Section A6.3.1 **Repeated dose toxicity (oral)**

Annex Point IIA6.3 **Rat**

47.3.1	Duration of treatment	4 weeks
47.3.2	Frequency of exposure	daily
47.3.3	Postexposure period	none; animals were sacrificed
47.3.4	**Oral**	
47.3.4.1	Type	in food
47.3.4.2	Concentration	0 ppm 1000 ppm 2000 ppm 4000 ppm
47.3.4.3	Vehicle	copper was uniformly mixed with the diet ration
47.3.4.4	Concentration in vehicle	0 mg/kg food 500 mg/kg food 1000 mg/kg food 2000 mg/kg food 4000 mg/kg food
47.3.4.5	Total volume applied	--
47.3.4.6	Controls	plain diet
47.4	**Examinations**	
47.4.1	Observations	
47.4.1.1	Clinical signs	yes (after 4 weeks exposure period)
47.4.1.2	Mortality	yes (during the 4 weeks exposure period)
47.4.2	Body weight	yes (average growth during the 4 weeks exposure period)
47.4.3	Food consumption	yes (average food intake during the 4 weeks exposure period)
47.4.4	Water consumption	no
47.4.5	Ophthalmoscopic examination	no
47.4.6	Haematology	yes number of animals: all animals receiving high-copper diets time points: end of study Parameters: Copper content of blood
47.4.7	Clinical Chemisty	yes number of animals: all animals receiving high-copper diets time points: end of study Parameters: Copper content of spleen and liver
47.4.8	Urinalysis	no
47.5	**Sacrifice and pathology**	

Section A6.3.1 **Repeated dose toxicity (oral)**

Annex Point IIA6.3 **Rat**

47.5.1	Organ Weights	yes organs: liver, spleen
47.5.2	Gross and histopathology	no
47.5.3	Other examinations	not stated
47.5.4	Statistics	not stated
47.6	**Further remarks**	

48 RESULTS AND DISCUSSION

48.1	**Observations**	
48.1.1	Clinical signs	The observations are summarised in table A6.3.1-1 and A6.3.1-2.
48.1.2	Mortality	3 mortalities at a dose of 4000 ppm occurred
48.2	**Body weight gain**	The rats receiving 500 ppm of copper showed a good growth. In the rats receiving larger doses of copper the average growth was markedly depressed.
48.3	**Food consumption and compound intake**	The rats receiving 500 ppm of copper showed a slightly subnormal food consumption. In the rats receiving larger doses of copper the average food intake was markedly depressed.
48.4	**Ophtalmoscopic examination**	not conducted
48.5	**Blood analysis**	
48.5.1	Haematology	not stated
48.5.2	Clinical chemistry	Analysis of copper content in the blood revealed a significant increase by increasing dosage. Detailed information is given in table A6.3.1-1.
48.5.3	Urinalysis	not stated
48.6	**Sacrifice and pathology**	
48.6.1	Organ weights	The weights of spleen and liver decreased by increasing dosages of copper. Detailed information is given in table A6.3.1-2.
48.6.2	Gross and histopathology	not stated
48.7	**Other**	--

49 APPLICANT'S SUMMARY AND CONCLUSION

49.1	**Materials and methods**	White rats were fed ad libitum diets which contained 0, 500, 1000, 2000 and 4000 ppm of added copper in the form of copper sulfate

Section A6.3.1		**Repeated dose toxicity (oral)**	
Annex Point IIA6.3		**Rat**	
49.2	**Results and discussion**	Slight toxicity was observed on 500 ppm with increasing toxicity on higher levels as indicated by growth records. Whereas the copper content of the blood and spleens was increased a maximum of 2 to 5 times, the liver increased to a maximum of 300 times normal.	
49.3	**Conclusion**		
49.3.1	LO(A)EL	The LO(A)EL was not calculated. Slight toxicity was observed on 500 ppm with increasing toxicity on higher levels.	X
49.3.2	NO(A)EL	The NO(A)EL was not calculated.	
49.3.3	Other	The results of this study can also be taken into account for the evaluation of copper hydroxide, since after oral administration of both copper sulfate and copper hydroxide , the metabolically available particle is the Cu^{2+} ion, which is formed in the acid medium in the stomach	
49.3.4	Reliability	3	
49.3.5	Deficiencies	Analysis of blood was conducted according to scientific standard at that time.	

Evaluation by Competent Authorities
Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
EVALUATION BY RAPPORTEUR MEMBER STATE

Section A6.3.1	**Repeated dose toxicity (oral)**
Annex Point IIA6.3	**Rat**

Date	12/01/05
Materials and Methods	Agree with applicant's version
Results and discussion	Agree with applicant's version
Conclusion	LO(A)EL: 500 ppm NO(A)EL: no NO(A)EL can be derived Large deficiencies due to the method of blood analysis conducted according to scientific standard at this time.
Reliability	3
Acceptability	Not acceptable Due to the large amount of data the repetition of the test is not required.
Remarks	

	COMMENTS FROM ... *(specify)*
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	

Section A6.3.2 Annex Point IIA6.3	Repeated dose toxicity (dermal)	
	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 a repeated dose toxicity study via dermal administration is considered to be not required since route-to-route extrapolation is not considered to be restricted, and dermal absorption has been shown to be minimal.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	12/01/2005	
Evaluation of applicant's justification	Agree with the applicant's version	
Conclusion	Agree with the applicant's version	
Remarks	See comments on 28-day inhalation study non-submission of data.	
	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 A6.3.3 Annex Point IIA6.3	Repeated dose toxicity (inhalation)	
	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 a repeated dose toxicity study via inhalative administration is considered to be not required since route-to-route extrapolation is not considered to be restricted, and an adequate 90d study (oral) is available.	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	12/01/2005
Evaluation of applicant's justification	Some concerns about the route-to-route extrapolation because of the entero-hepatic cycle which occur by oral route and not by inhalation route. As it was demonstrated, Cu is first accumulated by the liver, if it can goes though general circulation before reaching the liver at relatively high doses, other sites of accumulation could be possible (ie. Brain).
Conclusion	Agree with the applicant's version (if it can be demonstrated that inhalation exposure is minimal)
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 A6.4.1 Chronic oral toxicity

Annex Point IIA6.4 Dog

			Official use only
		50 REFERENCE	
50.1	**Reference**	**A6.4.1/03: Doc.No. URA-97-08740-057** Anonymous (1982): Joint FAO/WHO Expert Committee on food additives: Copper toxicological evaluation of certain food additives; WHO Food additives Series 17.	
50.2	**Data protection**	No	
50.2.1	Data owner	Published data	
50.2.2	Companies with letter of access	--	
50.2.3	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.	
		51 GUIDELINES AND QUALITY ASSURANCE	
51.1	**Guideline study**	No	
51.2	**GLP**	No The study was conducted prior to implementation of GLP.	
51.3	**Deviations**	Not applicable	
		52 MATERIALS AND METHODS	
52.1	**Test material**	Copper gluconate	
52.1.1	Lot/Batch number	Not stated	
52.1.2	Specification	Not stated	
52.1.3	Purity	Not stated	
52.1.4	Description	Not stated	
52.1.5	Stability	Not stated	
52.2	**Test animals**		
52.2.1	Species	Dog	
52.2.2	Strain	Beagle	
52.2.3	Source	Not stated	
52.2.4	Sex	Male and female	
52.2.5	Age/weight at study initiation	Not stated	
52.2.6	Number of animals per group	Not specified	
52.2.7	Control animals	Yes	
52.3	**Administration/ Exposure**	Oral	
52.3.1	Duration of treatment	up to 1 year	

Section A6.4.1 Chronic oral toxicity

Annex Point IIA6.4 Dog

52.3.2	Frequency of exposure	Daily
52.3.3	Post-exposure period	No specified (at least a 12-week withdrawal period in high dose animals)
52.3.4	Type	In food
52.3.5	Concentration	3, 15, 60 mg Cu/kg bw/day
52.3.6	Vehicle	Diet
52.3.7	Concentration in vehicle	0.012, 0.06, 0.24 % of the diet
52.3.8	Total volume applied	Not stated
52.3.9	Controls	Not specified
52.4	**Examinations**	
52.4.1	Observations	
	Clinical signs	Yes
	Mortality	Yes
52.4.2	Body weight	Yes
52.4.3	Food consumption	Yes
52.4.4	Water consumption	Not stated
52.4.5	Ophthalmoscopic examination	Not stated
52.4.6	Haematology	Yes
		Not specified
52.4.7	Clinical chemistry	Yes
		Not specified
52.4.8	Urinalysis	Yes
		Not specified
52.5	**Sacrifice and pathology**	
52.5.1	Organ weights	Not stated
52.5.2	Gross and histopathology	Yes
		Not specified
52.5.3	Other examinations	Accumulation of copper in liver, kidneys and spleen
52.5.4	Statistics	Not stated
52.6	**Further remarks**	The results cited in the reference are based on the following report, which is not publicly available: Shanaman et al. (1972): One year chronic oral toxicity of copper gluconate, W10219A, in beagle dogs. Warner-Lambert Res. Inst., Morris Plains, N.J.; Res. Rept. No. 955-0353.

