	combination of control plus anion at weeks 4 and 8. The HCO_3^- concentrations in Cu sources with anion except for the control plus anion group were decreased compared with combinations of Cu sources with cation at week 12. The reason for these interactions is not known. Effects of dietary Cu sources and DCAB on blood pCO_2 were not significant. There was interaction between Cu sources and DCAB on blood pCO_2 level (**Figure A6.2(33)-2**). Blood pH in the $CuSO_4$-supplemented group was higher than the CuOsupplemented group at week 4 and higher than the control group at week 8 but not different between any groups at week 12 (**Table A6.2(33)-2**). Treatment groups fed the cationic diet increased blood pH in comparison with groups fed the anionic diet at both wk 8 and 12. Blood pH (hydrogen ion concentration) is determined by pCO_2 and HCO_3^- concentration. Therefore, any changes in HCO_3^- concentration or $pC0_2$ will result in a change in pH in the blood. Examining changes in blood HCO_3^- concentration and pCO_2 showed that changes in blood pH were mainly a result of HCO_3^- changes. The effect of interactions between Cu sources and DCAB in the diet on blood pH is presented in **Figure A6.2(33)-3**. At weeks 4 and 8, the $CuSO_4$ plus anionic diet increased blood pH compared with CuO in combination with the anionic diet but not in combination with the cationic diet. At week 8, all Cu treatment groups with the cationic diet had higher blood pH than the control plus anionic diet. At week 12, all treatment combinations with the cationic diet had an increased blood pH compared with the control plus anionic diet. There was no interaction between Cu sources and the anionic diet. The mechanism involved in the interactions between Cu sources and DCAB is unclear. Blood pH in all treatment combinations between Cu sources and DCAB except for the control plus anionic diet increased consistently over time. The reason for this is not clear.
172.2.2 Growth performance	Sources and levels of Cu supplementation in the diet did not show any effect on the growth of calves. The cationic diet increased growth of calves compared with the anionic diet (**Table A6.2(33)-3**). This positive effect on the growth was significant only at week 12 of treatment but tended to start from week 8 of the experiment (**Figure A6.2(33)-4**). Based on the observations in this trial, calves did not start to consume starter until the 2nd week of treatment (approximately 3 weeks after birth), and average daily feed intake did not reach 1 kg until 8 week of treatment. Therefore, no dietary effects of DCAB on growth should be expected before a considerable amount of diet was consumed within the first 8 weeks after birth. No interaction between DCAB and Cu sources on calf growth was found in the experiment.
172.2.3 Blood superoxide dismutase	There was no difference of SOD activity between Cu sources or DCAB (**Table A6.2(33)-3**). Unaffected SOD activity probably indicated that Cu status of calves in this experiment was above physiological Cu deficiency range. No interaction between Cu sources and CAB was shown for SOD activity.
172.2.4 Liver Cu concentrations	Liver Cu concentration in the $CuSO_4$ group was higher ($P < 0.01$) than in the CuO-supplemented or control group at week 12 (**Table A6.2(33)-3**). However, there was no difference ($P > 0.10$) in liver Cu concentration between the CuO and control groups even though the level in the CuO group averaged 172% of the control. Because liver Cu concentration reflects amount of Cu taken up by the animal and biological availability of Cu sources, we conclude that $CuSO_4$ is highly available, whereas CuO (in powder form) is a fairly poor source of Cu in the diet for calves. Cation-anion balance did not affect liver Cu concentration. There was no significant interaction between Cu sources and DCAD in liver Cu concentration.

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/33	**A6.2(33), Bioavailability of copper**

172.3 Toxic effects, clinical signs

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

No effects.

5.11 Recovery of labelled compound

state percentage

Not applicable.

173 APPLICANT'S SUMMARY AND CONCLUSION

173.1 Materials and methods

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

A study was carried out to evaluate the biological availability of CuO as a Cu supplement in calf starter for dairy calves and to determine the effects of dietary cation-anion balance (DCAB) on Cu metabolism and acid-base balance. The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP.

