occur. Copper accumulation in the brain causes degeneration of the basal ganglia, resulting in defective movement, slurred speech, difficulty in swallowing, facial and other muscular spasms, dystonia and poor motor control. Depression and schizophrenia have been reported. Copper may also be deposited in the cornea (Kayser-Fleischer rings).

Menkes disease is an X-linked copper deficiency disease that is usually fatal in early childhood. It is usually present in males, but has been recorded in eight females (cases have been cited where genetic translocation was noted in a female). The frequency in the human population is stated as 1 in 100,000 to 1 in 250,000 live births. The symptoms result from a defect in the MNK protein, producing an inability to export copper from cells, particularly from the basal membrane of the small intestine, where copper is absorbed (see LEEMING, N.M., 2003; reference A6.2/01). This leads to very high concentrations of copper in sloughed intestinal cells, but the failure to export the 'absorbed' copper to the bloodstream results in an effective copper deficiency for the rest of the body. The disease shows progressive mental retardation, hypothermia, seizures, poor muscle tone, feeding difficulties, jaundice, diarrhoea and a general failure to thrive. There are abnormalities of connective tissue with deformities of the skull, long bones and ribs. The hair is abnormal with a wiry texture and a spiral twist.

Both diseases result from genetic defects where the subject is unable to produce respectively the copper ATPases ATP7B and ATP7A. These are members of the human cation-transporting P-type ATPase family. The P-type ATPases are a large group of membrane proteins that utilise the energy of ATP hydrolysis to transport various ions across cell membranes. During the catalytic cycle the γ-phosphate of ATP is transferred to the invariant aspartic acid residue within the nucleotide-binding site of ATPase with the formation of acylphosphate intermediate: this property distinguishes the P-type ATPases from other cation-transporting pumps. Over 100 P-type ATPases have been described. The loci of the encoding genes have been identified for both WD and MD. Both pump copper across cell membranes. The MD pump (ATP7A) is the pump that actually moves copper through the basal membrane of the intestinal epithelial cells so that copper enters the hepatic portal system where it binds to albumin, transcuprein and histidine to reach the liver. In the MD subject, ATP7A is inactive, and copper from the diet accumulates in the intestinal epithelial cells, bound to induced metallothionein. The presence of copper within the cell induces the production of more metallothionein, and the copper-metallothionein complex accumulates during the life of the cell. When the cells are sloughed off into the intestinal lumen, as is the normal course of events, the cells and the copper within them are excreted in the faeces, and the copper is lost to the body. Subjects with Menkes' disease can still absorb small amounts of copper. Copper accumulates in fibroblasts and in the kidney of Menkes' disease subjects, but there is no evidence of increased incidence of cancer in these tissues either. Menkes' disease is effectively a disease of copper deficiency. In terms of risk assessment of copper in the normal human, the accumulation of copper in the intestinal epithelium on Menkes' subjects can be considered as the equivalent of an excessive oral dose of copper to the epithelial cells.

Wilson's disease (WD) involves the other ATPase previously referred to, ATP7B. In normal humans, this enzyme is primarily active in hepatocytes. It is involved in the trans-Golgi network (TGN). Copper absorbed by the hepatocyte via the inbound membrane pump hCTR1 (human copper transporter protein 1, see LEEMING, N.M., 2003; reference A6.2/01) and is bound to metallothionein within the cell. It may be bound by ATP7B to ceruloplasmin (a protein that binds up to 6 copper ions tightly and transports them to various tissues for use, including the brain. If there is excess copper in the hepatocyte, ATP7B is induced to traffic to vesicular compartments (lysosomes) and directly to the apical membrane, where copper is secreted from the cell bound to a trypsin-independent fragment of ceruloplasmin and excreted in the bile. In WD, ATP7B is inactive and the absorbed copper accumulates in the hepatocytes bound to metallothionein. The bile of WD subjects does not contain copper. In the hepatocyte, excess copper may accumulate in mitochondria, in the cytoplasm and in lysosomes, bound to metallothionein. Eventually the cell's copper storage capacity is exceeded. Mitochondrial damage occurs and eventually the hepatocyte dies, whence the cell contents are released to the circulation, depositing copper in extrahepatic tissues.

Wilson's disease thus leads to massive accumulation of copper in the liver. The disease usually manifests in late adolescence, and is ultimately fatal if not treated from liver failure. Treatment involves administration of penicillamine, which forms a copper complex capable of urinary excretion. Accumulation of copper leads to cell death, but this is only in the presence of excessive copper concentrations, brought about by a genetic condition resulting in the disruption of the natural homeostatic mechanisms for copper. It should be noted that Wilson's disease is genetic, and the accumulation of copper and resulting liver failure occur under the natural levels of copper in the diet, not as a result of exposure to excessive levels of copper in the environment. However, the accumulation of copper in the liver may be taken as a model for accumulation of excess copper in a toxicity study.

As with short-term toxicology it is considered appropriate to present data on the active substance, the copper ion, rather than the formulated or technical materials. A metabolism/bioequivalence study has been performed to demonstrate that the ion, as present in the form of cupric sulphate pentahydrate, is similarly bioavailable from several different copper compounds, and other forms that liberate the copper ion may therefore also be used in the risk assessment process (HIMMELSTEIN, M., 2004; reference A6.2/02).

(5) No data are also presented on the mouse. However, short-term studies on the mouse show that the mouse is much more tolerant of higher doses of copper than the rat (HÉBERT, C.D., 1993; reference A6.4.1/01, and that the mouse does not show the histological changes in the liver and kidney that are seen in the rat. As the rat shows lower short term NOELs than the mouse, it is logical to assume that the NOELs from long term mouse studies would be higher than in the rat. Therefore, data from long term mouse studies would not be used in the risk assessment for setting values such as the ADI or the AOEL. As stated previously, there are human diseases that lead to chronic, lethal accumulations of copper in target tissues, but no evidence for tumour formation. Further carcinogenicity and chronic toxicity studies on

animals are considered unnecessary.

Conclusion for long-term toxicity:

Copper is an essential nutrient, naturally present in almost all foodstuffs. As such the population is exposed to copper in the diet every day. The various natural mechanisms for regulating copper in humans were described in Section 5.1. It is also instructive to consider the consequences and effects of genetic conditions that affect copper regulation, as these conditions lead to accumulation of copper in various tissues. Accumulation of chemicals in 'target organs', particularly the liver, can be associated with toxicity, and in the case of carcinogens, tumour formation. There are two genetic conditions in the human (Wilson's disease and Menkes' disease) that result in major alterations in copper absorption, distribution and excretion. Wilson's disease (where copper is absorbed in the intestine but cannot be pumped out of the liver to bile) leads to accumulation of copper in the principal target organ, the liver, and also in the kidney, brain and the cornea of the eye. People with Menkes' disease (where copper is absorbed by intestinal cells but cannot be pumped out of these cells to the hepatic portal system) can only absorb minimal amounts of copper, and show chronic accumulation of copper in the intestinal epithelium and high levels in kidney and in fibroblasts. Human subjects with these conditions may die of the condition itself (if untreated), but they do not show increased incidence of any cancer. This is significant for the risk assessment of copper. If abnormally high levels of copper are present over long periods in an organ or tissue, yet there is no association between the high copper levels and cancer in these organs or tissues, in chronic disease, then it is reasonable to conclude that copper is not carcinogenic in these tissues. It is also reasonable to conclude that as copper levels in normal humans are actively controlled by homeostatic mechanisms, copper will not accumulate in other organs or tissues. If it does not accumulate, it cannot cause any illness/ long-term toxic effects, including increased risk of cancer.

There are studies in rats that describe the effects of long term administration of copper in various forms. These studies include typical long-term toxicity studies, special studies to investigate effects of copper when administered together with known carcinogens, and special long term studies to investigate specific effects and adaptations to prolonged administration of high levels of copper. These are listed in Table A6.5- 7 to give a review.

Long-term toxic effects due to elevated copper intake are considered to be exhaustively covered by reference A6.5/01. For the reasons given above, as supported by the cited studies, long-term toxicity testing in a second species is not considered to be necessary.

Undertaking of intended data submission [ ]

Evaluation by Competent Authorities		
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)	
Date	10/06/05	
Evaluation of applicant's justification	Agree with applicant version	
Conclusion	Waving agreed	
Remarks		
	COMMENTS FROM ...	
Date		
Evaluation of applicant's justification		
Conclusion		
Remarks		

Table A6.5- 7: **Summary of long term toxicity.**

Study type	Species	Route	Test material, Dose levels	NOEL	Reference
Carcinogenicity	Rat	Diet	Copper sulphate, 530, or 1600 ppm (= 27 or 80 mg Cu/kg bw/day) Potassium sodium copper chlorophyllin 0.1, 1 or 3% (= 2.7, 27 or 80 mg Cu/kg bw/day) Copper gluconate 1600 ppm (= 80 mg Cu/kg bw/day)	Not carcinogenic. NOEL for Potassium sodium copper chlorophyllin 3% (=80 mg Cu/kg bw/day) NOAEL for copper sulphate = 27 mg Cu/kg bw/day	**A6.5/01**
Carcinogen co-administration	Rat	Diet	*p*-dimethylaminobenzene at 0.9% diet with or without 0.5% copper acetate or 2% ferric citrate	Co-administration of copper markedly reduced the incidence of liver tumours caused by *p*-dimethylaminobenzene	Cross-reference: **A6.7/01**
Special study	Rat	Diet	Copper sulphate 2000 ppm Cu (= 200 mg Cu/kg bw/day)	Toxicity after 6 weeks, followed by regeneration of tissues and recovery by 15 weeks	**A6.5/02:** Haywood S (1980) The effect of excess dietary copper on the liver and kidney of the male rat. J. Comp. Path. 90: 217–232 (published).
Special study	Rat	Diet	Copper sulphate 3000, 4000, 5000 or 6000 ppm Cu (= 150, 200, 250 or 300 mg Cu/kg bw/day)	6000 ppm showed unsustainable liver damage by 6 weeks. Lower doses showed toxicity followed by regeneration of tissues and recovery	**A6.5/03:** Haywood S (1985) Copper toxicosis and tolerance in the rat I – changes in copper content of the liver and kidney. J. Path. 145: 149–158 (published).
Special study	Rat	Diet	Copper sulphate 3000 ppm Cu (= 250 mg Cu/kg bw/day) for 1 year Copper sulphate 3000 ppm Cu (= 250 mg Cu/kg bw/day) for 15 weeks, followed by 6000 ppm Cu for 3 weeks. Naïve rats given 6000 ppm after 15 weeks on control diet	Not carcinogenic after 1 year administration at 3000 ppm. Increasing the dose from 3000 ppm to 6000 ppm after 15 weeks showed no adverse effects. Treatment of naïve rats showed hepatocellular necrosis.	Cross-reference: **A6.7/02**