Section A6.4.1 Chronic oral toxicity

Annex Point IIA6.4 Dog

		53 RESULTS
53.1	**Interim sacrifice**	After 6 month of exposure, 2 animals of each sex were sacrificed and necropsied. Weight gains and food consumption values were similar for the control and treated groups. Overall health, haematology and urinalysis were comparable to controls.
53.2	**Terminal sacrifice**	After 1 year, minimal liver function changes were observed in 1 of 12 dogs receiving the 0.24 % copper gluconate diet, a change that was reversed following a 12-week withdrawal period. Accumulation of copper in liver, kidneys and spleen was seen at the high dose. No compound-related effects were observed at the lowest dose and there were no compound-related deaths or gross or microscopic pathological lesions in any dog.
		54 APPLICANT'S SUMMARY AND CONCLUSION
54.1	**Materials and methods**	A 1-year chronic study was conducted with male and female Beagle dogs to evaluate the potential oral toxicity of copper gluconate administered at levels of 0.012, 0.06 and 0.24 % of the diet.
54.2	**Results and discussion**	Following 1 year of exposure to the highest tested dose, minimal liver function changes were observed in 1 of 12 dogs, a change that was reversed following a 12-week withdrawal period. Accumulation of copper in liver, kidneys and spleen was seen at the high dose. No compound-related effects were observed at the lowest dose and there were no compound-related deaths or gross or microscopic pathological lesions in any dog.
54.3	**Conclusion**	This report documents that there is no scientific justification to perform any further toxicity testing on dogs. Dogs have a different form of albumin to rats and humans, and cannot excrete copper in the bile as readily as most other species. One of the major copper transporter proteins of the blood, albumin, contains a histidine in position 3 which is essential for tight binding of copper. In the dog (and the pig), this histidine is replaced by a tyrosine, and the albumin does not have the same affinity for copper. Dog and pig albumins have several low-affinity sites for copper, and albumin is still an effective transporter in those species. Dogs have unusually high levels of copper in the liver, ten times the levels in other species (dog liver 67 ppm, compared to 6.2 in human and 4.6 in rat). While dog liver rapidly took up copper injected intravenously, dogs do not appear to be able to excrete copper via the bile as readily as other species. Dogs are unable to tolerate the kinds of repeated doses that can be administered to rats. The protein that excretes copper from the liver (WND) is the protein that is inactive in Wilson's disease. The Bedlington terrier suffers from copper toxicosis and has been cited as a model for Wilson's disease, although the genetic basis for this has not been proven. It is possible that dogs express the WND protein less than other species resulting in accumulation of copper in the liver. These differences in albumin structure and the liver of the dog mean that the dog is not a good animal model for human risk assessment of copper.
54.3.1	LOEL	15 mg Cu/kg bw/day
54.3.2	NOAEL	15 mg Cu/kg bw/day

Section A6.4.1 Chronic oral toxicity

Annex Point IIA6.4 Dog

54.3.3	Reliability	4 Not assignable, since only a short summary in secondary literature is available.
54.3.4	Deficiencies	Yes Insufficient reporting

Section A 6.4.1 Subchronic oral toxicity

Annex Point IIA 6.4 Rat

			Official use only
		55 REFERENCE	
55.1	**Reference**	A6.4.1/01 Doc. no. 00620-2-05-47a HÉBERT, C.D. (1993): NTP Technical Report on Toxicity Studies of Cupric Sulfate Administered in Drinking Water and Feed to F344/N Rats and B6C3F$_1$ mice. *NTP Toxicity Report Series* No. 29, NIH Publication 93-3352 A6.4.1/02 Doc. no. 00620-2-05-47b HÉBERT, C.D. *ET AL.* (1993): Subchronic Toxicity of Cupric Sulfate Administered in Drinking Water and Feed to Rats and Mice. *Fundam. Appl. Toxicol.* 21, 461 - 475	
55.2	**Data protection**	No	
55.2.1	Data owner	published data	
55.2.2	Companies with letter of access	--	
55.2.3	Criteria for data protection	No data protection claimed	
		56 GUIDELINES AND QUALITY ASSURANCE	
56.1	**Guideline study**	compliant with OECD 408 generally meets the requirements of EC B.26	
56.2	**GLP**	Yes The studies were performed in compliance with U.S. Food and Drug Administration Good Laboratory Practise regulations (p. 39)	
56.3	**Deviations**	Yes Some parameters for haematology and clinical chemistry differ slightly from the list proposed by the guideline, and ophthalmological examinations were not performed. The two publications referred to above both belong to the same series of studies, but some information is only presented in one of the two papers. In this study summary only the results for rats were considered because the sensitivity to copper is higher in rats than in mice.	X
		57 MATERIALS AND METHODS	
57.1	**Test material**	Copper sulphate pentahydrate JT Baker (Phillipsburg, NJ)	
57.1.1	Lot/Batch number	Lot 533344	
57.1.2	Specification	Deviating from specification given in section 2 as follows: Copper sulphate pentahydrate CAS No. 7758-99-8	

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
57.1.2.1 Description	blue, crystalline solid	
57.1.2.2 Purity	99 % cupric sulfate	
57.1.2.3 Stability	Literature references indicate that cupric sulfate is stable at normal storage temperatures when kept dry. At the study laboratory cupric sulfate was stored at room temperature.	
57.2 Test Animals		
57.2.1 Species	Rats and mice	
57.2.2 Strain	Fischer 344/N rats and B6C3F$_1$ mice	
57.2.3 Source	Simonsen Laboratories (Gilroy, CA)	
57.2.4 Sex	male and female	
57.2.5 Age/weight at study initiation	Age: 6 weeks Weight: 105 - 107 g	X
57.2.6 Number of animals per group	60 animals • 20 rats (10 male and 10 female) in the diet group 0, 500 1000, 2000, 4000, 8000 ppm for 92 days • 20 rats (10 male and 10 female) with the same diet were used for clinical pathological determination during the study • 20 B6C3F$_1$ mice (10 male and 10 female) in the diet group 0, 1000, 2000, 4000, 8000, 16000 ppm for 92 days	
57.2.7 Control animals	Yes	
57.3 Administration/ Exposure	Oral	
57.3.1 Duration of treatment	13 weeks	
57.3.2 Frequency of exposure	daily	
57.3.3 Postexposure period	none	
57.3.4 Oral		
57.3.4.1 Type	gavage in food	
57.3.4.2 Concentration	food consumption per day: 34, 68, 135, 267, 528 mg/kg/d drinking water ad libitum	X
57.3.4.3 Vehicle	NIH-07 Open Formulare Diet in pellet form	
57.3.4.4 Concentration in vehicle	0, 500 1000, 2000, 4000, 8000 ppm	
57.3.4.5 Total volume applied	10 - 11 g per day	X
57.3.4.6 Controls	not stated	
57.4 Examinations		
57.4.1 Observations		

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
57.4.1.1 Clinical signs	no	X
57.4.1.2 Mortality	none	X
57.4.2 Body weight	Study initiation: 105 - 107 g Final weight:: 179 - 199 g Final weight relative to Controls: 76 - 99 % (male) and 93 - 102 % (female)	X
57.4.3 Food consumption	10 - 11 g/day	X
57.4.4 Water consumption	water was available ad libitum	X
57.4.5 Ophthalmoscopic examination	no	
57.4.6 Haematology	yes number of animals: supplemental rats time points: day 5, 21 and 92 Parameters: haematocrit, haemoglobin concentration, erythrocyte count, reticulocyte count, mean cell volume, platelets, leukocyte count	
57.4.7 Clinical Chemisty	yes number of animals: supplemental rats time points: day 5, 21 and 92 Parameters: alanine aminotransferase, alkaline phosphatase, 5'-nucleotidase, sorbitol dehydrogenase, bile salts, total protein, albumin, creatinine, urea nitrogen	
57.4.8 Urinalysis	yes number of animals: supplemental rats time points: day 19 and 90 Parameters: creatinine, glucose, protein, aspartate aminotransferase, N-actetyl-β-D-glucosaminidase, volume and specific gravity	
57.5 Sacrifice and pathology		
57.5.1 Organ Weights	The content of copper and other metals were determined in liver, kidney, plasma and testis.	X
57.5.2 Gross and histopathology	yes Histopathological examination were conducted on the target organs liver, kidney and forestomach in all dose groups and controls	
57.5.3 Other examinations	Sperm morphology and motility were evaluated at necropsy and vaginal cytology evaluations were performed during the 12 days prior to termination from controls and the three highest exposure groups.	X
57.5.4 Statistics	Organ and body weight data: Parametric multiple comparisons procedures of Williams (1971.1972) or Dunnett (1955).	X