Fourteen Holstein and 10 Jersey calves (13 female), ranging from 4 to 11 days of age, were assigned to a 2 x 3 factorial arrangement in a split-plot, randomized design with 4 calves in each treatment group, 2 levels of DCAB, and 3 levels of Cu. Calves were blocked by breed when assigned to the treatment. All calves were housed in hutches, offered starter at the age of 1 week, and individually fed *ad libitum* throughout the trial. Two levels of DCAB in calf starters were achieved by manipulating dietary Na^+, K^+, Cl^- and S^{-2}. Treatments were 20 meq of DCAB and no Cu supplementation (control plus cationic), 20 meq of DCAB and 20 ppm of Cu supplemented as CuO (CuO plus cationic), 20 meq of DCAB and 20 ppm of Cu supplemented as $CuSO_4$ ($CuSO_4$ plus cationic), -10 meq of DCAB and no Cu supplementation (control plus anionic), -10 meq of DCAB and 20 ppm of Cu supplemented as CuO (CuO plus anionic), and -10 meq of DCAB and 20 ppm of Cu supplemented as $CuSO_4$ ($CuSO_4$ plus anionic).

For the purposes of this summary, only the sampling procedure and analytical method relevant to the determination of Cu concentrations in liver are described, as follows: At week 12, liver samples were taken by biopsy. Liver samples were dried at 100°C for 72 hours, wet ashed and analyzed for Cu by atomic absorption spectrophotometry.

173.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

For the purposes of this summary, only the results relevant to the determination of Cu concentrations in liver are described, as follows: Mean liver Cu concentration in the $CuSO_4$ group was higher (257 mg/kg dry weight) than in the CuO-supplemented (115 mg/kg dry weight) or control groups (67 mg/kg dry weight) at week 12. The difference in liver Cu concentration between the CuO and control groups was not statistically significant ($P>0.01$). Because liver Cu concentration reflects amount of Cu taken up by the animal and biological availability of Cu sources, it was concluded that $CuSO_4$ is highly available, whereas CuO is a poor source of Cu in the diet for calves.

Cation-anion balance did not affect liver Cu concentration. There was no significant interaction between Cu sources and DCAD in liver Cu concentration.

173.3 Conclusion

Copper oxide was a very poor source of Cu compared with $CuSO_4$ for calves at early age based on liver Cu concentrations.

173.3.1 Reliability

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

2

173.3.2 Deficiencies

Yes

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID 5.0/33	**A6.2(33), Bioavailability of copper**

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the relative bioavailability of copper. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary.

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

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

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

Section A6.2 **Metabolism in mammals**

Annex Point IIA6.2 *Specify section no., heading and species as appropriate*

IUCLID 5.0/33 **A6.2(33), Bioavailability of copper**

Table A6.2(33)-1. Ingredient and nutrient composition of calf starters.[1]

				Anionic Treatment		
Ingredients	**Control**	**CuO**	**$CuSO_4$**	**Control**	**CuO**	**$CuSO_4$**
Cracked corn	37.30	37.30	37.30	37.30	37.30	37.30
Crimped oats	33.30	33.30	33.30	33.30	33.30	33.30
Soybean meal, 44% CP	17.50	17.50	17.50	17.50	17.50	17.50
Molasses	5.00	5.00	5.00	5.00	5.00	5.00
Iodized salt	.70	.70	.70	.70	.70	.70
Dicalcium phosphate	.70	.70	.70	.70	.70	.70
Feed grain limestone	1.40	1.40	1.40	.00	.00	.00
Se premix	.10	.10	.10	.10	.10	.10
Vitamins A, D, E[2]	.15	.15	.15	.15	.15	.15
Trace-mineral premix	.02	.02	.02	.02	.02	.02
Rumensin premix[3]	2.50	2.50	2.50	2.50	2.50	2.50
KHCO3	.87	.87	.87	.00	.00	.00
$NaHC0_3$	.50	.50	.50	.00	.00	.00
Mg0	.00	.00	.00	1.36	1.36	1.36
$CaCl_2$	.00	.00	.00	1.37	1.37	1.37
CuO, g/100 kg	.00	3.23	.00	.00	3.23	.00
$CuSO_4 \cdot 5H_20$, g/100 kg	.00	.00	10.24	.00	.00	10.24
Composition						
CP	15.5	16.4	14.0	14.2	14.9	14.7
NE_L, Mcal/kg	1.78	1.78	1.78	1.78	1.78	1.78
ADF	7.6	7.6	7.1	8.6	7.5	7.2
NDF	19.4	16.3	17.3	17.5	16.3	15.2
Ca	.58	.64	.55	.66	.76	.72
P	.48	.49	.45	.47	.49	.48
Mg	.17	.18	.16	.19	.20	.20
S	.20	.19	.17	.18	.19	.20
Na	.27	.36	.34	.23	.31	.27
K	1.05	1.11	1.00	.87	.89	.90
Cl	.32	.57	.44	1.08	1.18	1.30
Cu, ppm	7.0	25.0	25.0	6.0	25.0	25.0
Cation-anion balance	17.13	16.09	17.39	-9.41	-8.87	-14.35