Section A6.6.1 *In-vitro* gene mutation study in bacteria

Annex Point IIA6.6

			Official use only
		56 REFERENCE	
56.1	**Reference**	[redacted] (1994): Study to determine the ability of copper II sulphate pentahydrate to induce mutation in five histidine-requiring strains of *Salmonella typhimurium*. [redacted]; Report no.: 456/31, June 21, 1994 (unpublished).	
		Doc.No. 456/31	
56.2	**Data protection**	Yes	
56.2.1	Data owner	Spiess-Urania Chemicals GmbH, Hamburg, Germany	
56.2.2	Companies with letter of access	--	
56.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		57 GUIDELINES AND QUALITY ASSURANCE	
57.1	**Guideline study**	Yes	
		OECD 471; EU method B14	
57.2	**GLP**	Yes	
		(Certified laboratory)	
57.3	**Deviations**	No	
		58 MATERIALS AND METHODS	
58.1	**Test material**	Copper II sulphate pentahydrate	
58.1.1	Lot/Batch number	A668269350	
58.1.2	Specification	Not specified	
58.1.3	Purity	99.0 – 100.5 %	
58.1.4	Description	Blue crystalline solid	
58.1.5	Stability	Not stated	
58.2	**Study type**	Bacterial reverse mutation test	
58.2.1	Organism/cell type	*S. typhimurium:* TA 1535, TA 1537, TA 98, TA 100, TA 102	
58.2.2	Deficiencies / Proficiencies	Histidine requiring strains	?
58.2.3	Metabolic activation system	S9 mix Mammalian liver post-mitochondrial fraction (S9) prepared from male Sprague-Dawley rats induced with Aroclor 1254.	
58.2.4	Negative control	Vehicle only	
58.2.5	Positive control	TA 98: 2-nitrofluorene (without metabolic activation) TA100, TA1535: Sodium azide (without metabolic activation) TA1537: 9-aminoacridine (without metabolic activation) TA102: Glutaraldehyde (without metabolic activation) At least 1 strain: 2-Aminoanthracene (with metabolic activation)	

58.3	**Application of test substance**	
58.3.1	Concentrations	Two independent experiments were performed using triplicate plates for each group: Exp. 1: 1.6, 8, 40, 200, and 1000 µg test item/plate (corresponding to 0.40722 – 254.51 µg Cu II/plate) Exp. 2: 50, 100, 200, 400, and 800 µg test item/plate (corresponding to 12.725 – 203.61 µg CU II/plate)
58.3.2	Way of application	The test substance was dissolved in sterile purified water. The platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46°C: 0.1 mL bacterial culture 0.1 mL test agent solution 0.5 mL 10 % S-9 mix or buffer solution followed by rapid mixing and pouring on to Minimal Davis agar plates. When set, the plates were inverted and incubated at 37°C in the dark for 3 days. As the results of the first experiment were negative, treatments in the presence of S-9 mix in experiment 2 included a pre-incubation step, where the quantities of test chemical or control solution, bacteria and S-9 mix were mixed and incubated for 1 hour at 37°C before the addition of 2.5 mL molten agar at 46°C followed by the normal plate-incorporation procedure.
58.3.3	Pre-incubation time	None (Experiment 1) 1 hour at 37°C (Experiment 2, with metabolic activation)
58.3.4	Other modifications	None
58.4	**Examinations**	Number of revertant colonies and background lawn

59 RESULTS

59.1	**Genotoxicity**	
59.1.1	Without metabolic activation	No The results are presented in Table A6.6.1- 1 and Table A6.6.1- 2.
59.1.2	With metabolic activation	No The results are presented in Table A6.6.1- 1 and Table A6.6.1- 2.
59.2	**Cytotoxicity**	Yes Evidence of toxicity was observed following all treatments at 1000 µg/plate in experiment 1 and at 800 µg/plate in experiment 2. In experiment 1, some evidence of toxicity was also observed following strain TA 102 treatments at 200 µg/plate in the presence of S9 mix only. In experiment 2, some treatments in the presence of S9 mix at test doses below 800 µg/plate also produced evidence of toxicity which was attributed to the use of a pre-incubation step.

60 APPLICANT'S SUMMARY AND CONCLUSION

60.1	**Materials and methods**	The mutagenic potential of Copper II sulphate pentahydrate was tested in five strains of *Salmonella typhimurium* according to OECD 471 and EU method B14 .
60.2	**Results and discussion**	All solvent control values were within the range of the historical control; positive control chemicals induced large increases in revertant colonies in the appropriate strains and less than 5 % of plates were lost. No Copper II sulphate pentahydrate treatment, either in the absence or presence of S9 mix, gave rise to a statistically significant increase in revertant numbers when the data were analysed at the 1 % level using Dunnett's test.
60.3	**Conclusion**	Copper II sulphate pentahydrate is not genotoxic under the conditions of the test. The extrapolation from copper sulphate to other copper compounds is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper sulphate will only lead to a more conservative but nevertheless valid assessment.
60.3.1	Reliability	1
60.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	29/11/2004
Materials and Methods	3.2.2 – It is normal to have HIS requiring strains of S Typhimurium. This statement has nothing to do in the section "Deficiencies / Proficiencies" Agree with the applicant's version
Results and discussion	Agree with the applicant's version
Conclusion	Agree with the applicant's version
Reliability	1
Acceptability	Acceptable
Remarks	
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Table A6.6.1- 1: Relative reverse mutation rates in *S. typhimurium* after treatment with Copper II sulphate pentahydrate (experiment 1)

Concentration [µg per plate]	Relative reverse mutation rates[1]							
	– S9					+ S9		
	TA98	TA100	TA1535	TA1537	TA102	TA98	TA100	TA1535
Historical control values mean (range)	24 (7–41)	107 (59–154)	12 (1-24)	10 (1-19)	285 (168-402)	29 (9–50)	124 (65-183)	16 (1-32)
Control (mean number of revertant colonies)	23.0 ± 4.6	104.4 ± 11.7	16.0 ± 2.5	16.2 ± 2.2	260.6 ± 23.7	26.6 ± 4.6	122.8 ± 6.1	24.8 ± 4.3
1.6	0.7	-	1.1^	1.1^	1.1^	1.2^	-	0.9
8.0	1.0	1.0^	0.9	1.1	1.1	1.0	1.1^	0.9
40	0.6	1.0	1.1	1.0	1.0	0.8	1.0	0.9
200	0.8	0.9	1.0	1.1	0.8	0.9	0.8	0.8
1000	0.4	0.7	0.9	0.7	0.4	0.7	0.5	0.6
2-nitrofluorene (50.0)	47.2	-	-	-	-	-	-	-
Sodium azide (2.0)	-	6.2	30.1	-	-	-	-	-
9-aminoacridine (50.0)	-	-	-	48.5	-	-	-	-
Glutaraldehyde (25.0)	-	-	-	-	2.2	-	-	-
2-aminoanthracene (5.0)	-	-	-	-	-	47.3	17.1	10.4

[1] results are expressed as ratio: Mean number of revertant colonies per treated plate / Mean number of revertant colonies per control plate
- not tested
^ represents maximum increase over control

Table A6.6.1- 2: Relative reverse mutation rates in *S. typhimurium* after treatment with Copper II sulphate pentahydrate (experiment 2)

Concentration [µg per plate]	Relative reverse mutation rates[1]							
	– S9					+ S9		
	TA98	TA100	TA1535	TA1537	TA102	TA98	TA100	TA1535
Historical control values mean (range)	24 (7–41)	107 (59–154)	12 (1-24)	10 (1-19)	285 (168-402)	29 (9–50)	124 (65-183)	16 (1-32)
Control (mean number of revertant colonies)	33.4 ± 1.3	132.2 ± 11.78	19.8 ± 2.9	10.2 ± 1.9	333.8 ± 12.9	42.4 ± 7.6	169.4 ± 11.3	20.0 ± 4.7
50	1.0	0.9	0.9	0.7	0.9	0.8	0.9	1.3^
100	0.8	0.9	1.0	0.8	0.8	0.5	0.7	1.1
200	0.8	0.9	0.8	0.7	0.7	0.4	0.6	1.1
400	0.7	0.8	0.9	0.4	0.7	0.5	0.5	0.7
800	0.6	0.5	0.7	0.6	0.6	0.1	0.4	0.7
2-nitrofluorene (50.0)	34.7	-	-	-	-	-	-	-
Sodium azide (2.0)	-	5.1	27.3	-	-	-	-	-
9-aminoacridine (50.0)	-	-	-	122.3	-	-	-	-
Glutaraldehyde (25.0)	-	-	-	-	1.8	-	-	-
2-aminoanthracene (5.0)	-	-	-	-	-	33.9	5.2	-