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
		Clinical chemistry and hematology data: Nonparametric multiple comparisons method by Shirley (1977) or Dunn (1964). Significance of dose-response trends: Jonckheere's test (1954).
57.6	**Further remarks**	no
		58 RESULTS AND DISCUSSION
58.1	**Observations**	
58.1.1	Clinical signs	No clinical signs of toxicity that could be directly attributed to cupric sulfate consumption were observed in male or female rats.
58.1.2	Mortality	Except for one female (1000 ppm) which was killed accidentally all rats survived until study termination.
58.2	**Body weight gain**	Body weights were significantly depressed in male rats of the 4000 ppm and 8000 ppm groups and in high dose females
58.3	**Food consumption and compound intake**	The average daily feed consumption in the 500 to 4000 ppm groups were similar to that of the control groups. Male and female rats in the high-dose group (8000 ppm) consumed slightly less food than the animals in the control group. Despite the slight decrease in feed consumption in high-dose rats, the average daily compound consumption increased proportionally with increasing concentrations of cupric sulfate in the feed.
58.4	**Ophtalmoscopic examination**	not performed
58.5	**Blood analysis**	
58.5.1	Haematology	Significant changes in haematology parameters were noted in male and female rats at all time points (see also table A6.4.1-1): At day 5: Increase of **hematocrit** (HCT) and **hemoglobin** (HGB) concentrations in high-dose male and female rats. By day 21 significant decrease of these parameters in the two/three highest dose groups (male/female). At day 92, HCT and HGB concentrations were significantly decreased. At day 5, significant increases in **erythrocyte** (RBC) counts were noted especially in males. On day 92 , the only significant increase in RBC count was noted in high-dose males. The **reticulocyte** counts decreased significant in the two highest dose groups in male and female at day 5. By day 21, reticulocyte counts were significant greater than those of the controls and at day 92 this parameter was significantly increased in the high-dose males. The only significant change note in **nucleated erythrocytes** was a marginal decrease in high-dose males at day 5. On day 5, **mean cell volume** (MCV) values were significantly decreased in the highest dose groups in male and female; **mean cell hemoglobin** (MCH) values were also significantly decreased for males in the highest dose groups. At day 21 and 92, decreases in MCV and MCH were noted in males and females in the three highest dose groups.

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
	The only significant changes in MCH concentrations were increase noted on day 21 in high-dose females and males in the two highest-dose groups. At day 5 and 21, significant increases in **platelet counts** were noted in males and females in the three highest-dose groups. The day 92 increases in platelet counts were noted for males and females in the two highest-dose groups, but significant increases only for males. **Leukocyte counts** were increased at all time points in male and female rats in the two highest dose groups, with significant increases occurring at day 5 in high-dose males, at day 92 in high-dose males and females. Significant increases in **lymphocytes** were noted at day 5 in high-dose males and at day 92 in high-dose females. The only other significant change noted in hematology parameters was an increase in **segmented neutrophils** at day 92 in high-dose male rats.	
58.5.2 Clinical chemistry	Significant changes in serum chemistry parameters occurred in male and female rats at all time points mainly in the two highest dose groups (see also table A6.4.1 -2): **Alanine aminotransferase** activities were significantly increased at all time points in male and female rats in the two highest dose groups: this parameter was also significantly increased at day 92 in males receiving 1000 of 2000 ppm cupric sulfate. At days 5 and 21, decreases in **alkaline phosphatase** (AP) activities were noted in males and females in the two highest dose groups; except for the day 21 AP activity in males in the 4000 ppm group all of these decreases were significant relative to the control values. Significant changes in **sorbitol dehydrogenase** (SDH) were limited to the day 21 and 92 time points. At both of these time points, SDH activities were significantly elevated in males in the two highest dose groups and in high-dose females; significant increases in SDH activities were also noted at day 92 in males in the 2000 ppm group and females in the 4000 ppm group. When compared to control values, **5'-nucleotidase** was significantly decreased in high-dose females at days 5 and 21 and in high-dose males at day 92, however, this parameters was significantly increased in males receiving 4000 of 8000 ppm cupric sulfate. At day 5, slight increases in **bile salts** were noted in males in the three highest dose groups; however, female bile salt values were decreased for all treated groups at this time point, with significant decreases in the 1000 and 8000 ppm groups. By day 21, no significant changes in this parameter were noted in females, but significant increases were noted in males in the two highest dose groups. At day 92, significant increases in bile salts were noted in high-dose males and in females receiving 2000 or 4000 ppm cupric sulfate. At all time points, **total protein** was significantly decreased in high-dose males and in females in the two highest dose groups: at day 5 and 21, total protein was also significantly decreased in males receiving 4000 ppm cupric sulfate and in females receiving 2000 ppm cupric sulfate. At day 5 and 21, decreases in albumin concentrations were noted. in males and females in the three highest dose groups, and all of these decreases were significant, excluding the day 21, albumin concentration for males receiving 2000 ppm cupric sulfate. At day 92, this parameter was significantly decreased in high dose males and in females in the two highest dose groups.	

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
	Urea nitrogen (UN) was significantly increased for males and females in the two highest dose groups at day 5, and by day 21, this parameter was significantly increased for males in males in the three highest dose groups and females in the highest dose group. At day 92, UN was significantly elevated in high-dose males and females as well as in females receiving 1000, 2000, of 4000 ppm cupric sulfate. The only significant change in creatinine was an increase noted in high-dose females. on day 92.	
58.5.3 Urinalysis	Significant changes in urinalysis parameters were noted in supplemental-study rats at day 19 and in base-study rats at day 90 (see also table A6.4.1-3). Significant increases in urinary **aspartate aminotransferase** (AST) activities expressed in IU/L or IU/mg creatinine, occurred at days 19 and 90 in male and female rats in the highest dose groups. Generally, increases in this parameter also occurred at both time points in males and female rats in the 4000 ppm groups, and most of these increases were significant. A few significant increases in AST activities occurred in animals in the lower dose groups (500 to 2000 ppm). Significant increases in N-acetyl-β-D-glucosaminidase activities expressed in IU/L or IU/mg creatinine were noted in high-dose males and female rats on day 90; at this time point, increases also occurred in males and females in the 4000 ppm groups, and the increases for the parameter expressed in IU/mg creatinine were significant. **Glucose output** (mg/mg creatinine) was significantly increased at day 19 in males in the 2000 ppm group, and at day 90, this parameter was significantly elevated in males in the two highest dose groups. A significant decrease in **protein output** (mg/mg creatinine) was noted in high-dose males at day 19; however, at day 90 evaluation in base-study rats, this parameter was significantly increased relative to the control in males in the two highest dose groups. No significant changes in glucose or protein output were noted in females at either time points.	
58.6 Sacrifice and pathology		
58.6.1 Organ weights	Dose-related accumulations of copper were observed in the liver and kidney accompanied by increases in zinc in the three highest groups. Copper levels were also elevated in plasma and the testis in the three highest groups. Detailed results for copper and other metal contents in tissues are given in table A6.4.1-4.	X
58.6.2 Gross and histopathology	Histopathologic findings given in table A6.4.1-5 corresponded to the gross lesions consisted of minimal to moderate hyperplasia of the squamous mucosa at the site of the limiting ridge. This lesion was characterized by a thickening and increased folding of the squamous mucosa; hyperkeratosis was a component of the squamous cell hyperplasia. The increased incidence and severity of this lesion were dose related. When this lesion was more severe (moderate grade), there was often an increase in the number of inflammatory cells and/or edema in the lamina propria of the limiting ridge. There was no evidence of erosion/ulceration, and no lesions were present in other areas of the squamous mucosa.	X

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
58.7 Other	**Sperm morphology and vaginal cytology**: There were no changes in testis, epididymis or cauda epididymis weight, or spermatid counts or sperm motility in males of either species at any dose level. Dose related data are given in table A6.4.1-6 Similarly, there were no changes in oestrous cycle length or in the timings in each phase of the cycle in females of either species. The study parameters are listed in table A6.4.1-7	
	59 APPLICANT'S SUMMARY AND CONCLUSION	
59.1 Materials and methods	Feeding studies with groups of 10 rats per sex were carried out in compliance with OECD guideline 408. Deviations from the guideline: • some parameters for haematology and clinical chemistry differ slightly from the list proposed by the guideline • ophtalmological examinations were not performed	
59.2 Results and discussion	No clinical signs of toxicity were observed. Body weights were significantly depressed in male rats in the high-dose groups. Effect on necropsy body weights, absolute organ weights and organ-to-body-weights ratios were seen in the two highest-dose groups. Gross macroscopic and histopathological examinations revealed lesions in the limiting ridge of the fore stomach at and above 2000 ppm in both sexes. A dose-related increase in chronic-active inflammation was seen in livers on most animals of the 4000 and 8000 ppm groups, and in one male of the 2000 ppm group. Positive staining for copper was observed in the two highest dose groups. Increased incidences of cytoplasmatic protein droplets were present in kidneys of animals of both sexes at and above 2000 ppm. However, one single incident with minimal severity was seen in females in the 1000 ppm group. Copper staining was positive only in the two highest dose groups. Changes in hematological, clinical chemistry parameters and urinalysis indicative for an ineffective hematopoesis resulting in microcytic anaemia, hepatocellular injury and renal tubular damage were observed mainly at 4000 and 8000 ppm with a few single incidents at 2000 ppm. Additionally, iron depletion was seen in spleens at and above 2000 ppm.	
59.3 Conclusion	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.	
59.3.1 LOAEL	129 mg/kg bw	X
59.3.2 NOAEL	1000 ppm	X