[1] Ingredients listed as a percentage of diet DM.
[2] Contains 4,000,000 IU vitamin A; 800,000 IU vitamin D3; and 500 IU vitamin E/.454 kg.
[3] Contains 1.32 kg of rumensin sodium/kg of premix.

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(33)-1. Ingredient and nutrient composition of calf starters.[1]

Ingredients	Cationic Treatment			Anionic Treatment		
	Control	CuO	$CuSO_4$	Control	CuO	$CuSO_4$
Cracked corn	37.30	37.30	37.30	37.30	37.30	37.30
Crimped oats	33.30	33.30	33.30	33.30	33.30	33.30
Soybean meal, 44% CP	17.50	17.50	17.50	17.50	17.50	17.50
Molasses	5.00	5.00	5.00	5.00	5.00	5.00
Iodized salt	.70	.70	.70	.70	.70	.70
Dicalcium phosphate	.70	.70	.70	.70	.70	.70
Feed grain limestone	1.40	1.40	1.40	.00	.00	.00
Se premix	.10	.10	.10	.10	.10	.10
Vitamins A, D, E[2]	.15	.15	.15	.15	.15	.15
Trace-mineral premix	.02	.02	.02	.02	.02	.02
Rumensin premix[3]	2.50	2.50	2.50	2.50	2.50	2.50
KHCO3	.87	.87	.87	.00	.00	.00
$NaHC0_3$	.50	.50	.50	.00	.00	.00
Mg0	.00	.00	.00	1.36	1.36	1.36
$CaCl_2$	.00	.00	.00	1.37	1.37	1.37
CuO, g/100 kg	.00	3.23	.00	.00	3.23	.00
$CuSO_4 \cdot 5H_20$, g/100 kg	.00	.00	10.24	.00	.00	10.24
Composition						
CP	15.5	16.4	14.0	14.2	14.9	14.7
NE_L, Mcal/kg	1.78	1.78	1.78	1.78	1.78	1.78
ADF	7.6	7.6	7.1	8.6	7.5	7.2
NDF	19.4	16.3	17.3	17.5	16.3	15.2
Ca	.58	.64	.55	.66	.76	.72
P	.48	.49	.45	.47	.49	.48
Mg	.17	.18	.16	.19	.20	.20
S	.20	.19	.17	.18	.19	.20
Na	.27	.36	.34	.23	.31	.27
K	1.05	1.11	1.00	.87	.89	.90
CI	.32	.57	.44	1.08	1.18	1.30
Cu, ppm	7.0	25.0	25.0	6.0	25.0	25.0
Cation-anion balance	17.3	16.09	17.39	-9.41	-8.87	-14.35

[1] Ingredients listed as a percentage of diet DM.

[2] Contains 4,000,000 IU vitamin A; 800,000 IU vitamin D3; and 500 IU vitamin E/.454 kg.

[3] Contains 1.32 kg of rumensin sodium/kg of premix.

Table A6.2(33) -2. Effects of dietary Cu sources and cation-anion balance (CAB) on blood acid-base parameters during 12 wk.