[1] results are expressed as ratio: Mean number of revertant colonies per treated plate / Mean number of revertant colonies per control plate
- not tested
^ represents maximum increase over control

Section A6.6.2 Annex Point IIA6.6	In-vitro cytogenicity in mammalian cells	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	Technically not feasible [ ] Scientifically unjustified [ ]	
Limited exposure [ ]	Other justification [ ]	
Detailed justification:	The performance of an *in vitro* cytogenicity test in mammalian cells is redundant because there is a higher level *in vivo* study performed with Copper II sulphate pentahydrate available for extrapolation (Riley 1994, refer to Section A6.6.4).	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	30/11/2004	
Evaluation of applicant's justification	Agree with applicant's version	
Conclusion	Agree with applicant's version	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.6.3 Annex Point IIA6.6	In-vitro gene mutation in mammalian cells	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [X]	**Technically not feasible [ ]** **Scientifically unjustified [ ]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The performance of an *in vitro* gene mutation test in mammalian cells is redundant because there is a higher level *in vivo* study performed with Copper II sulphate pentahydrate available for extrapolation (Ward 1994, refer to Section A6.6.5).	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	30/11/2004	
Evaluation of applicant's justification	Agree with applicant's version	
Conclusion	Agree with applicant's version	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.6.4 *In vivo* mammalian micronucleus test

Annex Point IIA6.6

			Official use only
		61 REFERENCE	
61.1	**Reference**	[redacted] (1994): Copper II sulphate pentahydrate: induction of micronuclei in the bone marrow of treated mice. [redacted] Report no.: 456/33, July 07, 1994 (unpublished).	
		Doc.No. 456/33	
61.2	**Data protection**	Yes	
61.2.1	Data owner	Spiess-Urania Chemicals GmbH	
61.2.2	Companies with letter of access	--	
61.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		62 GUIDELINES AND QUALITY ASSURANCE	
62.1	**Guideline study**	Yes	
		EU method B.12	
62.2	**GLP**	Yes	
		(Certified laboratory)	
62.3	**Deviations**	No	X
		63 MATERIALS AND METHODS	
63.1	**Test material**	Copper II sulphate pentahydrate	
63.1.1	Lot/Batch number	A668269 350	
63.1.2	Specification	Not stated	
63.1.3	Purity	99 – 100.5 %	
63.1.4	Description	Blue crystalline substance	
63.1.5	Stability	Not stated	
63.1.6	Maximum tolerable dose	A range-finding test was conducted at concentrations in a range of 142.8 to 2000 mg/kg using groups of 3 males and 3 females. The LD_{50} two days after the second dose was calculated at 745 mg/kg (*2). A dose equivalent to 50-80 % of the LD_{50} was considered acceptable as a maximum dose level, thus 447 mg/kg was chosen as the upper dose for the micronucleus assay.	
63.2	**Test animals**		
63.2.1	Species	Mouse	
63.2.2	Strain	CD-1 mice	
63.2.3	Source	[redacted]	
63.2.4	Sex	Male and female	
63.2.5	Age/weight at study initiation	Age: 35-42 days Body weight: m 24-30 g, f 21-26 g	

Section A6.6.4 *In vivo* mammalian micronucleus test

Annex Point IIA6.6

63.2.6	Number of animals per group	15 males and 15 females		
63.2.7	Control animals	Yes		
		10 males and 10 females (vehicle control), 5 males and 5 females (positive control, single administration)		
63.3	**Administration/ Exposure**	Oral		
63.3.1	Number of applications	Two		
63.3.2	Interval between applications	Dosed once daily for 2 consecutive days		
63.3.3	Post-exposure period	24 or 48 hours		
		(24 hours for the positive control)		
63.3.4	Type	By gavage		
63.3.5	Concentration	447 mg/kg bw/day		
		corresponding to 113.76 mg Cu/kg bw/day		
63.3.6	Vehicle	Purified water		
63.3.7	Concentration in vehicle	22.35 mg/mL		
63.3.8	Total volume applied	20 ml/ kg bw		
63.3.9	Controls	Purified water (vehicle control)		
		Cyclophosphamide (positive control, 80 mg/kg)		
63.4	**Examinations**			
63.4.1	Mortality	Yes		
63.4.2	Tissue	Bone marrow		
		Number of animals:	5 per sex at each of 2 time points	
		Time points:	24, 48 hours after treatment	
		Type of cells:	Bone marrow cells	
		Parameters:	PCE/NCE ratio	
		Number of cells:	at least 1000 cells	
		Parameters:	frequency of micronucleated PCE	
		Number of cells:	at least 2000 cells	
63.5	**Further remarks**	None		
		64 RESULTS		
64.1	**Mortality**	In the test item group, 5 male out of 15 and 2 females out of 15 died prior to sampling, indicating that it would not have been practicable to administer the test agent at an appreciably higher dose.		X

64.2	**Tissue examination**	The heterogeneity χ^2 test provided evidence of acceptable variability in the number of micro-nucleated PCE between animals within each group. The incidence of micro-nucleated PCE in the vehicle control was within the range of the historical control. At least 8 animals out of each group at both sampling times were available for analysis and the positive control chemical induced a statistically significant increase in the frequency of micro-nucleated PCE. Thus, the assay was considered to be valid. Decreased PCE/NCE ratios were observed in mice treated with Copper II sulphate pentahydrate at the 24 hour sampling time, indicating cellular toxicity and evidence of test substance penetration into the bone marrow. At 48 hours, PCE/NCE ratios in the test substance group were similar to those in vehicle controls. The numbers of micro-nucleated PCE seen at both sampling times were similar to those seen in controls and were not significantly different by χ^2 analysis. The results are summarised in Table A6.6.4- 1.
64.3	**Genotoxicity**	No
64.4	**Other**	No
		65 APPLICANT'S SUMMARY AND CONCLUSION
65.1	**Materials and methods**	Copper II sulphate pentahydrate was assayed *in vivo* in a mouse bone marrow micronucleus test at a single dose level according to EU method B.12.
65.2	**Results and discussion**	Decreased PCE/NCE ratios were observed in mice treated with Copper II sulphate pentahydrate at the 24 hour sampling time. At 48 hours, PCE/NCE ratios in the test substance group were similar to those in vehicle controls. For both sampling times, the test substance induced no significant effect on the number of micro-nucleated PCE when compared to the vehicle control.
65.3	**Conclusion**	Copper II sulphate pentahydrate was **not genotoxic** under the conditions of this test. The extrapolation from copper sulphate to other copper compounds is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper sulphate will only lead to a more conservative but nevertheless valid assessment.
65.3.1	Reliability	1
65.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	30/11/2004
Materials and Methods	Agree with applicant's version
Results and discussion	2.3 – Only one dose tested in the main study. Considering the substance, the test can, however, be consider as valid. Agree with applicant's version Rem. 4.1 - From the study, 4 out of 15 females died (1 due to dosing error, 1 before 24h sampling time, 2 before 48h sampling time).
Conclusion	Agree with applicant's version
Reliability	2
Acceptability	Acceptable
Remarks	
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Table A6.6.4- 1: Results of the micronucleus test with Copper II sulphate pentahydrate (*in vivo*)

Treatment group (mg/kg bw x 2)	Sampling time [h]	Sex	Mean ratio PCE/NCE	Group mean frequency of micronucleated PCE (per 1000) per sex	per treatment group
Historical control[1]	24 and 48	male	1.06 (0.67-1.83)	0.51 (0-1.29)	
		female	1.08 (0.70-1.45)	0.47 (0-1.10)	
Vehicle control	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	
Test item	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[2]	24	male	0.52	26.87	28.07
		female	0.48	29.27	

[1] Average of group means from 26 consecutive studies at October 23, 1992 (data from 24 and 48 hour sampling times are combined)
[2] administered as a single dose

Section A6.6.5 *In vivo* test of unscheduled DNA synthesis in rat liver

Annex Point IIA6.6

Official use only

		66 REFERENCE
66.1	**Reference**	[redacted] (1994): Copper II sulphate pentahydrate: measurement of unscheduled DNA synthesis in rat liver using an *in vivo/in vitro* procedure. [redacted]; Report no.: 456/32, July 20, 1994. Doc.No. 456/32
66.2	**Data protection**	Yes
66.2.1	Data owner	Spiess-Urania Chemicals GmbH, Hamburg, Germany
66.2.2	Companies with letter of access	--
66.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.
		67 GUIDELINES AND QUALITY ASSURANCE
67.1	**Guideline study**	No The conduct of the study was consistent in all important aspects to method B.39 (2000/32/EC).
67.2	**GLP**	Yes (Certified laboratory)
67.3	**Deviations**	No
		68 MATERIALS AND METHODS
68.1	**Test material**	Copper II sulphate pentahydrate
68.1.1	Lot/Batch number	A668269 350
68.1.2	Specification	Not stated
68.1.3	Purity	99 – 100.5 %
68.1.4	Description	Blue crystalline substance
68.1.5	Stability	Not stated
68.1.6	Maximum tolerable dose	2000 mg/kg bw (based on an initial range-finding test)
68.2	**Test animals**	
68.2.1	Species	Rat
68.2.2	Strain	Wistar
68.2.3	Source	[redacted]
68.2.4	Sex	Male
68.2.5	Age/weight at study initiation	Age: 41-51 days Body weight: 189-254 g
68.2.6	Number of animals per group	6