Section A 6.4.1	Subchronic oral toxicity	
Annex Point IIA 6.4	Rat	
	64 - 68 mg/kg bw	
59.3.3 Other	not stated	
59.3.4 Reliability	2	
59.3.5 Deficiencies	No	X

Section A 6.4.1	**Subchronic oral toxicity**
Annex Point IIA 6.4	**Rat**

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	12/01/2005
Materials and Methods	2.3 Deviations: histopathological examination did not include the aorta. 3.2.5 Range cited in this section is only for females. For males bw range was 119-120 3.3.4.2 Concentrations cited in this section were for females. For males the following compound consumption were reported: 32, 64, 129, 259 and 551 mg/kg bw/d for the 500, 1000, 2000, 4000 and 8000 ppm groups respectively. 3.3.4.5 Food consumption indicated in this section is for females only. For males the following range was reported: 14 – 17g/day 3.4.1.1 Clinical observation recorded weekly. 3.4.1.2 Mortality/morbidity recorded twice daily 3.4.2 Individual body weight were recorded prior the start of the study, on day 1 and weekly thereafter. Results given here should be placed in the proper section. 3.4.3 Food consumption was recorded weekly. 3.4.4 Water consumption not reported. 3.5.1 Organ weighted: liver, thymus, right kidney, right testis, heart, lung and brain. The sentence included in this section should be put in the 3.5.3 section 3.5.3 Add sentence cited in section 3.5.1. 3.5.4 Vaginal cytology data: Multivariate analysis of variance (Morrison, 1976).

Section A 6.4.1	**Subchronic oral toxicity**
Annex Point IIA 6.4	**Rat**
Results and discussion	4.6.1 Should be included in the 4.7 section. Significant changes in absolute organ weights were observed in the high dose group animals: decreases in absolute brain, heart, kidney, liver, lung and thymus weight in males and absolute kidney weight in females. 4.6.2 Liver and kidney lesions were also observed. In the liver a dose-related increase of chronic inflammation was observed in males from 2000 ppm and in females from 4000 ppm. It consisted in multiple foci of a mixture of mononuclear inflammatory cells, primarily macrophages often associated with hepatocyte necrosis in or around the foci. These lesions primarily occurred in the periportal portion of the lobules. In the kidneys, cytoplasmic alteration (increase in size and number of cytoplasmic protein droplets) was observed from 2000 ppm (also in one out of 10 females of the 1000 ppm group). This lesion was less severe in females but of greater incidence in females in the 2000 ppm group (see table6.4.1-5). After staining the identity of the contents of the droplets could not be ascertained. In high dose males karyomegaly of tubule cells was also observed. Degeneration of the renal tubule epithelium was present in 3 females of the high dose group. 4.7.1 Cu accumulation on organs: add sentence cited in 4.6.1. Moreover, Cu concentrations were increased in the liver and kidneys of **all** treated groups (not only the three highest groups), this was not clear in the §.
Conclusion	LO(A)EL: - 2000 ppm for forestomach lesion (males and females) - 2000 ppm for liver damages in males and 4000 ppm in females - 1000 ppm for kidney damages in females and 2000 ppm in males NO(A)EL: - 1000 ppm for forestomach lesions (68 mg/kg bw/d for males and 64 mg/kg bw/d for females) - 1000 ppm (68 mg/kg bw/d) for hepatic damages in males and 2000 ppm (135 mg/kg bw/d for females) - 500 ppm (34 mg/kg bw/d) for renal damages in females and 1000 ppm (68 mg/kg bw/d) in males. The NOAEL of 500 ppm is very conservative as minimal kidneys effects were seen in only one female at 1000 ppm. 5.3.5 deficiencies: see previous comments
Reliability	2
Acceptability	Acceptable
Remarks	
	COMMENTS FROM ... *(specify)*
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*

Section A 6.4.1	**Subchronic oral toxicity**
Annex Point IIA 6.4	**Rat**

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

Table A6.4.1-1: Results of haematology during the study in rats

Parameter changed		Concentration of cupric sulfate																	
		Controls			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
day of treatment		5	21	92	5	21	92	5	21	92	5	21	92	5	21	92	5	21	92
males	Hematocrit (%)	39.2	46.0	47.9								↓			⇓	⇓	⇑	⇓	⇓
	Hemoglobin (g/dL)	13.1	14.2	14.3											⇓	⇓	⇑	⇓	⇓
	Erythrocytes (10^6/µL)	6.6	7.89	8.88							↓			⇑			⇑	↓	⇑
	Reticulo-cytes (10^6/µL)	0.45	0.2	0.15			↑							⇓	⇑	⇑	⇓	⇑	⇑
	Mean cell volume (fL)	59.4	58.3	54.0								⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓
	Mean cell hemoglobin (pg)	19.9	18.0	16.0								⇓	⇓	⇓	⇓	⇓	⇓	⇓	⇓
	Platelets (10^3/µL)	836	735	631	↑	↑	↑	⇑	↑	↑	⇑	↑	↑	⇑	↑	⇑	⇑	↑↑	⇑
	Leukocytes (10^3/µL)	5.93	6.15	8.39							↑	⇑		↑	⇑	↑	⇑	↑	⇑
females	Hematocrit (%)	43.3	49.3	48.6								⇓			⇓		⇑	⇓	⇓
	Hemoglobin (g/dL)	13.9	15.2	14.5								⇓			⇓		⇑	⇓	⇓
	Erythrocytes (10^6/µL)	7.25	8.27	8.48													⇑		
	Reticulo-cytes (10^6/µL)	0.34	0.12	0.13										⇓	⇑		⇓	⇑	
	Mean cell volume (fL)	59.9	59.7	57.2								⇓	⇓		⇓	⇓	⇓	⇓	⇓
	Mean cell hemoglobin (pg)	19.2	18.4	17.0								⇓	⇓		⇓	↓		⇓	⇓
	Platelets (10^3/µL)	823	696	700							⇑			⇑	↑	↑	⇑	↑	↑
	Leukocytes (10^3/µL)	5.49	6.81	7.78						↓				↑	↑	↑	↑	↑	⇑

Table A6.4.1-2: Results of clinical chemistry in the study on rats

Parameter changed		Concentration of cupric sulfate																	
		Controls			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
day of treatment		5	21	92	5	21	92	5	21	92	5	21	92	5	21	92	5	21	92
males	Alanine aminotrans-ferase (IU/L)	42	44	51			↑		↑	⇑		↑	⇑	⇑	⇑	⇑	⇑	⇑	⇑
	Alkaline phos-phatase (IU/L)	1596	1131	503			↑							⇓	↓		⇓	⇓	↑
	5'-nucleoti-dase (IU/L)	36.5	31.8	33.6										↓		⇑	⇓	↑	⇑
	Sorbitol de-hydrogenase (IU/L)	18	22	22									⇑		⇑	⇑		⇑	⇑
	Bile salts (µmol/L)	15.8	15.6	14.1	↑	↓	↓	↓		↓	↑			↑	⇑	↑	↑	⇑	⇑
	Total protein (g/dL)	5.6	5.9	6.6										⇓	⇓		⇓	⇓	⇓
	Albumin (g/dL)	4.2	4.3	4.6							⇓	↓		⇓	⇓		⇓	⇓	⇓
	Urea nitrogen (mg/dL)	21.1	20.0	21.6								⇑		⇑	⇑		⇑	⇑	⇑
females	Alanine aminotrans-ferase (IU/L)	39	37	44									↓	⇑	⇑	⇑	⇑	⇑	⇑
	Alkaline phos-phatase (IU/L)	1226	893	408						↑			↑	⇓	⇓		⇓	⇓	↓
	5'-nucleoti-dase (IU/L)	39.0	35.4	34.5		↑				↑				↓			⇓	⇓	
	Sorbitol de-hydrogenase (IU/L)	24	22	16				↓			↓					⇑		⇑	⇑
	Bile salts (µmol/L)	19.3	17.5	13.4	↓			⇓	↓		↓	↓	⇑	↓		⇑	⇓	↑	
	Total protein (g/dL)	5.7	5.9	6.6							⇓	⇓		⇓	⇓	⇓	⇓	⇓	⇓
	Albumin (g/dL)	4.3	4.4	4.8							⇓	⇓		⇓	⇓	⇓	⇓	⇓	⇓
	Urea nitrogen (mg/dL)	21.9	22.1	17.1						⇑			⇑	⇑		⇑	⇑	⇑	⇑