	CU SOURCE				CAB		
	Control	CuO	$CuSO_4$	SEM	Anionic	Cationic	SEM
Bicarbonate, meq/L							
Week							
0	28.15	26.93	27.88	.71	26.82	28.04	.59
4	26.51^{ab}	24.66^{b}	27.94^{a}	.71	24.59^{b}	28.15^{a}	.58
8	25.96^{ef}	25.39^{f}	27.50^{e}	.53	25.05^{b}	27.51^{a}	.44
12	26.26	25.36	25.06	.75	23.67^{b}	27.45^{a}	.62
pCO_2, [1]mm Hg							
Week							
0	61.46	62.50	54.25	2.50	60.87	57.90	1.38
4	53.59	53.14	52.61	1.64	50.88	54.95	1.32
8	51.46	50.13	50.47	1.13	50.50	50.87	.90
12	51.92	45.46	47.13	1.44	47.44	48.90	1.16
pH							
Week							
0	7.26	7.24	7.31	.02	7.24	7.29	.02
4	7.30^{ab}	7.27^{b}	7.34^{a}	.01	7.31	7.34	.01
8	7.30^{b}	7.32^{ab}	7.34^{a}	.01	7.31^{b}	7.34^{a}	.01
12	7.31	7.35	7.33	.01	7.30^{b}	7.35^{a}	.01

[ab] Means with different superscripts in the same row within copper source or CAB main effect differ ($P < .05$)
[ef] Means with different superscripts in the same row within copper source or CAB main effect differ ($P < .06$).
[1] pCO_2 = Partial pressure of $C0_2$.

Table 3. Main effects of dietary Cu sources and cation-anion balance (CAB) on growth, superoxide dismutase (SOD) activity and liver Cu at wk 12.

	Cu Source				CAB		
Parameter	Control	CuO	$CuSO_4$	SEM	Anionic	Cationic	SEM
BW, kg	89.5	82.0	85.8	3.9	82.8^{b}	89.6^{a}	3.2
SOD Activity, units/ml	26.1	32.4	26.7	2.0	28.0	28.8	1.6
Liver Cu, mg/kg DM	67^{b}	115^{b}	257a	20	142	151	16

[ab] Means with different superscripts in the same row within Cu source or CAB differ ($p < .01$).

Figure A6.2(33)-1

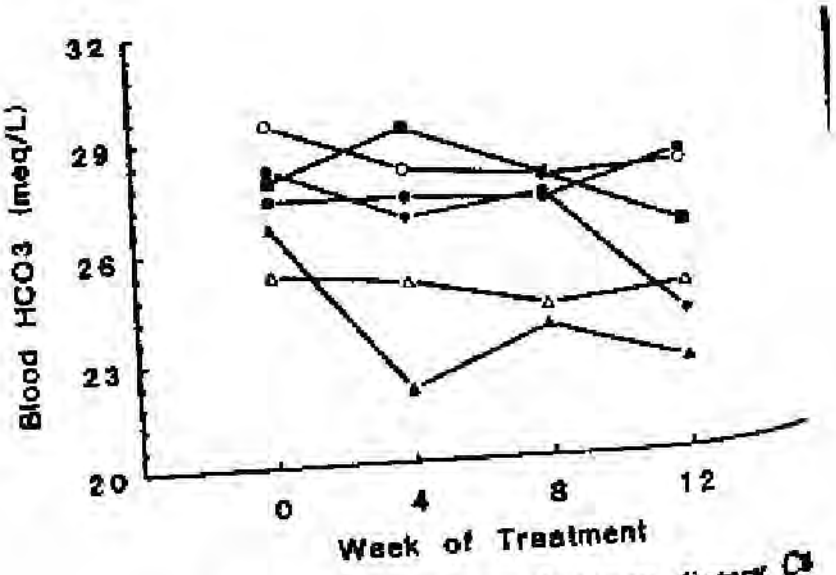

Figure 1. Effect of interactions between dietary Cu sources and cation-anion balance on blood bicarbonate (HCO_3^-) concentration by month throughout the trial; Δ = control + anion; O = control + cation; ▲ = CuO + anion; ● = CuO + cation; ♦ = $CuSO_4$ + anion; ■ = $CuSO_4$ + cation.

