A have normal fertility, it is not surprising that the weights of their reproductive organs are comparable to that of the animals in group B.

No gross anatomical deformities were noted at autopsy in F1 females. Histological examination of tissues showed no deviation from normal.

Hamster: There was no apparent effect on fertility of offspring of treated and untreated mothers in females of the F1 generation. Twelve of the 16 females in group A and 7 of 7 females in group B were mated and delivered normally. The average number of pups was 7.9 ± 0.8 in group A and 7.8 ± 0.9 in group B. These differences are not significant. X

F1 generation females were autopsied at the age of 145-150 days. Weights of the reproductive organs are shown in **Table A6.8.1(04)**. There were no significant differences in organ weights of female offspring of copper wire treated and untreated mothers.

At autopsy, there were no demonstrable macroscopic anatomical deformities in F1 females. Histological examination of the tissues of F1 females showed no abnormalities.

Rabbit: The fertility of F1 generation females was tested when the animals reached the age of 7V2 - 8 months by mating with normal animals. The average number of implantation sites in A females was 7.5 (range 5 - 10). This represents a normal degree of fertility.

Some of the F1 generation animals were autopsied at either the age of 3 or 6 months. No teratogenic effects were observed in these animals and their growth rate was normal. The reproductive organs were fixed for histological examination. The remaining animals were autopsied at the

	age of 8V2 months. There were no significant differences in body weight and organ weights between the female F_1 generation animals from copper treated and untreated mothers. Histological examination of the female reproductive tissues of F_1 generation animals showed no deviations from normal.
220.6.5 F_2 males	*No effects / describe significant effects referring to data in results table*

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.8.2/04	**A6.8.1(04), Teratogenicity of copper**

Rat: The F_2 generation males were examined grossly at birth for possible malformations. The animals were weaned at 25 days of age and some were eliminated when they reached the age of 60 days. Some were autopsied and the reproductive organs preserved for histological study. The remaining animals were used for fertility testing at age 120 days. The mating procedures for F_2 generation animals were the same as those of the F_1 generation. Offspring of F_1A' and F_1B' males were designated A'A' and B'B' females and A'A' and B'B' males, respectively.

All F_2 generation animals used for assessment of fertility, were mated. The average number of implantation sites in each group was as follows: A'A' females, 10.0 ± 1.0; B'B' females, 11.5 ± 0.5; A'A' males, 10.5 ± 0,3; and B'B' males, 10.7 ± 0.4. These results show that there were no significant differences in fertility among F_2 generation descendants of copper wire treated and untreated animals.

The organ weights of F_2 generation males are shown in **Table A6.8.1(04)**. There were no significant differences in body weights or organ weights. The autopsy data obtained for F_2 male rats were in the normal range.

No gross anatomical deformities were noted at autopsy in F_2 males. Histological examination of tissues showed no deviation from normal.

Hamster: F_2 generation males were examined macroscopically at birth and at weaning for possible malformations. At weaning, some animals were eliminated and others were eliminated when they reached the age of 45-50 days. Some were autopsied and the reproductive organs were fixed for histological examination. Some of the remaining animals were used for fertility testing when the males reached 120 days of age. The mating procedures were the same as in the F_1 generation.

The results show that there were no differences in the fertility of AA or A'A' males. However, the average number of pups delivered in AA males (descendants of copper treated Parent) was significantly lower than that of normal animals (3.1 ± 0.6 vs. 7.9 ± 0.8). The cause for this difference in the F_2 generation is not known.

The body weight and organ weights of F_2 generation males are shown in **Table A6.8.1(04)**. There were no significant differences in the body weight and organ weights, with the exception of the adrenal weights. The adrenal weights of the A'A' males were significantly increased. At autopsy, there were no demonstrable macroscopic anatomical deformities in F_2 males. Histological examination of the tissues of F_2 males showed no abnormalities.

Rabbit: Not applicable

220.6.6 F_2 females

No effects / describe significant effects referring to data in results table

Rat: The F_2 generation females were examined grossly at birth for possible malformations. Animals were weaned at 25 days of age and some were eliminated when they reached the age of 60 days. Some were autopsied and the reproductive organs were preserved for histological study. The remaining animals were used for fertility testing

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~~at an age of 90 days. The mating procedures for the F2 generation~~ animals were the same as those of the Fl generation. Offspring of F_1A and F_1B females were designated as AA and BB females, and AA and BB males, respectively.

All F_2 generation animals used for assessment of fertility, were mated. The average number of implantation sites in each group was as follows: AA females, 12.8 ± 0.7; BB females, 10.0 ± 1.1; AA males, 10.9 ± 0.5; BB males, 11.1 ± 0.5. These results show that there were no significant differences in fertility among F_2 generation descendants of copper wire treated and untreated animals.

The organ weights of F_2 generation females are shown in **Table A6.8.1(04)**. There were no significant differences in body weights or organ weights. The autopsy data obtained for F_2 female rats were in the normal range.