Table A6.4.1-3: Results of urinalysis during the study in rats

Parameter changed		Concentration of cupric sulfate											
		Controls		500 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm	
day of treatment		19	90	19	90	19	90	19	90	19	90	19	90
males	Creatinine (mg/dL)	52.5	109	↓				↑	↓	↓	↓		↓
	Glucose (mg/dL)	13	19					↑	↓				
	Glucose output (mg/mg creatinine)	0.25	0.18					⇑			⇑		⇑
	Protein (mg/dL)	225	282	↓						↓		↓	
	Protein output (mg/mg creatinine)	4.1	2.6								⇑	⇓	⇑
	Aspartate aminotransferase (IU/L)	4	8							↑	↑	⇑	⇑
	Aspartate aminotransferase (IU/mg creatinine)	0.09	0.08							↑		⇑	⇑
	N-acetyl-β-D-glucosaminidase (IU/L)	6.7	8.9								↑		⇑
	N-acetyl-β-D-glucosa-minidase (IU/mg creatinine)	0.13	0.09								⇑		⇑
	Volume (mL/16 h)	9.4	7.9					↓				↓	
	Specific gravity	1.02	1.03										
females	Creatinine (mg/dL)	27.2	63.7		↓	↓			↑			↓	
	Glucose (mg/dL)	7	7			↓			↑			↓	
	Glucose output (mg/mg creatinine)	0.23	0.11										
	Protein (mg/dL)	34	67			↓	↓		↑		↓	↓	↓
	Protein output (mg/mg creatinine)	0.86	0.94							↑			
	Aspartate aminotransferase (IU/L)	2	3						↑		↑	⇑	⇑
	Aspartate aminotransferase (IU/mg creatinine)	0.08	0.04								↑	⇑	⇑
	N-acetyl-β-D-glucosaminidase (IU/L)	4.2	6.3			↓			↑		↑	↓	⇑
	N-acetyl-β-D-glucosaminidase (IU/mg creatinine)	0.17	0.1								⇑		⇑
	Volume (mL/16 h)	11.5	7.3						↓				
	Specific gravity	1.02	1.02										

Table 6.4.1-4: Tissue metal concentrations (ppm) in male rats

	Dose level (ppm)					
	0	500	1000	2000	4000	8000
Copper						
Liver	0.24	1.83*	6.11*	17.90*	127.31*	372.12*
Kidney	0.62	4.81*	3.45*	7.65*	52.89*	181.03*
Plasma	0.09	0.09	0.02	0.18	0.29*	0.85*
Testis	0.10	0.11	0.26	1.25*	1.24*	1.21*
Calcium						
Kidney	15.48	14.24	12.23	10.59	12.25	9.78
Plasma	3.15	2.31	2.49	0.74	0.62	0.17*
Magnesium						
Plasma	0.00	0.09	0.11	0.16	0.05	0.86*
Zinc						
Liver	0.31	0.63	0.07	3.68*	2.71*	4.43*
Kidney	3.22	4.34	3.97	5.35*	5.48*	6.38*

* $P < 0.01$

Table 6.4.1-5: Histopathological findings (incidence and severity) in rats

	Incidence and mean severity ( ) at dose level (ppm)					
	0	500	1000	2000	4000	8000
Male						
Forestomach, hyperplasia and hyperkeratosis	0	-	-	10 (1.6)	10 (2.8)	10 (2.8)
Liver, inflammation	0	-	0	1 (1.0)	10 (1.0)	10 (1.9)
Kidney, droplets	0	-	0	3 (1.0)	10 (2.0)	10 (2.5)
Kidney, karyomegaly	0	-	0	0	0	10 (1.0)
Female						
Forestomach, hyperplasia and hyperkeratosis	0	-	-	7 (1.3)	10 (2.5)	10 (2.5)
Liver, inflammation	0	-	0	0	6 (1.2)	10 (1.9)
Kidney, droplets	0	-	1 (1.0)	9 (1.0)	10 (1.0)	10 (1.0)
Kidney, karyomegaly	0	-	0	0	0	10 (1.1)
Kidney, degeneration	0	-	0	0	0	3 (1.3)

Mean severity (in brackets) based on number of animals with lesions 1, minimal; 2, mild; 3, moderate; 4, marked

Table 6.4.1-6: Evaluation of reproduction tissues in male rats

	Concentration of cupric sulfate (ppm)			
	0 ppm	500 ppm	2000 ppm	4000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	361 ± 5	345 ± 9	352 ± 11	339 ± 5
Left epididymis	0.440 ± 0.009	0.428 ± 0.004	0.440 ± 0.013	0.432 ± 0.007
Left cauda epididymis	0.145 ± 0.006	0.139 ± 0.005	0.146 ± 0.004	0.138 ± 0.004
Left Testis	1.51 ± 0.02	1.49 ± 0.03	1.52 ± 0.04	1.59 ± 0.08
Spermatid measurements				
Spermatid heads (10^7/g testis)	10.83 ± 0.42	11.39 ± 0.83	12.66 ± 0.49	10.76 ± 0.57
Spermatid heads (10^7/testis)	8.05 ± 0.27	8.20 ± 0.62	9.20 ± 0.39	8.10 ± 0.36
Spermatid count (mean 10^{-4}/mL suspension)	80.48 ± 2.74	82.03 ± 6.16	92.03 ± 3.89	81.03 ± 3.60
Spermatozoal measuremetns				
Mobility (%)	71.44 ± 1.95	72.98 ± 1.60	67.14 ± 2.16	70.09 ± 2.02
Concentration (10^6/g cauda epididymal tissue)	885.6 ± 66.5	810.7 ± 48.2	773.3 ± 37.3	782.2 ± 25.0

Table 6.4.1-7: Evaluation of estrous cycle parameters in female rats

	Concentration of cupric sulfate (ppm)			
	0 ppm	500 ppm	2000 ppm	4000 ppm
n	10	10	10	10
Necropsy body weight (g)	196 ± 2	194 ± 3	196 ± 3	190 ± 3
Estrous cycle length (days)	4.85 ± 0.11	4.75 ± 0.11	4.95 ± 0.09	5.20 ± 0.13
Estrous stages (% of cycle)				
Diestrus	33.3	37.5	36.7	42.5
Proestrus	10.8	11.7	10.0	10.8
Estrus	33.3	31.7	31.7	25.8
Metestrus	225	19.2	20.8	20.0
Uncertain diagnoses (%)	0.0	0.0	0.8	0.8

Section A6.4.2 Annex Point IIA6.4	Subchronic dermal toxicity test	
	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 a subchronic dermal toxicity test is considered to be not required since route-to-route extrapolation is not considered to be restricted.	

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	12/01/2005
Evaluation of applicant's justification	See previous comments on 28-day inhalation study. Non submission data here is justify because dermal penetration is minimal.
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 A6.4.3 Annex Point IIA6.4	Subchronic inhalation toxicity test	
	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 a subchronic inhalation toxicity test is considered to be not required since route-to-route extrapolation is not considered to be restricted.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	12/01/2005	
Evaluation of applicant's justification	See comments made for the 28-day inhalation study non submission of data.	
Conclusion	See comments made for the 28-day inhalation study non submission of data. If inhalation exposure is possible at significant levels, this study could be required.	
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 A 6.5 Chronic toxicity

Annex Point IIA 6.5 **– Rat, oral –**

Official use only

60 REFERENCE

60.1	**Reference**	**A6.5/01:**
		HARRISON, J.W.E. et al. (1954): The safety and fate of potassium sodium copper chlorophyllin and other copper compounds; J. Am. Pharm. Ass. 43, 722-737.
		Doc. no. 97-08740-067
60.2	**Data protection**	No
60.2.1	Data owner	Published data
60.2.2	Companies with letter of access	--
60.2.3	Criteria for data protection	Not applicable

61 GUIDELINES AND QUALITY ASSURANCE

61.1	**Guideline study**	No
		The conduct of the study was similar to method B.30 (88/303/EEC), except that adrenals were not weighed upon necropsy and that no detailed results were reported for haematology, clinical chemistry and urinalysis.
61.2	**GLP**	No
		The study was conducted prior to implementation of GLP.
61.3	**Deviations**	Not applicable

62 MATERIALS AND METHODS

62.1	**Test material**	Potassium sodium copper chlorophyllin (Trial 1) Copper sulphate anhyd. (Trial 2) Copper gluconate (Trial 2)
62.1.1	Lot/Batch number	Not stated
62.1.2	Specification	Not specified
62.1.2.1	Description	Not specified
62.1.2.2	Purity	Not stated
62.1.2.3	Stability	Not stated

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 – Rat, oral –

62.2	**Test Animals**	
62.2.1	Species	Rat
62.2.2	Strain	Sprague-Dawley
62.2.3	Source	Not stated
62.2.4	Sex	Male and female
62.2.5	Age/weight at study initiation	Age: not specified (weanling rats) Weight: f 49–52 g; m 53–54 g (Trial 1) f 67–75 g; m 71–81 g (Trial 2)
62.2.6	Number of animals per group	25 females and 25 males
62.2.7	Control animals	Yes, concurrently for each trial
62.3	**Administration/ Exposure**	Oral
62.3.1	Duration of treatment	Up to 104 weeks (Trial 1) up to 40–44 weeks (Trial 2)
62.3.2	Frequency of exposure	Not specified
62.3.3	Post-exposure period	None
62.3.4	**Oral**	
62.3.4.1	Type	In food
62.3.4.2	Concentration	53, 530 or 1600 ppm of copper as potassium sodium copper chlorophyllin 530 or 1600 ppm of copper as copper sulphate 1600 ppm of copper as copper gluconate
62.3.4.3	Vehicle	Diet (Rockland rat meal)
62.3.4.4	Concentration in vehicle	0.1, 1.0, 3.0 % of potassium sodium copper chlorophyllin in the diet 0.135, 0.406 % copper sulphate in the diet 1.147 % copper gluconate in the diet
62.3.4.5	Total volume applied	Not specified
62.3.4.6	Controls	Vehicle only
62.4	**Examinations**	
62.4.1	Observations	
62.4.1.1	Clinical signs	Yes (at least three times each week)
62.4.1.2	Mortality	Yes (at least three times each week)
62.4.2	Body weight	Yes (weekly)
62.4.3	Food consumption	Yes (weekly)