Figure A6.2(33)-2

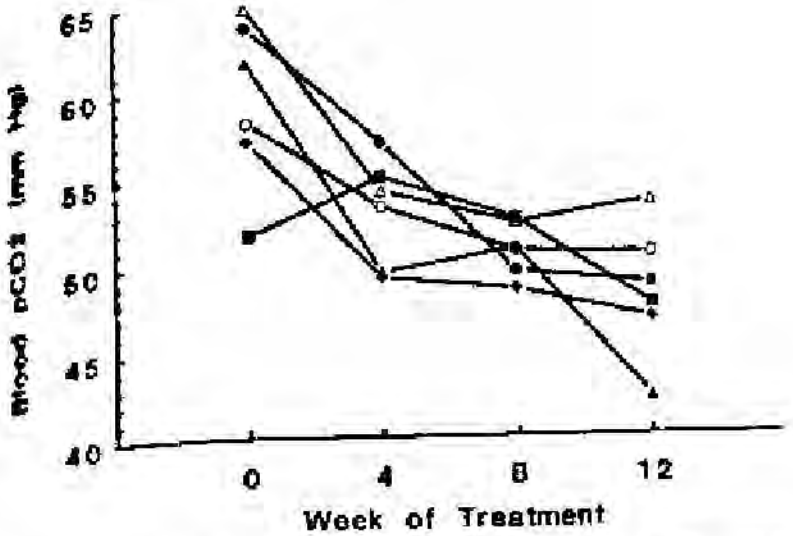

Figure 2. Effect of interactions between dietary Cu source and cation-anion balance on blood partial pressure of carbon dioxide (pCO_2) by month throughout the trial; Δ = control + anion; ○ = control + cation; ▲ = CuO + anion; ● = CuO + cation; ♦ = $CuSO_4$ + anion; ■ = $CuSO_4$ + cation.

Figure A6.2(33)-3

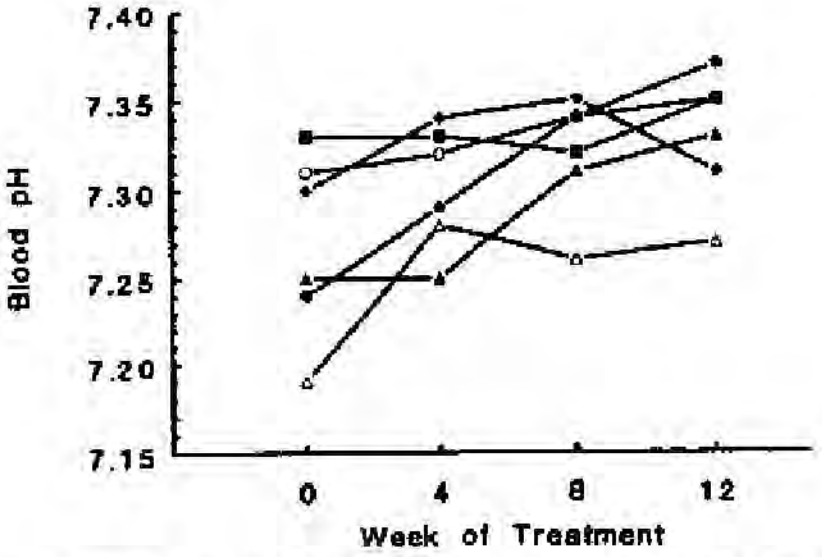

Figure 3. Effect of interactions between dietary Cu sources and cation-anion balance on blood pH by month throughout the trial; Δ = control + anion; O = control + cation; ▲ = CuO + anion; ● = CuO + cation; ♦ = $CuSO_4$ + anion; ■ = $CuSO_4$ + cation.

Figure A6.2(33)-4

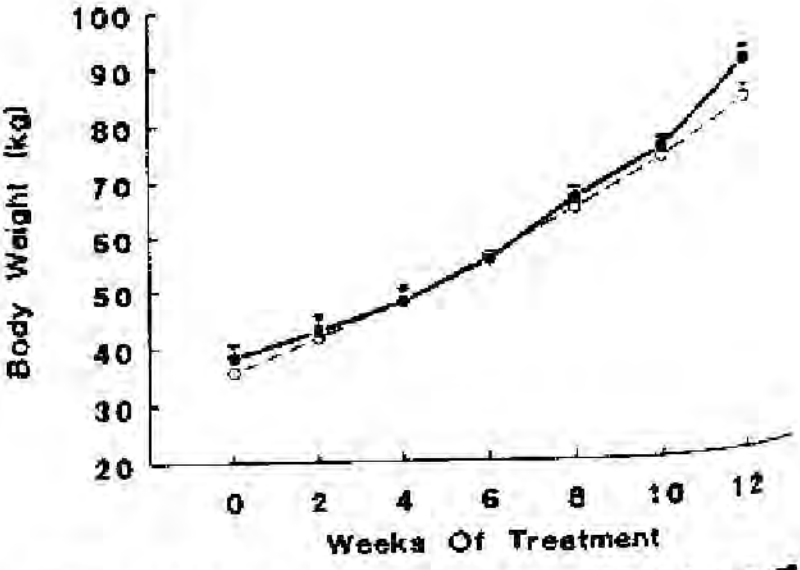

Figure 4. Effect of dietary cation-anion balance on growth performance biweekly throughout the trial; ● = cationic diet; ○ = anionic diet.