No gross anatomical deformities were noted at autopsy in F_2 females. Histological examination of tissues showed no deviation from normal.

Hamster: F_2 generation females were examined macroscopically at birth and at weaning for possible malformations. At weaning, some animals were eliminated and others were eliminated when they reached the age of 45-50 days. Some were autopsied and the reproductive organs were fixed for histological examination. Some of the remaining animals were used for fertility testing when the females reached 90 days of age. The mating procedures were the same as in the F_1 generation.

The results show that there were no differences in the fertility of AA or A'A' females. However, the average number of pups delivered in BB females (descendants of control Parent) was significantly lower than that of normal animals (2.0 ± 1.0 vs. 7.8 ± 0.9 for the female Parents). The cause for this difference in the F_2 generation is not known.

The body weight and organ weights of F_2 generation females are shown in **Table A6.8.1(04)**. There were no significant differences in the body weight and organ weights.

At autopsy, there were no demonstrable macroscopic anatomical deformities in F_2 females. Histological examination of the tissues of F_2 females showed no abnormalities.

Rabbit: Not applicable.

220.7 Other

Describe any other significant effects

None.

221 APPLICANT'S SUMMARY AND CONCLUSION

221.1 Materials and methods

Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines

A study was carried out to determine whether copper wire, placed within the uterus after implantation and kept in situ throughout pregnancy, produced any teratogenic effects on the embryo, or altered in any way the development and subsequent growth of the offspring of rats, hamsters and rabbits. The potential for adverse effects on the fertility of treated animals was also assessed.

Section A6.8.1 **Teratogenicity Study**

Annex Point IIA6.8.1 *Specify section no., heading, route and species as appropriate*

IUCLID: 5.8.2/04 **A6.8.1(04), Teratogenicity of copper**

Nulliparous female rats of the Holtzman strain (bodyweights in the range 180-220 g); adult cycling female hamsters (bodyweights in the range 100 - 120 g); and adult albino New Zealand female rabbits (age 9 to 91/2 months old) were used. The animals were housed in temperature (24.5 – 26.7°C) and illumination (14 hr light and 10 hr dark) controlled rooms and maintained on standard laboratory chow specific to each species. Tap water was provided *ad libitum*.

In rats and hamsters, positive matings were verified by the presence of sperm in vaginal smears. The day of insemination was designated as Day 1 of pregnancy. In rabbits, visual observation only was used to confirm copulation, and that day was designated as Day 0 of pregnancy.

Copper wire (99.9% pure, 0.1 mm in diameter) was inserted into the endometrial cavities of both uterine horns of rats and hamsters on Day 6 of pregnancy. The wire was inserted by means of a half-circle suture needle through the antimesometrial surface about 5 mm below the uterotubal. junction and led through the uterine lumen and brought out 2-3 mm below the original entry. The two ends were tied together, thus making a ring 5-7 mm in diameter. The surface area of copper wire within the uterine cavity was approximately 3 mm^2. It was estimated that the rate of dissolution of the wire used in the cycling rat was approximately 2.75 µg per day.

In rabbits, the wire was inserted into the uterine horns on Day 7 of pregnancy at a position approximately 2 cm below the utero-tubal junction and a ring of 1-1.5 cm in diameter was made. This provided a surface area of the copper wire within the uterus of approximately 6 mm^2. The amount of copper released in 24 hrs from the wire was estimated to be approximately 5.50 µg on the assumption that the rate of dissolution of the wire used in the rabbit is similar to that in the rat.

The wire was left *in situ* during pregnancy and lactation, and the gestation period was recorded. The mothers were sacrificed at the time of weaning and the ovaries, uteri and adrenals were fixed with Bouin's solution for histological examination. The number and sex of the pups of rats and hamsters were recorded at birth and the offspring were observed for gross abnormalities. The body weight of F_1 generation rats was recorded at 5-day intervals from the age of 5 days through 60 days. The offspring of rats and hamsters were weaned at the age of 25 days and the number of surviving F_1 generation was recorded. In the meantime, the females were separated from the males and maintained in separate cages to raise F_2 and F_3 generations. In rabbits, laparotomy was done on Day 15 of pregnancy and the number of implantation sites was recorded. The offspring were weaned when 30-35 days of age. Some of the F_1 generation rabbits were sacrificed at the age of either 3 or 6 months.

When the F_1 generation rat and hamster females reached the age of 90 days and the males 120 days, each female was cohabited with one fertile male and each male with 2 virgin cycling females for 10 days. The fertility of the F_1 generation animals was evaluated by the following regimens: a) the ratio of the animals mated over the animals used and b) the number of implantation sites or the number of pups delivered. Some of the animals delivered by the F_1 generation were eliminated at the time of weaning and examined for gross malformations. The remaining

Section A6.8.1	**Teratogenicity Study**
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animals were used for fertility testing when they reached maturity. The fertility of F_2 generation animals was tested in a manner similar to that described for the F_1 generation.