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 – Rat, oral –

62.4.4	Water consumption	Yes (weekly)
62.4.5	Ophthalmoscopic examination	Not stated
62.4.6	Haematology	Yes (Trial 1 and 2) Number of animals: not specified Time points: not specified Parameters: routine haematological examinations (not specified) and oxygen carrying capacity
62.4.7	Clinical Chemistry	Yes (Trial 1 and 2) Number of animals: not specified Time points: not specified Parameters: not specified and non-protein nitrogen (Trial 1 and 2), plasma concentration of potassium sodium copper chlorophyllin and of copper (Trial 1)
62.4.8	Urinalysis	Yes (Trial 1 and 2) Number of animals: not specified Time points: not specified Parameters: routine urine examinations (not specified)
62.5	**Sacrifice and pathology**	
62.5.1	Organ Weights	Yes (at 52 and ca. 104 weeks for Trial 1; 33 and 42 weeks for Trial 2) Organs: liver, lungs, kidneys, testes, seminal vesicles, uterus, ovaries, spleen, brain, heart, stomach
62.5.2	Gross and histopathology	Yes (at 10, 52 and ca. 104 weeks for Trial 1; ca. 33 and ca. 42 weeks for Trial 2) Histopathology (52 weeks, Trial 1): kidneys, liver, stomach, small intestine and spleen . Histopathology (104 weeks, control and high dose group, Trial 1): oesophagus, stomach, large intestines, liver, pancreas, kidneys, adrenals, spleen, heart, uterus, ovaries, sciatic nerve tissue, testes and seminal vesicles. Histopathology (ca. 42 weeks, low dose, Trial 2): liver, kidneys and testes. Histopathology (ca. 33 weeks, high dose groups and control, Trial 2): spleen, adrenals, small and large intestines, stomach, sciatic nerve, kidneys, liver, ovaries, and testes.
62.5.3	Other examinations	Tissue stored copper and iron was determined in liver, kidneys and spleen (Trial 1 and 2). Mating trial: Five males and five females from each group from trial 1 were paired for mating for a period of one week. The females were allowed to litter and rear pups to maturity. Numbers of pups born and the number raised to maturity were counted.
62.5.4	Statistics	Not specified

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 – Rat, oral –

62.6	**Further remarks**	In order to hold a reasonably consistent ratio of test substance intake per gram of animal weight over the wide life cycle weight range of the test animals, a moving percentage in the diet was maintained. During the first fourteen days on test when food intake is highest per gram of animal weight, 25 % of the stated concentrations were fed, and during the second fourteen days 50 % of the stated concentrations were administered. Thereafter the specified test concentrations in the diet were maintained. 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.

63 RESULTS AND DISCUSSION

63.1	**Observations**	
63.1.1	Clinical signs	Not stated.
63.1.2	Mortality	The majority of deaths occurred during the last few weeks of trial 1, resulting in a total of 30 % mortality in the control group, 22 % and 18 % mortality in the 3 % and 0.1 % diet groups, respectively. In contrast, 90 % of animals administered with 1600 ppm copper as copper gluconate died between the 4^{th} and 8^{th} month of trial 2.
63.2	**Body weight gain**	After 20 month of administration of potassium sodium copper chlorophyllin, body weight gain in male and female rats was similar to the control. After this period, death and emaciation due to age affected all groups including the control group. In contrast, animals receiving 1600 ppm copper as copper gluconate or copper sulphate were adversely affected in growth. This retardation became readily discernible at the 26^{th} week, when male control animals and males receiving 530 ppm of copper as copper sulphate weighed at least 50 % more than those animals upon the 1600 ppm copper intake, either as gluconate or as sulphate. The results of trial 1 and 2 are presented in Table A6.5- 1 and Table A6.5- 2 , respectively.
63.3	**Food consumption and compound intake**	During the first trial, the net food intake over a ninety-three week period averaged within 3 % for all groups, an average net daily food use of approximately 20 g for males and 16 g for females.
63.4	**Ophthalmoscopic examination**	No data

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 – Rat, oral –

63.5	**Blood analysis**	Routine haematological and urine examinations were performed at intervals during both trials and were reported to be within normal expected ranges, with exception of blood non-protein nitrogen levels during trial 2. Non-protein nitrogen levels exceeding the expected range of 50 – 70 mg. % were noted in males of the high dose copper sulphate group (83 mg. %) as well as males of the high dose copper gluconate group (109 mg. %). No treatment-related effects on the oxygen carrying capacity were observed.
63.6	**Sacrifice and pathology**	
63.6.1	Organ weights	For animals administered with potassium sodium copper chlorophyllin, no significant differences in organ weights were observed when compared to the control upon necropsy after 52 and 104 weeks. In contrast, hypertrophied uteri, ovaries or seminal vesicles were observed in animals dosed with copper gluconate and enlarged stomachs were noted in females of the high dose copper sulphate group and animals of both sexes of the copper gluconate group. The organ weights are summarised in Table A6.5- 3.
63.6.2	Gross and histopathology	Gross necropsy of animals sacrificed at the 10^{th}, 52^{nd} and approx. 104^{th} week revealed no treatment-related changes (Trial 1). In contrast, findings observed in animals receiving 1600 ppm copper as copper sulphate or copper gluconate, included 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 ulcer, some blood; bloody mucous in the intestinal tract. In some animals receiving copper gluconate, flabby and distended stomachs were noted. During trial 1, histopathological examinations revealed no evidence of adverse effects of the administered substance upon the organs examined, aside from minor adrenal cortical findings. Changes of a cystic and old haemorrhagic nature in the cortex of two high dose (3 %) animals and a small adenoma in one high dose animal were observed, which could well be associated with old age. In contrast, liver sections of animals receiving high levels of copper sulphate or gluconate revealed well defined abnormalities of a toxic nature in both sexes in that their icteric pigmentation was increased and cytoplasmic staining properties were abnormal. In addition, kidney sections of high dose animals (copper sulphate or gluconate) exhibited minor changes. Varying degrees of testicular degeneration were noted in both the high and low levels of the copper sulphate animals, although mean testis weights at 530 ppm were not adversely affected.

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 **– Rat, oral –**

63.7 Other

Tissue stored copper and iron:

Less than 2 mg Cu/100 g was detected in liver tissue of animals fed with the control diet or diets containing 0.1 % or 1.0 % of potassium sodium copper chlorophyllin. Slightly higher, non-significant, concentrations of liver copper were found for animals fed at the 3 % level for a period of 2 years. Animals receiving 530 ppm Cu as copper sulphate stored more copper than observed for high dose animals in trial 1. The highest amount of copper was deposited in livers of rats administered with 1600 ppm Cu as copper gluconate, which correlates with the high death rate of these animals, the high blood non-protein nitrogen as well as gross pathological and histopathological findings. The same pattern of copper storage was observed in kidney and spleen. Concurrent examinations of iron contents showed that a high storage of copper seems to depress the storage of iron.

The results are summarised in Table A6.5- 4, Table A6.5- 5 and Table A6.5- 6.

Mating trial:

(Chlorophyllin treated animals, 5 pairs of animals per group) Not all females were pregnant, although the period allowed for mating was only 1 week. Mean numbers of pups born were 7.2 for controls and 6.5 to 9 for the treated groups. The number of pups raised to maturity was 5.2 for the controls and 4.5 to 6.2 for the treated groups. There were no differences that could be attributed to treatment. The report does not state the duration of pre-mating treatment.

64 APPLICANT'S SUMMARY AND CONCLUSION

64.1 Materials and methods

Groups of male and female rats were administered with 53, 530 or 1600 ppm of copper as potassium sodium copper chlorophyllin for up to 104 weeks. In a second trial, male and female rats received 530 or 1600 ppm of copper as copper sulphate or 1600 ppm of copper as copper gluconate for up to 42 weeks. Although not a guideline study, the conduct of the study was similar to method B.30 (88/303/EEC), with the exception that adrenals were not weighed upon necropsy and that no detailed results were reported for haematology, clinical chemistry and urinalysis.