Section A6.6.5 *In vivo* test of unscheduled DNA synthesis in rat liver

Annex Point IIA6.6

68.2.7	Control animals	Yes	
68.3	**Administration/ Exposure**	Oral	
68.3.1	Number of applications	One	
68.3.2	Interval between applications	Not applicable	
68.3.3	Post-exposure period	12-14 hours (experiment 1)	
		2-4 hours (experiment 2)	
68.3.4	Type	By gavage	
68.3.5	Concentration	632.5, 2000 mg/kg bw (corresponding to 161, 509 mg Cu II/kg bw)	
68.3.6	Vehicle	Purified water	
68.3.7	Concentration in vehicle	63.25, 200 mg/mL	
68.3.8	Total volume applied	10 mL/ kg bw	
68.3.9	Controls	Purified water (vehicle control)	
		2-Acetamidofluorene (suspended in corn oil, positive control, 75 mg/kg)	
		Dimethylnitrosamine (suspended in water, positive control, 10 mg/kg)	
68.4	**Examinations**		
68.4.1	Clinical signs	Yes	
68.4.2	Tissue	Mammalian liver cells	
		Number of animals:	5 per group
		Time points:	12-14 hours (exp. 1); 2-4 hours (exp. 2)
		Type of cells:	Hepatocytes (treated with [^{3}H] thymidine)
		Parameters:	Cytoplasmic and nuclear grain count
		Number of cells:	100 cells per animal
68.5	**Further remarks**	None	

Section A6.6.5 *In vivo* test of unscheduled DNA synthesis in rat liver

Annex Point IIA6.6

		69 RESULTS
69.1	**Clinical signs**	No deaths were observed in an initial range finding study at doses of up to 2000 mg/kg bw. Lethargy was observed at or above 250 mg/kg bw. During the main test, deaths occurred in the 2000 mg/kg bw group of experiment 2 and animals of a spare dose group were included to obtain hepatocytes from a total of 5 animals.
69.2	**Tissue examination**	The group mean net grain count was less than 0 in the vehicle control group. The positive control chemicals 2-AAF and DMN induced increases in the group mean net grain count of 5 or more and the percent of cells in repair was above 50 %. Thus, the experiment was considered to be valid. Following treatment with Copper II sulphate pentahydrate at doses of up to 2000 mg/kg bw the group mean net nuclear grain counts (NG) were well below the value of 5 NG required for a positive response. In addition, no more than 1.0 % cells were seen in repair. The results are summarised in Table A6.6.5- 1.
69.3	**Genotoxicity**	No
69.4	**Other**	No
		70 APPLICANT'S SUMMARY AND CONCLUSION
70.1	**Materials and methods**	Copper II sulphate pentahydrate was administered orally to male rats at 632.5 or 2000 mg/kg bw. Unscheduled DNA synthesis was investigated in hepatocytes isolated approximately 12 – 14 or 2 – 4 hours after dosing. Although not a guideline study, the conduct of the study was consistent in all important aspects to method B.39 (2000/32/EC).
70.2	**Results and discussion**	Following treatment with Copper II sulphate pentahydrate at doses of up to 2000 mg/kg bw the group mean net nuclear grain counts (NG) were well below the value of 5 NG required for a positive response. In addition, no more than 1.0 % cells were seen in repair.
70.3	**Conclusion**	Copper II sulphate pentahydrate was **not genotoxic** under the conditions of this test. The extrapolation from copper sulphate to other copper compounds is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper sulphate will only lead to a more conservative but nevertheless valid assessment.
70.3.1	Reliability	1
70.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
Date	EVALUATION BY RAPPORTEUR MEMBER STATE (*) 30/11/2004

Materials and Methods	Agree with applicant's version
Results and discussion	Agree with applicant's version
Conclusion	Agree with applicant's version
Reliability	2
Acceptability	Acceptable
Remarks	
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Table A6.6.5- 1: Group mean net grain count values

Sampling time [hours]	Dose (mg/kg bw)	Net nuclear grain count (NG) mean	SD	Net grain count of cells in repair mean	SD	Percent of cells in repair (NG ≥ 5) mean	SD
12 – 14	0	-1.3	0.6	0	-	-	-
	632.5	-1.3	0.3	10.2	6.4	0.6	0.9
	2000	-1.0	0.3	5.5	0.9	1.0	1.0
	2-AAF[1] (75)	12.7	0.9	13.7	0.8	90.0	4.0
2 – 4	0	-2.2	0.3	0	-	-	-
	632.5	-2.2	0.2	0	-	-	-
	2000	-3.2	0.5	0	-	-	-
	DMN[1] (10)	17.2	2.8	17.3	2.7	99.6	0.9
Historical control	vehicle control	-1.5	1.4	-	-	0.7	0.9
	(range)	(-7.2 – 0)				(0 – 3.6)	
	2-AAF	11.2	3.2	-	-	80.7	12.9
	(range)	(5.4 – 18.7)				(45.6 – 98.4	
	DMN	11.5	4.3	-	-	79.7	14.9
	(range)	(5.6 – 21.9)				(51.8 – 98.8)	

[1] positive control

Section A6.6.6

Annex Point IIA6.6.6

Genotoxicity *in vivo*
Mouse
Bone marrow chromosome aberration assay
Micronucleus assay
Sperm abnormality assay

			Official use only
		71 REFERENCE	
71.1	**Reference**	BHUNYA, S.P., PATI, P.C. (1987): Genotoxicity of an Inorganic Pesticide, Copper Sulphate in Mouse *in vivo* Test System. *Cytologia* 52, 801- 808 Doc.No.: 00620B-IIA-666	
71.2	**Data protection**	No	
71.2.1	Data owner	published data	
71.2.2	Companies with letter of access	--	
71.2.3	Criteria for data protection	No data protection claimed	
		72 GUIDELINES AND QUALITY ASSURANCE	
72.1	**Guideline study**	No	
72.2	**GLP**	No	
72.3	**Deviations**	Only three animals per group were used; no positive control group	
		73 MATERIALS AND METHODS	
73.1	**Test material**	Copper Sulphate, analytical grade	
73.1.1	Lot/Batch number	not stated	
73.1.2	Specification	$CuSO_4*5H_2O$ (BHD)	
73.1.2.1	Description	not stated	
73.1.2.2	Purity	not stated	
73.1.2.3	Stability	not stated	
73.1.2.4	Maximum tolerable dose	20 mg/kg body weight	
73.2	**Test Animals**		
73.2.1	Species	mouse	
73.2.2	Strain	swiss	
73.2.3	Source	not stated	
73.2.4	Sex	sex not stated	X
73.2.5	Age/weight at study initiation	10 -12 weeks old, average body weight of 15 g	X

Section A6.6.6 Annex Point IIA6.6.6		Genotoxicity *in vivo* Mouse Bone marrow chromosome aberration assay Micronucleus assay Sperm abnormality assay	
73.2.6	Number of animals per group	3 animals per group in all three tests	
73.2.7	Control animals	yes	
73.3	**Administration/ Exposure**	oral, intraperitoneal, subcutaneous	X
73.3.1	Number of applications	5 applications	X
73.3.2	Interval between applications	24 h	X
73.3.3	Postexposure period	6, 24, 48 h for acute treatment 24 h for chronic treatment	X
		Oral	
73.3.4	Type	Usually gavage	
73.3.5	Concentration	20 mg/kg bw	
73.3.6	Vehicle	Moistened with glass double distilled water	
73.3.7	Concentration in vehicle	not stated	
73.3.8	Total volume applied	20 mg $CuSO_4$/kg bw	
73.3.9	Controls	Vehicle (glass double distilled water)	
		Intraperitoneal/subcutaneous	
73.3.10	Vehicle	glass double distilled water	
73.3.11	Concentration in vehicle	not stated	
73.3.12	Total volume applied	not stated	
73.3.13	dose applied	5, 10, 20 mg/kg bw	
73.3.14	Substance used as Positive Control	glass double distilled water	
73.3.15	Controls	Vehicle (glass double distilled water)	

Section A6.6.6 Annex Point IIA6.6.6		Genotoxicity *in vivo* Mouse Bone marrow chromosome aberration assay Micronucleus assay Sperm abnormality assay		
73.4	**Examinations**			
73.4.1	Clinical signs	No		
73.4.2	Tissue	Bone marrow chromosome aberration assay Sperm abnormality assay Micronucleus assay		
		Number of animals:	all animals	
		Number of cells:	4600 cells	
		Time points:	6, 24, 48, 120 h after treatment	
		Type of cells:	bone marrow cells	
			spermatogonia	X
		Parameters:	Frequency of chromosomal aberrations (chromatid and isochromatid gaps, isochromatid breaks, fragments, double minutes, exchanges and rings)	
			Incidence of sperm abnormality	
73.5	**Further remarks**	--		
		74 RESULTS AND DISCUSSION		
74.1	**Clinical signs**	not stated		
74.2	**Haematology / Tissue examination**	The results of the tissue examination are given in • Table A6.6.6-1: Micronucleus test • Table A6.6.6-2: Sperm abnormalities • Table A6.6.6-3: Chromosome aberration		
74.3	**Genotoxicity**	Effect dose not stated		
74.4	**Other**	See table A6.6.6-1, A6.6.6-2 and A6.6.6-3		

Section A6.6.6

Annex Point IIA6.6.6

Genotoxicity *in vivo*
Mouse
Bone marrow chromosome aberration assay
Micronucleus assay
Sperm abnormality assay