At autopsy, the body weight and the weights of the following organs were determined: ovaries, uteri and adrenals in the females; testes, seminal vesicles, epididymus (in the rabbit only), ventral prostate and adrenals in the males. All tissues were fixed in Bouin's solution. Histological sections were stained with haematoxylin and eosin. All the results obtained were analysed statistically using Student's t-test. A probability of less than 0.05 was considered as statistically significant.

221.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Rats: There was no difference in gestation periods between the mothers bearing the wire in the uterus and controls. All copper wire treated and control mothers delivered normally. However, a comparison of the average number of pups delivered from treated mothers to those from untreated rats showed that the copper wire treated females delivered 6.5 ± 0.7 pups, a number significantly lower than that of the untreated controls (8.6 ± 0.6) at the 5% confidence level. Since rat blastocysts are spaced along the longitudinal axis of the endometrial wall and may implant in the immediate vicinity of the utero-tubal junction, it is considered likely that the incidence of fewer pups in the treated group was due to manipulation of the uterus and damage to the embryos at or near the site when the copper wire was inserted.

No teratogenic effects were evident in offspring. No abnormalities were observed at birth, at weaning or at the time of the fertility test. There was no effect of copper wire on survival rates of the F_1 generation animals at the time of weaning. Survival rates of the descendants of treated and untreated mothers indicates that lactation was not interrupted by the wire. F_1 generation animals of both sexes grew normally, as evidenced by the increases in body weight. There were no significant differences in fertility of offspring of copper treated and untreated mothers of either sex in the F_1 generation. There were no significant differences in organ weights of offspring of copper wire treated and untreated mothers in either sex of the F_1 generation.

There were no significant differences in fertility among F_2 generation descendants of copper wire treated and untreated animals. There were no significant differences in body weights or organ weights in either sex of the F_2 generation.

At autopsy, there were no gross anatomical deformities noted in Parent, F_1 or F_2 generations. Histological examination of the ovaries, uteri and adrenals of Parent females, and of female and male tissues of F_1 and F_2 generations did not show deviations from normal.

Hamsters: There was no difference in the average number of pups born between the group bearing copper wire and the control group. The gestation period for treated animals was not different from the controls. Lactation in treated mothers was considered to be normal, based on the average body weights of pups and the percentage lost at weaning. No teratogenic effects were observed in the F_1 generation animals at birth and at weaning. Histological examination of the ovaries, uteri and adrenals of mothers with copper wire showed no deviation from normal.

Section A6.8.1	**Teratogenicity Study**	
Annex Point IIA6.8.1	*Specify section no., heading, route and species as appropriate*	
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	There was no apparent effect on the fertility of offspring of treated and untreated mothers in either sex of the F_1 generation. There were no significant differences in organ weights of offspring of copper wire treated and untreated mothers in either sex of the F_1 generation.	
	There were no significant differences in fertility among F_2 generation descendants of copper wire treated and untreated animals. There were no significant differences in body weights or organ weights in either sex of the F_2 generation, other than an unexplained increase in the adrenal weights of control males.	X
	At autopsy, there were no gross anatomical deformities noted in Parent, F_1 or F_2 generations. Histological examination of the ovaries, uteri and adrenals of Parent females, and of female and male tissues of F_1 and F_2 generations did not show deviations from normal.	
	Rabbits: At the time of insertion of the copper wire (Day 7 of pregnancy), there was no difference in the average number of implantation sites between the animals which were to be exposed to copper wire and the controls. However, at laparotomy on Day 15 of pregnancy, the number of implantation sites was significantly less than that observed on Day 7 of pregnancy. The number of pups subsequently delivered from these animals was reduced as compared to that in the control animals. This difference was thought to be due to manipulation of the uterus at the time of insertion of the copper wire.	
	There were no gross anatomical deformities noted in F_1 generation at birth, at weaning or at autopsy. The fertility of F_1 generation was normal.	
	The Parent females were autopsied after weaning. Histological examination of the ovaries, uteri and adrenals showed no deviations from normal.	
	No teratogenic effects were observed in F_1 generation animals and their growth rate was normal. There were no significant differences in body weight and organ weights between the F_1 generation animals of either sex from copper treated and untreated mothers. Histological examination of the female and male reproductive tissues of F_1 generation animals showed no deviations from normal.	
221.3 Conclusion	The fertility of rats, hamsters and rabbits of the parent, F_1 and F_2 generations was unaffected by exposure of parent animals to copper from wire placed into the uterus after implantation of embryos. Similarly, no adverse effects (teratogenicity or growth and development) attributable to the exposure of parent females to copper were seen in F_1 or F_2 animals.	
221.3.1 LO(A)EL	*Give critical effect and dose/concentration*	
	Non-entry field	