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/34	**A6.2(34), Bioavailability of copper**

Official use only

174 REFERENCE

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

Allen, M.M., Barber, R.S., Braude, R. and Mitchell, K.G. (1961). Further studies on various aspects of the use of high-copper supplements for growing pigs. Brit. J. Nutr., **15:** 507 – 522 (published).

174.2 Data protection No
(indicate if data protection is claimed)

174.2.1 Data owner *Give name of company*
Public domain

174.2.2 Criteria for data protection Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:
No data protection claimed

175 GUIDELINES AND QUALITY ASSURANCE

175.1 Guideline study No. This was a non-regulatory study carried out to compare the effects of 250 ppm copper, given in the diet as the sulphate or the carbonate. No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

175.2 GLP No. This was a non-regulatory study conducted before GLP was compulsory.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

175.3 Deviations Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

176 MATERIALS AND METHODS

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

176.1 Test material Cu^{2+} as copper sulphate ($CuSO_4.5H_2O$).
Cu^{2+} as basic copper carbonate ($CuCO_3.Cu(OH)_2.H_2O$).

176.1.1 Lot/Batch number Not available

176.1.2 Specification Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/34	**A6.2(34), Bioavailability of copper**

176.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Not stated.
176.1.2.2	Purity	*Give purity in % of active substance* [redacted]
176.1.2.3	Stability	*Describe stability of test material* Not stated.
176.1.2.4	Radiolabelling	*give structural location of radio labelling, give reason if not labelled* Not deemed necessary for the purposes of this study.
176.2 Test Animals		Non-entry field
176.2.1	Species	Pigs.
176.2.2	Strain	Large White
176.2.3	Source	Shinfield, virus pneumonia-free.
176.2.4	Sex	Male and female.
176.2.5	Age/weight/height at study initiation	*Young adults recommended* Age: weaners.
176.2.6	Number of animals	8 pigs on each of 6 treatments.
176.2.7	Controls	Yes
176.3 Administration/ Exposure		*(fill in respective route in the following, delete other routes)* Oral administration of the test substances in the diet.
176.3.1	Duration of treatment	Until bacon weight was reached.
176.4 Procedures		Non-entry field
176.4.1	Experimental design	The study consisted of 4 separate experiments; only experiment 2 (consisting of 6 separate treatments) is relevant to the purposes of this summary and is reported herein. Experiment 2 was designed as a 3 x 2 factorial. There were randomised blocks, blocks corresponding to litters, and treatments were allocated at random to the pens. There was no direct communication between pigs on different treatments. Pigs on treatments 4, 5 and 6 were given twice daily as much meal as they would consume within 30 minutes up to a maximum of 61/2 lb/day, water at the rate of 3 lb to every 1 lb meal being added immediately before feeding. This system of feeding was termed semi-*ad lib*. Pigs on treatments 1, 2 and 3 were also given meal twice daily, 3 lb water per 1 lb of meal again being added immediately before feeding, but the amount of meal given was based on live weight and according to a scale, a daily maximum of 61/2 lb/pig being given to an animal weighing 170 lb.

Section A6.2 **Metabolism in mammals**

Annex Point IIA6.2 *Specify section no., heading and species as appropriate*

IUCLID: 5.0/34 **A6.2(34), Bioavailability of copper**

176.4.2 Treatments

Details of the treatments given were as follows:

Treatment No.	Feeding system	Protein supplement	Copper supplement (250 ppm)
1	TS	WFM	None
2	TS	WFM	As sulphate
3	TS	WFM	As carbonate
4	S	WFM	None
5	S	WFM	As sulphate
6	S	WFM	As carbonate

S, Semi-*ad lib*., wet; WFM, white-fish meal; TS, to scale, wet.