		75 APPLICANT'S SUMMARY AND CONCLUSION	
75.1	**Materials and methods**	Mutagenicity of copper sulphate was evaluated in vivo by chromosome aberration, sperm abnormality and micronucleus tests in mice. For the three tests different doses (5,10 and 20 mg/kg bw) were administered intraperitoneal, oral and subcutaneous.	
75.2	**Results and discussion**	Dose, route and time influenced significantly the frequency of chromosome aberration, incidence of micronucleus and sperm abnormality. The relative sensitivity of three assays are: sperm abnormality > chromosome aberration > micronuclei formation. No statement is given on signs of mortality, The PCE//NCE ratio was increased in comparison to control. Disregarding "gaps", the effect of treatment is only marginal, and the test result may be considered as ambiguous.	X
75.3	**Conclusion**	Results indicated that copper sulphate solution administered by intra-peritoneal injection caused mutagenic activity in bone marrow cells and in sperm. Dose, route and time influenced significantly the frequency of chromosomal aberration, incidence of micronucleus and sperm abnormality. The extrapolation from copper sulphate to basic copper carbonate is considered not to be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper sulphate will only lead to a more conservative but nevertheless valid assessment.	X
75.3.1	Reliability	3	X
75.3.2	Deficiencies	Yes several deficiencies render this a publication of limited validity	X

Section A6.6.6 **Annex Point IIA6.6.6**	**Genotoxicity *in vivo*** **Mouse** **Bone marrow chromosome aberration assay** **Micronucleus assay** **Sperm abnormality assay**

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	01/12/2004
Materials and Methods	3.2.4 and 3.2.5 Average body weight of 25g. Male mice of sperm abnormality assay (!) 3.3 – Experimental procedure is not clearly reported in the sections 3.3.x Three different experiments are reported in this publication. - Bone marrow chromosome aberration assay: Acute treatment with 20 mg/kg i.p. on three animals with fixation times of 6, 24 and 48 h. Two other doses (10 mg/kg and 5 mg/kg) were administered to 2 more animals with fixation time of 24 h. The highest dose was also given orally or subcutaneously to 2 animals with fixation time of 24 h. Chronic administration of 5x4 mg/kg was also performed on one animal (24 h between each injection and sacrifice 24 h after the last treatment). - Sperm abnormality assay: 3 animals/dose were treated with 20 mg/kg, 10 mg/kg and 5 mg/kg. Doses were fractionated into 5 equal parts and injected i.p. 5 times with 24 h between each dose. Animals were sacrificed **35 days** after the first injection. Sperms were collected from caudae epididymides, and 500 sperms were examined per animal for sperm abnormalities. - Micronucleaus assay: each dose (20, 10, 5 mg/kg) was injected i.p. twice at an interval of 24 h and the animals were sacrificed 6 h after the second injection. 3.4.2 – Spermatogonies are the target cells of this study but spermatozoids are the observed cells at the end of the observation period of 35 days.
Results and discussion	Quite low doses, mortality is not expected at these levels. Results and experimental data is not enough reported to have a clear opinion of these experiments.
Conclusion	Some concerns are raised by this series of studies. These results are not confirmed by other studies (mutagenicity and reproductive toxicity). Moreover insufficient reporting weaken the validity of the results. But these effects cannot be disregarded and should be kept in mind when assessing other studies.
Reliability	4

Section A6.6.6	**Genotoxicity *in vivo*** **Mouse** **Bone marrow chromosome aberration assay** **Micronucleus assay** **Sperm abnormality assay**
Annex Point IIA6.6.6	

Acceptability	Not acceptable Other data exist, no further testing is required.
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*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A6.6.6-1: Mouse bone marrow findings (Micronucleus test and lysis) induced by $CuSO_4$

State mean ± standard deviation state individual numbers for critical findings		control group	low dose 5 mg/kg	mid dose 10 mg/kg	high dose 20 mg/kg
Number of cells evaluated		**3000**	**3000**	**3000**	**3000**
Sampling time (h)		**6 h**	**6 h**	**6 h**	**6 h**
Number of erythrocytes	**normochromatic erythrocytes with micronuclei**	**0.10 ± 0.05**	**0.66 ± 0.27**	**1.03 ± 0.72**	**1.46 ± 0.72**
	polychromatic erythrocytes with micronuclei	**0.20 ± 0.05**	**1.30 ± 0.47**	**1.80 ± 0.94**	**2.06 ± 0.72**
	poly- and normochromatic erythrocytes with micronuclei	**0.15 ± 0.05**	**0.98 ± 0.27**	**1.41 ± 0.27**	**1.76 ± 1.44**
Ratio of erythrocytes	**polychromatic with micronuclei/ normochromatic with micronuclei**	**0.88**	**1.1**	**1.1**	**1.1**
Immature white cells with MN		**0.06 ± 0.03**	**0.4 ± 0.27**	**0.88 ± 0.27**	**1.23 ± 0.72**
Nuclei in lysis		**--**	**0.2 ± 0.05**	**0.30 ± 0.15**	**0.46 ± 0.27**

Table A6.6.6-2: Incidence of sperm abnormality in mice by $CuSO_4$ treated intraperitoneal

Dose [mg/kg]	No. of animals / no. of sperm studies	No. of abnormal sperms	Mean [%]	'Z' value
20	**3 / 1500**	**231**	**15.40 ± 0.81**	**17.09**
10	**3 / 1500**	**166**	**11.60 ± 0.98**	**12.97**
5	**3 / 1500**	**87**	**5.80 ± 1.41**	**6.59**
Control	**3 / 3000**	**62**	**2.06 ± 0.54**	**--**

The result is statistically relevant when $Z \geq 1.96$

Table A6.6.6-3: Frequency of chromosomal aberration induced in bone marrow cells by $CuSO_4$ administered through different routes

Dose [mg/kg]	Route	Interval of fixation	No. of cells studied	Chro-matid gap	Chro-matid break	Isochro-matid gap	Frag-ments	Double minutes	Ex-change/ ring	Total	Aber-ration [%]
Acute											
20	**i.p.**	**6**	**300**	**4**	**3**	**1**	**1**	**2**	**1**	**12**	**4.00 ± 0.57**
20	**i.p.**	**24**	**300**	**6**	**5**	**1**	**3**	--	--	**15**	**5.00 ± 1.15**
20	**i.p.**	**48**	**300**	**9**	**2**	**1**	**1**	--	--	**13**	**4.33 ± 0.33**
10	**i.p.**	**24**	**300**	**11**	--	--	**2**	--	**1**	**14**	**4.66 ± 0.33**
5	**i.p.**	**24**	**300**	**8**	**3**	--	**1**	--	--	**12**	**4.00 ± 1.00**
Chronic											
5 x 4	**i.p.**	**120**	**300**	**9**	--	**1**	**1**	**1**	--	**12**	**4.00 ± 0.57**
Composite											
Control	**i.p.**	--	**1000**	**5**	**2**	--	--	--	--	7	**0.70 ± 0.26**
20	**or**	**24**	**300**	**8**	--	--	**2**	**2**	--	**12**	**4.00 ± 0.57**
Control	**or**	**24**	**600**	**3**	**1**	--	--	--	--	**4**	**0.66 ± 0.33**
20	**sc**	**24**	**300**	**9**	**2**	--	**1**	**1**	**1**	**14**	**4.66 ± 0.88**
Control	**sc**	**24**	**600**	**4**	--	--	--	--	--	**4**	**0.66 ± 0.33**

i.p. = intraperitoneal, or = oral, sc = subcutaneous

Section A6.6.7 Annex Point IIA6.6.7	Further testing if metabolites of concern are formed in mammals	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ]	**Technically not feasible [ ]** **Scientifically unjustified [X]**	
Limited exposure [ ]	**Other justification [ ]**	
Detailed justification:	The performance of further genotoxicity testing is considered to be not required since no metabolites are formed by basic Copper carbonate.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	01/12/2004	
Evaluation of applicant's justification	Agree with applicant's version	
Conclusion	Agree with applicant's version	
Remarks		
	COMMENTS FROM OTHER MEMBER STATE *(specify)*	
Date	*Give date of comments submitted*	
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*	
Conclusion	*Discuss if deviating from view of rapporteur member state*	
Remarks		

Section A6.7 Carcinogenicity

Annex Point IIA6.7

	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
	Other existing data [X] **Technically not feasible [ ]** **Scientifically unjustified [ ]**	
	Limited exposure [ ] **Other justification [ ]**	
Detailed justification:	In subchapter 6.7 of the TNsG on data requirements according to Directive EC98/8/EEC, carcinogenicity testing is required for one rodent and one other mammalian species. The carcinogenicity study should identify the carcinogenicity potential of the substance in laboratory animals in order to facilitate the extrapolation of potential risks to humans. The studies must be sufficient to establish the species specificity and organ specificity of tumours induced, to establish the dose-response relationship and for non-genotoxic carcinogens to identify doses eliciting no adverse effects (threshold dose). However, the applicant is of the opinion that the conduct and submission of a conventional carcinogenicity study is not required, for the following reasons: (1)Copper is an essential micronutrient, and its use, and incorporation in many enzyme systems in the human has been researched in great depth. The absorption, distribution and excretion of copper is described in Section A6.2, using data from several species, including the human. Sections A6.5 and A6.7 contain summaries of several long-term animal studies from peer-reviewed journals in the public domain. None of these meets exactly the requirements of the guideline B.30/32/33, but they do show conclusively that copper has no carcinogenic activity. Also, there is no need to perform additional animal studies, because there are human data. (2) Two rare genetic diseases of copper in the human provide evidence that copper is not carcinogenic following systemic absorption. These are Wilson's disease (WD) and Menkes' disease (MD): Wilson's disease is a defect in the ATPase for copper transport ATP7B (or WND), expressed mainly in the liver (LEEMING, N.M., 2003; reference A6.2/01), resulting in faulty copper transport, impaired incorporation of copper into ceruloplasmin, impaired copper biliary excretion, and copper accumulation in the liver and brain. Frequency in the human population is stated as 1 in 300,000 live births. Hepatic copper levels range from 200 to 800 µg/g dry weight (normal range 20 to 50 µg/g), and patients present with hepatic cirrhosis and fatty infiltration of the liver. Urinary copper is much higher than normal (as in rats given sufficiently high oral doses to cause liver toxicity). Treatment is by chelation therapy using D-penicillamine, such that intestinal absorption is reduced, and chelated copper complexes are excreted in the urine, and liver and body levels are kept below levels at which liver disease occurs. Zinc therapy (orally as zinc sulphate) acts to induce excess metallothionein in the intestinal cells. Metallothionein has a stronger affinity for copper than zinc. The copper remains bound in the gut cells, which are then sloughed off, and the copper is lost. In the second or third decade of the disease, neurological symptoms can occur. Copper accumulation in the brain causes degeneration of the basal ganglia, resulting in defective movement, slurred speech, difficulty	X

Supprimé : [

in swallowing, facial and other muscular spasms, dystonia and poor motor control. Depression and schizophrenia have been reported. Copper may also be deposited in the cornea (Kayser-Fleischer rings).