Section A 6.5 Chronic toxicity

Annex Point IIA 6.5 **– Rat, oral –**

64.2	**Results and discussion**	Administration of potassium sodium copper chlorophyllin at levels up to 3% (equivalent to 1600 ppm copper, or approximately 80 mg/kg bodyweight/day) for up to 104 weeks resulted in no adverse reactions to treatment. Bodyweight gains were similar to controls. There was no metal toxicity and the livers, kidneys and spleen did not store increased amounts of copper compared with that stored in tissues of animals receiving equivalent amounts of copper sulphate or copper gluconate. A small sub-sample of controls and animals treated with copper chlorophyllin mated successfully. Copper as sulphate at doses equivalent to 1600 ppm in the diet showed increased mortality after 40 weeks, and this phase of the study was terminated at 42/44 weeks. Animals receiving 1600 ppm of copper as gluconate and males receiving 1600 ppm copper as sulphate showed reduced body weights gains compared to controls. Males and females receiving 530 ppm copper as sulphate (equivalent to approximately 27 g/kg bw/day) showed similar bodyweight gains to controls, and no indications of systemic toxicity. Copper levels in liver and to a lesser extent in kidney and spleen were higher than concurrent controls. Copper gluconate was more readily absorbed and deposited in tissues when administered orally than an equivalent amount of copper sulphate. There were no observations of increased tumour incidence in rats receiving copper as chlorophyllin at 104 weeks. The No Observed Effect level (NOEL) for potassium sodium copper chlorophyllin was 3% dietary inclusion (equivalent to approximately 80 mg Cu/kg bw/day), and the No Observed Adverse Effect Level (NOAEL) for copper sulphate was 530 ppm diet (approximately equivalent to 27 mg Cu/kg bw/day)
64.3	**Conclusion**	
64.3.1	LOAEL	Not stated.
64.3.2	NOAEL	530 ppm of copper as copper sulphate, (approx. equivalent to 27 mg Cu/kg b.w./d)
64.3.3	Reliability	2
64.3.4	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	12/01/2005
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 A6.5- 1: **Average body weights of rats receiving Potassium sodium copper chlorophyllin in diet.**

	Average body weight [g] (N)						
Treatment	**Initial**	**4 weeks**	**8 weeks**	**12 weeks**	**6 months**	**1 year**	**99 weeks**
Females (Trial 1, Potassium sodium copper chlorophyllin)							
Controls	52 ± 3.0 (20)	168 ± 3.5 (20)	212 ± 3.6 (20)	235 ± 3.5 (16)	261 ± 4.2 (15)	286 ± 5.8 (15)	359 ± 33.0 (11)
53 ppm Cu	52 ± 1.6 (20)	168 ± 3.4 (20)	210 ± 9.3 (20)	237 ± 4.0 (15)	267 ± 4.7 (15)	288 ± 4.8 (15)	328 ± 13.4 (11)
530 ppm Cu	50 ± 2.4 (22)	167 ± 3.8 (22)	229 ± 5.1 (22)	227 ± 3.7 (18)	258 ± 4.5 (18)	290 ± 4.2 (18)	308 ± 43.4 (11)
1600 ppm Cu	49 ± 3.0 (20)	165 ± 3.3 (20)	202 ± 3.6 (20)	220 ± 4.1 (16)	248 ± 4.7 (16)	287 ± 8.0 (16)	299 ± 28.9 (9)
Males (Trial 1, Potassium sodium copper chlorophyllin)							
Controls	53 ± 3.4 (20)	223 ±21.0 (20)	335 ± 4.6 (20)	382 ± 27.2 (16)	422 ± 24.1 (15)	500 ± 15.4 (15)	440 ± 92.1 (8)
53 ppm Cu	54 ± 3.6 (20)	228 ± 21.1 (20)	320 ± 20.9 (20)	366 ± 5.8 (16)	421 ± 6.3 (16)	533 ± 10.0 (16)	519 ± 60.6 (8)
530 ppm Cu	54 ± 2.6 (18)	227 ± 5.5 (18)	294 ± 7.3 (18)	360 ± 5.6 (14)	403 ± 10.8 (14)	481 ± 15.9 (14)	461 ± 8.8 (7)
1600 ppm Cu	54 ± 3.7 (20)	223 ± 7.4 (20)	311 ± 6.0 (20)	352 ± 8.0 (16)	393 ± 10.0 (16)	500 ± 15.8 (16)	495 ± 15.5 (7)

Table A6.5- 2: **Average body weights of rats receiving copper sulphate or copper gluconate in diet.**

	Average body weight [g] (N)					
Treatment	**Initial**	**4 weeks**	**8 weeks**	**12 weeks**	**20 weeks**	**35 weeks**
Females						
Controls	73 ± 2.3 (25)	172 ± 3.2 (24)	204 ± 4.0 (24)	220 ± 3.9 (24)	261 ± 4.5 (24)	265 ± 4.3 (24)
Copper sulphate, 530 ppm Cu	67 ± 3.3 (25)	154 ± 2.8 (25)	207 ± 3.5 (25)	232 ± 3.2 (25)	270 ± 3.5 (25)	260 ± 5.1 (25)
Copper sulphate, 1600 ppm Cu	73 ± 2.2 (25)	153 ± 3.4 (25)	198 ± 2.7 (25)	224 ± 3.1 (25)	220 ± 4.2 (24)	257 ± 3.6 (20)
Copper gluconate, 1600 ppm Cu	75 ± 2.5 (25)	170 ± 2.9 (25)	200 ± 3.1 (25)	235 ± 4.1 (25)	204 ± 3.8 (23)	182 ± 11.7 (6)
Males						
Controls	81 ± 2.3 (23)	218 ± 7.2 (23)	310 ± 6.2 (23)	382 ± 7.0 (23)	438 ± 17.3 (23)	459 ± 17.3 (22)
Copper sulphate, 530 ppm Cu	72 ± 3.4 (25)	194 ± 6.5 (25)	279 ± 1.3 (25)	358 ± 5.8 (25)	425 ± 10.7 (24)	481 ± 3.7 (23)
Copper sulphate, 1600 ppm Cu	71 ± 9.3 (23)	174 ± 5.7 (23)	247 ± 6.3 (23)	280 ± 9.1 23)	282 ± 10.6 (20)	335 ± 9.5 (16)
Copper gluconate, 1600 ppm Cu	75 ± 2.7 (22)	198 ± 7.5 (22)	272 ± 7.6 (22)	327 ± 6.5 (22)	268 ± 8.3 (15)	219 ± 11.0 (2)

Table A6.5- 3: **Average organ weights (g tissue/100 g bw).**

Treatment	N	Heart	Lungs	Liver	Spleen	Kidneys	Uterus (seminal vesicles)	Ovaries (testes)	Stomach	Brain	Approx. weeks on test
Females (Trial 1, Potassium sodium copper chlorophyllin)											
Controls	6	0.384	0.554	3.902	0.203	0.816	0.256	0.040	0.634	0.614	104
0.1 % in diet (53 ppm Cu)	10	0.367	0.555	3.559	0.240	0.799	0.285	0.078	0.643	0.616	104
1.0 % in diet (530 ppm Cu)	9	0.371	0.590	4.375	0.213	0.855	0.263	0.040	0.708	0.615	104
3.0 % in diet (1600 ppm Cu)	7	0.389	0.670	3.632	0.232	0.953	0.346	0.042	0.758	0.712	104
Males (Trial 1, Potassium sodium copper chlorophyllin)											
Controls	4	0.358	0.526	3.564	0.208	0.837	0.227	0.737	0.601	0.488	104
0.1 % in diet (53 ppm Cu)	6	0.415	0.532	3.946	0.179	0.872	0.189	0.688	0.686	0.584	104
1.0 % in diet (530 ppm Cu)	5	0.366	0.701	4.419	0.177	1.190	0.193	0.537	0.725	0.506	104
3.0 % in diet (1600 ppm Cu)	4	0.347	0.404	4.015	0.170	0.921	0.225	0.699	0.770	0.506	104
Females (Trial 2)											
Controls	9	0.317	0.500	3.214	0.203	0.717	0.274	0.038	0.615	0.656	42
Copper sulphate, 530 ppm Cu	15	0.295	0.553	3.250	0.182	0.714	0.212	0.037	0.628	0.630	42
Copper sulphate, 1600 ppm Cu	10	0.301	0.564	3.778	0.209	0.799	0.179	0.040	0.821	0.684	42
Copper gluconate, 1600 ppm Cu	4	0.329	0.651	4.825	0.255	0.804	0.078	0.024	1.127	0.824	42
Males (Trial 2)											
Controls	8	0.208	0.495	3.586	0.169	0.798	0.827	0.350	0.518	0.424	42
Copper sulphate, 530 ppm Cu	12	0.282	0.487	3.074	0.189	0.792	0.666	0.357	0.383	0.423	42
Copper sulphate, 1600 ppm Cu	6	0.301	0.488	4.072	0.198	0.889	0.839	0.403	0.686	0.505	42
Copper gluconate, 1600 ppm Cu	2	0.419	0.967	3.940	0.198	1.052	0.760	0.157	1.227	1.136[a]	42
Females (Trial 2)											
Controls	4	0.336	0.770	3.524	0.188	0.753	0.230	0.039	0.645	0.668	33
Copper sulphate, 1600 ppm Cu	4	0.333	0.569	3.767	0.185	0.670	0.135[a]	0.024[b]	0.705	0.669	33
Copper gluconate, 1600 ppm Cu	4	0.378	0.676	4.465	0.215	0.782	0.081	0.022	1.020	0.648	33
Males (Trial 2)											
Controls	4	0.301	0.713	3.556	0.173	0.777	0.923	0.359	0.531	0.479	33
Copper sulphate, 1600 ppm Cu	4	0.297	0.518	3.492	0.170	0.720	0.700	0.255	1.061	0.572	33
Copper gluconate, 1600 ppm Cu	4	0.328	0.553	3.963	0.205	0.891	1.008	0.286	1.013	0.664	33