176.4.3 Sampling procedures and Analytical methods

All pigs were weighed once weekly throughout the experiment, the rations of the pigs in treatments 1, 2 and 3, which were fed to scale, being adjusted after each weekly weighing. All the pigs were sent to slaughter individually when their live weight at the weekly weighing exceeded 203 lb.

A sample of liver tissue adjacent to the bile duct was taken at slaughter from each pig and stored at –20°C prior to determination of Cu.

176.4.4 Statistical analysis

Standard errors were calculated from randomised block analyses of variance, no adjustments being made for variation in either live weight or cold dead weight. The term 'treatment' is confounded with 'pen' but the pen effect was considered to be negligible.

177 RESULTS AND DISCUSSION

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

177.1 Results

The mean results for daily weight gain, food conversion efficiency, rate of food consumption and Cu content of the liver are shown in **Table A6.2(34)-1**, together with appropriate standard errors.

Supplementation of the diet with either copper sulphate or copper carbonate resulted in large increases in the amount of Cu present in the livers at bacon weight, relative to unsupplemented controls. Mean liver copper concentrations of pigs fed diets to scale were 52, 843 and 624 mg/kg dry weight for control group, copper sulphate-treated and copper carbonate-treated animals, respectively. Corresponding Values for animals fed the semi-*ad lib* diet were 61, 779 and 383 mg Cu/kg dry weight.

177.2 Discussion

It was considered that there was some indication that the increase in liver copper stores was not so great when copper was given as the carbonate instead of as the sulphate, particularly with semi-*ad lib* feeding.

177.3 Toxic effects, clinical signs

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

No effects. The general health of the pigs in this experiment was satisfactory.

5.12 Recovery of labelled compound

state percentage

Not applicable.

178 APPLICANT'S SUMMARY AND CONCLUSION

178.1 Materials and methods

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

Section A6.2	**Metabolism in mammals**
Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/34	**A6.2(34), Bioavailability of copper**

A study was carried out to evaluate the biological availability in pigs of Cu derived from basic copper carbonate, relative to that of Cu from copper sulphate pentahydrate. The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP.

Fourty eight weaners were assigned to a 3 x 2 factorial arrangement in randomised blocks, with 8 animals in each of 6 treatment groups. Pigs in groups 1 and 3 (controls) received no supplementary copper in their diets; those in groups 2 and 5 received diets supplemented with 250 ppm copper sulphate pentahydrate and those in groups 3 and 6 received diets supplemented with 250 ppm basic copper carbonate.

The manner of administration of these diets was varied as follows: Pigs on treatments 4, 5 and 6 were given twice daily as much meal as they would consume within 30 minutes up to a maximum of 6V2 lb/day, water at the rate of 3 lb to every 1 lb meal being added immediately before feeding. This system of feeding was termed semi-*ad lib*. Pigs on treatments 1, 2 and 3 were also given meal twice daily, 3 lb water per 1 lb of meal again being added immediately before feeding, but the amount of meal given was based on live weight and according to a scale, a daily maximum of 6V2 lb/pig being given to an animal weighing 170 lb.

All pigs were weighed once weekly throughout the experiment, the rations of the pigs in treatments 1, 2 and 3, which were fed to scale, being adjusted after each weekly weighing. All the pigs were sent to slaughter individually when their live weight at the weekly weighing exceeded 203 lb. A sample of liver tissue adjacent to the bile duct was taken at slaughter from each pig and stored at –20°C prior to determination of Cu.

178.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Supplementation of the diet with either 250 ppm copper sulphate or 250 ppm copper carbonate resulted in large increases in the amount of Cu present in the livers at bacon weight, relative to unsupplemented controls. Mean liver copper concentrations of pigs fed diets to scale were 52, 843 and 624 mg/kg dry weight for control group, copper sulphate-treated and copper carbonate-treated animals, respectively. Corresponding Values for animals fed the semi-*ad lib* diet were 61, 779 and 383 mg Cu/kg dry weight. It was therefore considered that there was some indication that the increase in liver copper stores was not so great when copper was given as the carbonate instead of as the sulphate, particularly with semi-*ad lib* feeding.