Menkes disease is an X-linked copper deficiency disease that is usually fatal in early childhood. It is usually present in males, but has been recorded in eight females (cases have been cited where genetic translocation was noted in a female). The frequency in the human population is stated as 1 in 100,000 to 1 in 250,000 live births. The symptoms result from a defect in the MNK protein, producing an inability to export copper from cells, particularly from the basal membrane of the small intestine, where copper is absorbed (see LEEMING, N.M., 2003; reference A6.2/01). This leads to very high concentrations of copper in sloughed intestinal cells, but the failure to export the 'absorbed' copper to the bloodstream results in an effective copper deficiency for the rest of the body. The disease shows progressive mental retardation, hypothermia, seizures, poor muscle tone, feeding difficulties, jaundice, diarrhoea and a general failure to thrive. There are abnormalities of connective tissue with deformities of the skull, long bones and ribs. The hair is abnormal with a wiry texture and a spiral twist.

Both diseases result from genetic defects where the subject is unable to produce respectively the copper ATPases ATP7B and ATP7A. These are members of the human cation-transporting P-type ATPase family. The P-type ATPases are a large group of membrane proteins that utilise the energy of ATP hydrolysis to transport various ions across cell membranes. During the catalytic cycle the γ-phosphate of ATP is transferred to the invariant aspartic acid residue within the nucleotide-binding site of ATPase with the formation of acylphosphate intermediate: this property distinguishes the P-type ATPases from other cation-transporting pumps. Over 100 P-type ATPases have been described. The loci of the encoding genes have been identified for both WD and MD. Both pump copper across cell membranes. The MD pump (ATP7A) is the pump that actually moves copper through the basal membrane of the intestinal epithelial cells so that copper enters the hepatic portal system where it binds to albumin, transcuprein and histidine to reach the liver. In the MD subject, ATP7A is inactive, and copper from the diet accumulates in the intestinal epithelial cells, bound to induced metallothionein. The presence of copper within the cell induces the production of more metallothionein, and the copper-metallothionein complex accumulates during the life of the cell. When the cells are sloughed off into the intestinal lumen, as is the normal course of events, the cells and the copper within them are excreted in the faeces, and the copper is lost to the body. Subjects with Menkes' disease can still absorb small amounts of copper. Copper accumulates in fibroblasts and in the kidney of Menkes' disease subjects, but there is no evidence of increased incidence of cancer in these tissues either. Menkes' disease is effectively a disease of copper deficiency. In terms of risk assessment of copper in the normal human, the accumulation of copper in the intestinal epithelium on Menkes' subjects can be considered as the equivalent of an excessive oral dose of copper to the epithelial cells. Carcinogens of the intestine may act by irritation or some other means to cause proliferation of the intestinal epithelium that eventually results in hyperplasia and tumour formation. MD subjects do not suffer from increased incidence of cancer of the intestine. This

shows conclusively that excess copper in the intestinal cells does not cause cancer or long-term toxicity in that tissue.

Wilson's disease (WD) involves the other ATPase previously referred to, ATP7B. In normal humans, this enzyme is primarily active in hepatocytes. It is involved in the trans-Golgi network (TGN). Copper absorbed by the hepatocyte via the inbound membrane pump hCTR1 (human copper transporter protein 1, see LEEMING, N.M., 2003; reference A6.2/01) and is bound to metallothionein within the cell. It may be bound by ATP7B to ceruloplasmin (a protein that binds up to 6 copper ions tightly and transports them to various tissues for use, including the brain. If there is excess copper in the hepatocyte, ATP7B is induced to traffic to vesicular compartments (lysosomes) and directly to the apical membrane, where copper is secreted from the cell bound to a trypsin-independent fragment of ceruloplasmin and excreted in the bile. In WD, ATP7B is inactive and the absorbed copper accumulates in the hepatocytes bound to metallothionein. The bile of WD subjects does not contain copper. In the hepatocyte, excess copper may accumulate in mitochondria, in the cytoplasm and in lysosomes, bound to metallothionein. Eventually the cell's copper storage capacity is exceeded. Mitochondrial damage occurs and eventually the hepatocyte dies, whence the cell contents are released to the circulation, depositing copper in extrahepatic tissues.

Wilson's disease thus leads to massive accumulation of copper in the liver. The disease usually manifests in late adolescence, and is ultimately fatal if not treated, but death is from liver failure, not from cancer. Treatment involves administration of penicillamine, which forms a copper complex capable of urinary excretion. There is no evidence of increased incidence of liver cancer in WD subjects. This shows that even massive accumulation of copper in the target organ, the liver, does not result in cancer in the human. Accumulation of copper leads to cell death, but this is only in the presence of excessive copper concentrations, brought about by a genetic condition resulting in the disruption of the natural homeostatic mechanisms for copper. It should be noted that Wilson's disease is genetic, and the accumulation of copper and resulting liver failure occur under the natural levels of copper in the diet, not as a result of exposure to excessive levels of copper in the environment. However, the accumulation of copper in the liver may be taken as a model for accumulation of excess copper in a toxicity study, and the conclusion drawn that chronic high liver levels do not result in increased incidence of cancer.

As with short-term toxicology it is considered appropriate to present data on the active substance, the copper ion, rather than the formulated or technical materials. A metabolism/bioequivalence study has been performed to demonstrate that the ion, as present in the form of cupric sulphate pentahydrate, is similarly bioavailable to the five forms defended, such that data from studies with the pentahydrate, and other forms that liberate the copper ion, may be used in the risk assessment process (HIMMELSTEIN, M., 2004; reference A6.2/02).

(3) No data have been presented on the mouse. Short-term studies on the mouse show that the mouse is much more tolerant of higher doses of copper than the rat (HÉBERT, C.D., 1993; reference A6.4.1/01), and that the mouse does not show the histological changes in the liver and kidney that are seen in the rat. As the rat shows lower short term NOELs than

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	the mouse, it is logical to assume that the NOELs from long term mouse studies would be higher than in the rat. Therefore, data from long term mouse studies would not be used in setting values such as the ADI. As stated previously, there are human diseases that lead to chronic, lethal accumulations of copper in target tissues, but no evidence for tumour formation. In summary, submission of further carcinogenicity studies on animals are considered unnecessary, in view of the argumentation set forth above, and the studies cited and summarised further below.	
Undertaking of intended data submission [ ]		

	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)	
Date	01/12/2004	
Evaluation of applicant's justification	Agree with applicant's version But it can remain some concerns if inhalation exposure of copper carbone dusts could occur. (see previous comments on chronic toxicity document IIIA 6.5)	
Conclusion	Acceptable if it is demonstrated that inhalation exposure is negligible.	
Remarks		
	COMMENTS FROM ...	
Date		
Evaluation of applicant's justification		
Conclusion		
Remarks		

Section A6.7 Carcinogenicity in rats

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			Official use only
		The following studies are considered to contain further information concerning carcinogenicity and are thus presented as supportive data.	
		76 REFERENCE	
76.1	**Reference**	A6.7/01: Doc.No. 00620B-IIA-67a HOWELL, J.S. (1958): The effect of copper acetate on *p*-dimethylamino-azobenzene carcinogenesis in the rat; Br. J. Cancer 12, p594-610.	
76.2	**Data protection**	No	
76.2.1	Data owner	published data	
76.2.2	Companies with letter of access	--	
76.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		77 GUIDELINES AND QUALITY ASSURANCE	
77.1	**Guideline study**	No Special investigation of the effect of copper acetate on DMAB carcinogenesis, which does not meet exactly the requirements of method B32 (88/303/EEC).	
77.2	**GLP**	No The study was conducted prior to implementation of GLP.	
77.3	**Deviations**	Not applicable	
		78 MATERIALS AND METHODS	
78.1	**Test material**	(i) *p*-dimethylaminoazobenzene (DMAB) (ii) copper acetate (iii) ferric citrate	
78.1.1	Lot/Batch number	Not stated	
78.1.2	Specification	Not specified	
78.1.3	Purity	Not stated	
78.1.4	Description	(i) dry crystalline (ii), (iii) powder	
78.1.5	Stability	DMAB was mixed with maize and copper acetate and stored for 2 month. Determination of DMAB by column chromatography showed that DMAB was stable in the diet mixture and did not underwent chemical alteration.	
78.2	**Test animals**		
78.2.1	Species	Rat	
78.2.2	Strain	Exp. A: entirely out-bred laboratory stock, not further specified Exp. B: Birmingham strain (Laboratory Animals Bureau Catalogue of Uniform Strains, No. 626, 1953), a heterozygous strain	