[a] 1 animal only [b] 3 animals only

Table A6.5- 4: **Copper content of tissues of rats receiving potassium sodium copper chlorophyllin in diet.**

	Average copper content [mg Cu/100 g tissue (wet basis)] ± S.E. (N)							
	Control		53 ppm		530 ppm		1600 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver								
10 weeks	0.41 ± 0.04 (4)	0.48 ± 0.08 (4)	0.47 ± 0.026 (4)	0.57 ± 0.09 (4)	0.58 ± 0.035 (4)	0.74 ± 0.065 (4)	0.56 ± 0.06 (4)	0.56 ± 0.08 (4)
52 weeks	0.78 ± 0.020 (3)	1.09 ± 0.052 (3)	1.46 ± 0.64 (3)	1.14 ± 0.29 (3)	0.81 ± 0.064 (3)	2.43 ± 1.12 (3)	1.06 ± 0.42 (3)	2.14 ± 0.711 (3)
104 weeks	1.82 ± 0.58 (4)	1.10 ± 0.152 (6)	1.47 ± 0.304 (6)	1.85 ± 0.251 (10)	1.85 ± 0.504 (5)	2.02 ± 0.51 (9)	2.18 ± 0.61 (4)	3.71 ± 1.28 (7)
Kidney								
10 weeks	1.07 ± 0.15 (4)	1.72 ± 0.57 (4)	1.47 ± 0.27 (4)	1.52 ± 0.11 (4)	1.58 ± 0.51 (4)	1.57 ± 0.16 (4)	1.48 ± 0.32 (4)	1.65 ± 0.22 (4)
52 weeks	2.08 ± 0.17 (3)	4.46 ± 2.20 (2)	1.52 ± 0.27 (3)	2.44 ± 0.55 (3)	1.83 ± 0.364 (3)	3.79 ± 0.847 (3)	2.11 ± 0.015 (3)	2.97 ± 0.11 (3)
104 weeks	3.45 ± 0.91 (4)	2.25 ± 0.23 (6)	2.03 ± 0.709 (5)	2.55 ± 0.19 (10)	2.35 ± 0.727 (5)	3.19 ± 0.393 (9)	2.48 ± 0.63 (4)	3.22 ± 0.416 (6)
Spleen								
10 weeks	0.96 ± 0.42 (2)	1.59 ± 0.05 (2)	0.52 ± 0.3 (2)	0.46 ± 0.03 (2)	0.40 ± 0.48 (2)	0.72 ± 0.38 (2)	0.68 ± 0.11 (2)	0.52 ± 0.18 (2)
52 weeks	1.83 ± 0.58 (2)	4.00 ± 1.02 (3)	2.92 ± 1.45 (3)	3.26 ± 1.02 (3)	3.05 ± 1.36 (3)	3.46 ± 0.817 (3)	2.36 ± 1.03 (2)	3.61 ± 1.89 (3)
104 weeks	3.38 ± 1.44 (4)	6.96 ± 2.22 (6)	3.34 ± 0.408 (6)	1.92 ± 0.396 (10)	2.75 ± 0.513 (5)	2.34 ± 0.386 (9)	3.01 ± 0.775 (4)	2.96 ± 0.685 (7)

Table A6.5- 5: **Iron content of tissues of rats receiving potassium sodium copper chlorophyllin in diet.**

	Average copper content [mg Fe/100 g tissue (wet basis)] ± S.E. (N)							
	Control		53 ppm		530 ppm		1600 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver								
10 weeks	2.36 ± 0.83 (2)	2.50 ± 0.27 (2)	2.25 ± 0.12 (2)	3.56 ± 0.43 (2)	1.57 ± 0.53 (2)	2.26 ± 0.37 (2)	3.22 ± 0.59 (2)	2.09 ± 0.82 (2)
52 weeks	2.64 ± 0.458 (3)	7.79 ± 0.341 (3)	2.14 ± 0.258 (3)	11.20 ± 1.92 (3)	5.15 ± 2.50 (3)	7.83 ± 2.35 (3)	3.17 ± 0.441 (3)	8.70 ± 0.452 (3)
104 weeks	17.7 ± 1.9 (4)	24.7 ± 8.44 (6)	16.6 ± 2.32 (6)	27.0 ± 3.07 (10)	15.7 ± 2.04 (5)	24.9 ± 3.90 (9)	18.0 ± 2.85 (4)	31.3 ± 6.45 (7)
Kidney								
52 weeks	7.45 ± 1.32 (3)	11.22 ± 1.95 (2)	10.82 ± 2.40 (3)	16.70 ± 3.59 (3)	13.86 ± 2.40 (3)	19.69 ± 1.01 (3)	10.73 ± 2.41 (3)	16.21 ± 2.67 (3)
104 weeks	19.9 ± 1.4 (4)	32.4 ± 7.7 (6)	24.7 ± 1.88 (6)	25.6 ± 2.12 (10)	17.4 ± 4.0 (5)	31.1 ± 2.83 (9)	23.5 ± 2.61 (4)	28.3 ± 4.88 (6)
Spleen								
104 weeks	219.0 ± 24.6 (4)	229.4 ± 32.7 (6)	162.6 ± 30.4 (6)	190.9 ± 17.3 (10)	160.5 ± 27.5 (5)	206.8 ± 27.9 (9)	235.4 ± 13.0 (4)	279.6 ± 41.2 (7)

Table A6.5- 6: **Copper and iron content of tissues of rats receiving copper sulphate or gluconate in diet.**

	Control		Copper sulphate				Copper gluconate	
			530 ppm Cu		1600 ppm Cu		1600 ppm Cu	
	Male	Female	Male	Female	Male	Female	Male	Female
Average copper content[mg Cu/100 g tissue (wet basis)] ±S.E. (N)								
Liver	1.16 ± 0.31 (6)	1.78 ± 0.39 (6)	12.47 ± 2.52 (6)	32.36 ± 14.6 (5)	38.28 ± 13.85 (6)	45.77 ± 5.18 (6)	75.1 ± 12.07 (6)	56.6 ± 6.10 (6)
Kidney	2.48 ± 0.20 (6)	3.53 ± 0.33 (6)	3.49 ± 0.54 (6)	6.91 ± 0.48 (6)	15.83 ± 6.21 (6)	12.11 ± 4.80 (6)	50.57 ± 14.75 (5)	54.1 ± 21.5 (5)
Spleen	3.34 ± 0.63 (6)	4.83 ± 0.33 (6)	5.63 ± 1.5 (6)	5.12 ± 1.3 (6)	13.91 ± 7.50 (6)	6.07 ± 1.72 (6)	12.39 ± 3.9 (6)	13.77 ± 3.29 (6)
Average iron content [mg Fe/100 g tissue (wet basis)] ±S.E. (N)								
Liver	9.7 ± 2.5 (6)	14.74 ± 4.0 (6)	18.0 ± 9.6 (6)	16.5 ± 1.6 (5)	14.1 ± 6.3 (6)	10.5 ± 5.2 (6)	5.9 ± 2.3 (6)	8.5 ± 5.0 (6)
Kidney	16.4 ± 1.4 (6)	17.44 ± 1.74 (6)	12.6 ± 1.97 (6)	15.0 ± 0.98 (6)	11.8 ± 1.7 (6)	14.8 ± 1.5 (6)	10.6 ± 1.02 (5)	9.0 ± 2.0 (5)
Spleen	128.1 ± 18.9 (6)	191.7 ± 37.3 (6)	120.3 ± 13.6 (6)	292.1 ± 12.4 (6)	108.9 ± 18.7 (6)	148.7 ± 41.7 (6)	49.7 ± 11.4 (6)	86.1 ± 41.7 (6)

Section A6.5 Chronic toxicity

Annex Point IIA6.5 **– Second species –**

	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 6.5 of the TNsG on data requirements according to Directive EC98/8/EEC, chronic toxicity testing is required for one rodent and one other mammalian species. It is further recommended to study the rat first, and based on this result more testing in another mammalian species may be necessary. A test should be performed in a rodent, the rat being the preferred species. However, the TNsG also explicitly state that the long-term-toxicity of an active substance may not be required where a full justification demonstrates that these tests are not necessary based on the sub-chronic toxicity test in the same species. The applicant is of the opinion that the conduct and submission of further chronic toxicity studies in excess of the key study on the rat (A6.5/01, peer-reviewed study from the public domain) 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. (2) 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. (3) It is also not considered required to perform additional animal studies because there are also human data available, which are preferable for the risk assessment. (4) Finally, two rare genetic diseases of copper in the human provide information based upon which long-term exposure to excessive copper may be assessed. 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	

Supprimé : [

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

Section A6.5 Chronic toxicity

Annex Point IIA6.5 **– Second species –**

	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		