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78.2.3	Source	Not specified
78.2.4	Sex	Male and female
78.2.5	Age/weight at study initiation	Age: 2 to 6 month Body weight: not stated
78.2.6	Number of animals per group	5 males and 5 females
78.2.7	Control animals	No
78.3	**Administration/ Exposure**	Oral
78.3.1	Duration of treatment	Exp. A: lifespan Exp. B: until presence of palpable liver tumours and subsequent sacrifice
78.3.2	Frequency of exposure	Daily
78.3.3	Post-exposure period	None
78.3.4	Type	In food
78.3.5	Concentration	DMAB: 0.09 % w/w Copper acetate: 0.5 % Ferric citrate: 2.0 % The test design is outlined in Table A6.7- 1.
78.3.6	Vehicle	Finely ground maize or standard laboratory diet (Thompson diet, Heygate and Sons)
78.3.7	Concentration in vehicle	DMAB: 0.09 % w/w Copper acetate: 0.5 % Ferric citrate: 2.0 %
78.3.8	Total volume applied	The amount of food provided was calculated on the basis of 10 g per rat per day.
78.3.9	Controls	Not applicable
78.4	**Examinations**	
78.4.1	Body weight	Yes (regularly in experiment B)
78.4.2	Food consumption	Yes (at intervals in experiment B)
78.4.3	Water consumption	No
78.4.4	Clinical signs	Not stated
78.4.5	Macroscopic investigations	After the 3rd month of treatment all animals were examined at approx. 14 day intervals for the presence of palpable liver tumours.
78.4.6	Ophthalmoscopic examination	No
78.4.7	Haematology	No
78.4.8	Clinical chemistry	No

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78.4.9	Urinalysis	No
78.5	**Pathology**	
78.5.1	Organ weights	Yes (Experiment B) All spleens were weighed during post-mortem examination.
78.5.2	Histopathology	Yes from: all animals Organs: liver, spleen and any other tissue which showed pathological changes
78.5.3	Other examinations	In experiment A rats were subjected to liver biopsy under anaesthesia. They were biopsied in rotation at monthly intervals from the second to the tenth months of the experiment. During experiment B, liver function was assessed by means of the bromsulphalein excretion test.
78.6	**Statistics**	Not stated
		79 RESULTS
79.1	**Body weight**	In experiment B, all animals gained weight during the study. Animals in group 6 showed initial loss of weight during the 1st month of treatment most likely due to abstention of food.
79.2	**Food consumption**	Food consumption was determined at intervals in experiment B. Over periods of one month the quantity of food consumed by the animals was estimated by weighing the residues daily, without determination of food spillage and individual variations in consumption. However, it was found that the animals in the various dietary groups consumed roughly the same amounts of food.

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79.3	**Macroscopic investigations**	The minimum induction period was set at 6 month of administration, since the first animal to develop tumours in both experiments died during the 6th month. Animals that died before this time have been excluded since they were considered not 'at risk'. During experiment A, animals fed with a diet containing copper acetate and ferric citrate showed lower incidences of liver tumours (4 animals with liver tumours out of 8 rats for the standard diet), especially when incorporated into a maize diet (0 rats with liver tumours out of 8 animals). In addition, survival was 2.25 month (maize) or 0.4 month (standard diet) longer for diets with copper acetate than for respective diets without copper acetate and the time to first tumour was also increased. These findings were confirmed by the results of group 5 to 7 of experiment B. Group 6 receiving copper acetate in the diet showed lower incidences of tumours, a longer time to first tumour and an increased average time to death than animals of the other two groups which received no copper acetate in the diet. The results of the alternating feeding experiments (groups 8 to 11) were more difficult to interpret, as the lower incidence of tumours may also have been influenced by lower DMAB consumption. The results are presented in Table A6.7- 1.
79.4	**Organ weights**	During experiment B all spleens were weighed at post-mortem examination. Differences in splenic weight were significant ($0.02 > p > 0.01$) for the directly comparable groups 5 (2.3 g) and 6 (1.1 g), with spleens of animals on DMBA-maize diet without copper acetate being about 1 g heavier than the mean weight of the spleen in group 6 receiving DMBA in maize diet with copper acetate. Enlarged spleens were also observed for animals receiving the alternated diets, with group 9 and 10 receiving the smallest quantity of copper and the highest quantity of DMBA showing the most enlarged spleens.
79.5	**Histopathology**	

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79.5.1	Liver	In experiment A rats were subjected to liver biopsy under anaesthesia. They were biopsied in rotation at monthly intervals from the second to the tenth months of the experiment. The incidence of liver changes are summarised in Table A6.7- 2. The results showed that hepatic injury developed and progressed rapidly in groups 1 and 2 feed on diets without copper acetate. Animals of group 4 (maize diet with copper acetate) showed a very considerable retardation in the severity of the lesions and a considerable prolongation in the time required to produce them. No animals with regenerative hyperplasia or any degree of cirrhosis were observed in this group and it was not until the eighth month that marked changes were observed, consisting of fusiform cells and chronic inflammatory cells tending to encircle lobules. Liver histopathology in animals dying without developing tumours during both experiments revealed that the microscopic changes in the DMAB, copper treated rats were of the same nature as those which result from DMAB alone, but there was delay in the rate of development and progression of the lesions especially marked during the first 12 month of treatment.
79.5.2	Spleen	Gross and microscopic changes of the spleen typically induced by DMAB were also observed in copper acetate treated rats, but the changes in these animals were always much less advanced than in those animals given DMAB without copper acetate.
79.6	**Time to tumours**	The results are presented in Table A6.7- 1.
		80 APPLICANT'S SUMMARY AND CONCLUSION
80.1	**Materials and methods**	During experiment A, groups of 5 male and 5 female rats received the known carcinogen *p*-dimethylaminoazobenzene in either standard laboratory diets or maize supplemented with ferric acid and copper acetate for their whole lifespan. Liver biopsies were performed regularly. Experiment B was performed to confirm the inhibitory effect of copper acetate. Groups of 5 male and 5 female rats received DMAB in maize with or without ferric acid or copper acetate. In addition, groups with alternating feeding were included to reduce the likelihood of copper acetate interfering with DMAB absorption in the gut. The animals were sacrificed when palpable liver tumours were observed. Spleen weights were determined and histopathology of liver and spleen was conducted. The study does not exactly meet the requirements of method B32 (88/303/EEC), since it was a special investigation of the effect of copper acetate on DMAB carcinogenesis.
80.2	**Results and discussion**	Copper, when added to rat diets containing the known carcinogen p-dimethylaminobenzene significantly reduced the incidence of liver tumours, and delayed the onset of histological changes leading to cirrhosis and hyperplasia.

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80.3	**Conclusion**	The design of the study did not permit assessment of tumour incidence of copper administered alone, and in the context of a risk assessment of the carcinogenic potential of copper the study (and the numerous other studies in the literature that also show a beneficial effect of copper when administered together with known carcinogens) must only be considered illustrative. However, if copper were to have any carcinogenic action either alone, or as a co-carcinogen, this type of study would certainly have shown an increased incidence of tumours, and an earlier onset. It did neither; the authors concluded that copper has a beneficial effect in reducing the action of the carcinogen. The study indicates that copper has no carcinogenic potential when administered in the diet. Other similar studies from the literature have not been included in this dossier as none of them meet the regulatory guideline for carcinogenicity, although most do show that the co-administration of copper with known carcinogens reduces the onset and/or incidence of the anticipated tumours, and where a copper-only control has been part of the design, there were no adverse effects of copper administration. The extrapolation from copper acetate to copper hydroxide is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake.
80.3.1	Reliability	2
80.3.2	Deficiencies	Yes Copper acetate was only investigated in combination with the carcinogen DMAB.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	10/06/05
Materials and Methods	Agree with applicant's version
Results and discussion	Agree with applicant's version
Conclusion	Data not suitable for cancer hazard assessment of copper carbonate
Reliability	3
Acceptability	Not acceptable
Remarks	Data available is sufficient to allow a hazard assessment of Copper carbonate by oral route. No more tests are needed.
	COMMENTS FROM ...
Date	

Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	

Table A6.7- 1: Tumour incidence

Group	Diet	Days on diet/week	No. surviving 6 month	Month to first tumour	No. of rats with liver tumour	Average induction time (month)	Average time to death (month)
Experiment A							
1	CP + Fe cit + DMAB	7	10	9	8	13.6 (9-16)	13.0 (9-16)
2	M + Fe cit + DMAB	7	9	6	8	11.25 (6-16)	11.0 (6-16)
3	CP + Fe cit + **Cu ac** + DMAB	7	8	11	4	14.0 (11-16)	12.5 (8-16)
4	M + Fe cit + **Cu ac** + DMAB	7	8	0	**0**	-	13.5 (10-16)
Experiment B							
5	M + DMAB	7	8	6	8	8.5 (6-10)	8.5 (6-10)
6	M + DMAB + **Cu ac**	7	8	18	1	-	13.3 (10-18)
7	M + DMAB + Fe cit	7	10	6	10	8.5 (6-12)	8.5 (6-12)
8	M + DMAB + Fe cit M + Cu ac	4 3	10	15	3	15	15.1 (8-19)
9	M + DMAB + Fe cit M + Cu ac	5 2	8	9	6	11.3 (9-15)	10.5 (8-15)
10	M + DMAB + Fe cit M + Cu ac	6 1	10	9	8	12 (9-15)	11.4 (9-15)
11	M + DMAB + Fe cit CP	4 3	9	15	5	16.2 (15-18)	14.1 (9-18)

CP = standard laboratory diet, M = maize, Fe cit = 2.0 % Ferric citrate, Cu ac = 0.5 % Copper acetate, DMAB = 0.09 % *p*-Dimethylaminoazobenzene
Remark: Groups 8 to 11 were included to reduce the likelihood of copper acetate interfering with DMAB absorption or of combining chemically with it in the gut. Therefore alternating feeding was devised.

Table A6.7- 2: Number of animals showing regenerative hyperplasia and cirrhosis of the liver upon biopsy (Experiment A)

Group	No. of biopsies	Regenerative hyperplasia	Degree of cirrhosis			
			Absent	Incipient	Early	Advanced
1	8	4	3	2	3	-
2	8	6	1	3	3	1
3	9	3	8	-	1	-
4	8	0	8	-	-	-

Section A6.7 Copper toxicosis and tolerance in rats

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		81 REFERENCE
81.1	**Reference**	A6.7/02: Doc.No. 00620B-IIA-67b HAYWOOD, S.; LOUGHRAN, M. (1985): Copper toxicosis and tolerance in the rat, II Tolerance – a liver protective adaptation. Liver 5, p267-275.
81.2	**Data protection**	No
81.2.1	Data owner	published data
81.2.2	Companies with letter of access	--
81.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.
		82 GUIDELINES AND QUALITY ASSURANCE
82.1	**Guideline study**	No Special investigation on copper toxicosis and tolerance in the rat, which does not meet exactly the requirements of methods B.32 or B.33 (88/303/EEC).
82.2	**GLP**	No The study was conducted prior to implementation of GLP.
82.3	**Deviations**	Not applicable
		83 MATERIALS AND METHODS
83.1	**Test material**	Copper sulphate
83.1.1	Lot/Batch number	Not stated
83.1.2	Specification	Not specified
83.1.3	Purity	Not stated
83.1.4	Description	Not stated
83.1.5	Stability	Not stated
83.2	**Test animals**	
83.2.1	Species	Rat
83.2.2	Strain	Wistar
83.2.3	Source	Not stated
83.2.4	Sex	Male
83.2.5	Age/weight at study initiation	Age: not specified (weanling rats of uniform age) Body weight: uniform weight, not specified
83.2.6	Number of animals per group	Exp. 1: 4 per group and sacrifice time Exp. 2: 3 to 4 per group and sacrifice time Exp. 3: 16 per group
83.2.7	Control animals	Yes

Section A6.7 Copper toxicosis and tolerance in rats

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83.3	**Administration/ Exposure**	Oral
83.3.1	Duration of treatment	Exp. 1: 15 weeks Exp. 2: 52 weeks Exp. 3: 3 weeks (pre-treatment for 15 weeks)
83.3.2	Interim sacrifice(s)	Exp. 1: 4 animals from each group sacrificed in intervals up to 6 weeks Exp. 2: groups of 3 to 4 rats were sacrificed at 15, 20, 29 or 52 weeks Exp. 3: 4 rats/ group sacrificed at 15 weeks (after pre-treatment)
83.3.3	Final sacrifice	Exp. 1: at 6 weeks (6000 mg/kg), at 15 weeks (other treatments) Exp. 2: up to 52 weeks Exp. 3: after 3 weeks of 'challenge'
83.3.4	Frequency of exposure	Daily
83.3.5	Post-exposure period	None
83.3.6	Type	In food
83.3.7	Concentration	Exp. 1: 3000, 4000, 5000, 6000 mg Cu/kg diet Exp. 2: 3000 mg Cu/kg diet Exp. 3: 6000 mg Cu/kg diet (animals previously fed control or 3000 mg Cu/kg diet for 15 weeks)
83.3.8	Vehicle	Powdered diet (Labsur animal diet, RHM Agricultur South Ltd.) containing 10-20 mg/kg of copper
83.3.9	Concentration in vehicle	Exp. 1: 3000, 4000, 5000, 6000 mg Cu/kg diet Exp. 2: 3000 mg Cu/kg diet Exp. 3: 6000 mg Cu/kg diet
83.3.10	Total volume applied	*ad libitum*
83.3.11	Controls	Vehicle only
83.4	**Examinations**	
83.4.1	Body weight	Yes (upon study termination)
83.4.2	Food consumption	No
83.4.3	Water consumption	No
83.4.4	Clinical signs	Yes
83.4.5	Ophthalmoscopic examination	No
83.4.6	Haematology	No
83.4.7	Clinical chemistry	No
83.4.8	Urinalysis	No
83.5	**Pathology**	

Section A6.7 Copper toxicosis and tolerance in rats

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83.5.1	Organ weights	No
83.5.2	Histopathology	Yes from: all animals Organs: liver
83.5.3	Other examinations	Copper analysis of livers by means of an atomic absorption spectrophotometer.
83.6	**Statistics**	Student's t-test, Pearson Product-Moment correlation
		84 RESULTS
84.1	**Experiment 1**	Effects of different levels of copper supplementation on growth rate, liver copper content and hepatic pathology were investigated.
84.1.1	Growth rate and clinical condition	All animals on the copper-supplemented diets showed a severe reduction in growth rate compared to the control. Animals receiving 3000, 4000 or 5000 mg Cu/kg diet gained body weight during the study and appeared active and sleek upon termination of the experiment. Animals receiving 6000 mg Cu/kg diet lost body weight and appeared ruffled, lethargic and had diarrhoea. Therefore, they were sacrificed after 6 weeks of administration.
84.1.2	Liver copper content	The liver copper concentration of rats on the 3000 mg/kg copper diet increased to a peak concentration of 3986 ± 297 µg/g at 4 weeks and declined thereafter. A similar pattern of liver copper distribution was noted for rats on the 4000 or 5000 mg/kg copper diets, except that peak copper concentrations occurred at 3 weeks of administration. Maximum liver copper concentrations occurred at 2 weeks on the 6000 mg/kg copper diet and showed no decrease upon sacrifice at 6 weeks of administration.
84.1.3	Histopathology	Liver necrosis first occurred as randomly distributed necrotic foci progressing to a more diffuse form which occasionally involved whole lobules. The onset of liver necrosis varied with the magnitude of copper loading, and was succeeded by regeneration in animals on dietary copper up to 5000 mg/kg. Hepatocellular damage persisted in animals receiving the highest dietary level of copper.
84.2	**Experiment 2**	Effects of prolonged exposure to a high copper diet on growth rate and liver copper content were investigated. Animals receiving 3000 mg Cu/kg diet gained body weight steadily during weeks 15 to 52. Body weight approximated 80 % of that of the control group upon study termination. Liver copper concentrations decreased over the same time period from 1303 ± 68 µg/g at 15 weeks to 440 ± 44 µg/g at 52 weeks (control: 23 ± 4 µg, 15 and 52 weeks). There was a negative correlation between liver copper concentration and body weight.

Section A6.7 Copper toxicosis and tolerance in rats

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84.3	**Experiment 3**	The effect of copper challenge on liver copper concentration and pathological response of copper-primed rats was investigated. Animals receiving the control diet prior to challenge with 6000 mg Cu/kg diet for 3 weeks appeared lethargic with ruffled coats. In contrast, animals receiving 3000 mg Cu/kg diet for 15 weeks prior to challenge with 6000 mg Cu/kg diet for 3 weeks were very active and did not appear to be affected by the additional challenge. Liver copper contents increased after challenge of un-primed animals while the copper contents in primed rats did not significantly alter. Concurrently no histopathological changes in livers of the primed rats were observed after challenge, while moderate to severe hepatocellular necrosis with an associated inflammatory response was observed after challenge of un-primed rats.
		85 APPLICANT'S SUMMARY AND CONCLUSION
85.1	**Materials and methods**	Three experiments were performed with male weanling Wistar rats. In the first experiment dietary levels of 3000, 4000, 5000 or 6000 mg Cu/kg diet and a concurrent vehicle control were administered for up to 15 weeks. In a second experiments rats received either control diet or 3000 mg/kg copper diets for up to 1 year. The third experiment investigated effects of copper challenge (6000 mg Cu/kg diet) in primed (3000 mg Cu/kg diet for 15 weeks) or un-primed (control diet) rats .Body weights, liver copper contents and histopathological changes of livers were determined during all experiments. The study does not exactly meet the requirements of methods B.32 or B.33 (88/303/EEC), since it was a special investigation on copper toxicosis and tolerance in rats.
85.2	**Results and discussion**	Animals treated with copper at 3000 ppm for one year showed no long-term evidence of liver toxicity: an adaptive response was shown similar to the earlier shorter study, and at 52 weeks, copper concentrations were lower than at 15 weeks. Animals previously treated with copper at 3000 ppm for 15 weeks that were then given 6000 ppm (double the dose) for three weeks did not show altered liver copper concentrations, whereas previously untreated rats of the same age and strain given 6000 ppm copper showed moderate to severe hepatocellular necrosis. The extrapolation from copper sulphate to copper hydroxide is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper sulphate will only lead to a more conservative but nevertheless valid assessment.
85.3	**Conclusion**	
85.3.1	Reliability	2
85.3.2	Deficiencies	Yes No haematological, clinical chemistry or urine parameters were recorded. Full gross necropsy and histopathology were not reported. Tumour incidences were also not reported.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE (*)
Date	10/06/05
Materials and Methods	Agree with applicant's version
Results and discussion	Agree with applicant's version
Conclusion	Data not suitable for cancer hazard assessment of copper carbonate
Reliability	3
Acceptability	Not acceptable
Remarks	Data available is sufficient to allow a hazard assessment of Copper carbonate by oral route. No more tests are needed.
	COMMENTS FROM ...
Date	
Materials and Methods	
Results and discussion	
Conclusion	
Reliability	
Acceptability	
Remarks	