The mean measured concentration resulting from copper carbonate supplementation for animals fed the diet to scale was 74% of that resulting from copper sulphate supplementation. The mean measured concentration resulting from copper carbonate supplementation for animals fed the diet semi-*ad lib* was 49% of that resulting from copper sulphate supplementation.

178.3 Conclusion

Copper derived from basic copper carbonate fed in the diet was somewhat less bioavailable than that from copper sulphate, when assessed in terms of liver copper concentration.

178.3.1 Reliability

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

2

178.3.2 Deficiencies

Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a

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IUCLID: 5.0/34	**A6.2(34), Bioavailability of copper**

peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the relative bioavailability of copper. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary.

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

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

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

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

Table 1.

Effect of supplementation with either copper sulphate or copper carbonate of diets given either to scale, wet (TS) or semi-ad lib., wet (S) (see p. 507) on mean daily weight gain, food conversion, rate of food consumption, dressing percentage and liver copper stores

	Treatment no. and dietary supplement							
	1 (TS) None	2 (TS) 250 p.p.m. Cu as sulphate	3 (TS) 250 p.p.m Cu as carbonate	4 (S) None	5 (S) 250 p.p.m. Cu as sulphate	6 (S) 250 p.p.m. Cu as carbonate	Standard error of mean†	Significance of treatment mean square‡
No. of pigs	8§	8§	8	8	8	8§	—	—
Initial weight (lb)	49·9	52·1	51·3	52·5	50·4	53·2	—	—
Final weight (lb)	207·6	211·3	211·8	206·5	208·6	207·6	—	—
Daily weight gain (lb)	1·36	1·44	1·47	1·43	1·58	1·56	0·033	***
Food conversion (lb meal/lb live-weight gain)	3·33	3·22	3·17	3·42	3·17	3·15	0·071	N.S.
Rate of food consumption (lb/day)	4·49	4·61	4·67	4·84	5·00	4·92	0·089	**
Dressing percentage	72·6	74·5	73·5	73·4	75·0	72·9	0·46	**
Cu in liver (mg/kg, dry tissue):								
Value‖	52 (7)	843 (7)	624 (8)	61 (7)	779 (6)	383 (5)	—	—
Range	31–76	496–1571	313–1061	34–108	358–2194	296–561	—	

† Based on 32 degrees of freedom.
‡ N.S., $P > 0{\cdot}05$; ** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.
§ One pig on each of treatments 1, 2 and 6 died or was taken off experiment shortly after the beginning of the trial for reasons unconnected with the experiment, and missing values, calculated by the missing-plot technique (Yates, 1933), were substituted.
‖ Five samples lost. Numbers of livers are shown in parentheses.

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179 REFERENCE

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

Rojas, L.X., McDowell, L.R., Cousins, R.J., Martin, F.G., Wilkinson, N.S., Johnson, A.B. and Velasquez, J.B. (1996). Interaction of different organic and inorganic zinc and copper sources fed to rats. J. Trace Elements Med. Biol. **10:** 139-144 (published).

179.2 Data protection No
(indicate if data protection is claimed)

179.2.1 Data owner *Give name of company*
Public domain

179.2.2 Criteria for data protection: Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed

180 GUIDELINES AND QUALITY ASSURANCE

180.1 Guideline study No. This was a non-regulatory study carried out to compare bioavailability, interactions and retention of complexed and inorganic sources of Zn and Cu fed to rats. No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

180.2 GLP No. This was a non-regulatory study.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)

180.3 Deviations Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

181 MATERIALS AND METHODS

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

181.1 Test material Cu^{2+} as copper sulphate ($CuSO_4.5H_2O$).
Cu^{2+}as copper oxide (CuO).
Cu^{2+}as copper lysine (CuLys).
Zn^{2+} as zinc methionine (ZnMet).
Zn^{2+} as zinc lysine (ZnLys). Zn^{2+} as zinc sulphate ($ZnSO_4$).

181.1.1 Lot/Batch number: Not available

181.1.2 Specification: Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

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IUCLID: 5.0/35	**A6.2(35), Bioavailability of copper**

181.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Organic Zn and Cu sources from Zinpro Corporation, Edina, Minnesota; inorganic Zn and Cu sources from Southeastern Minerals, Bainbridge, Georgia.
181.1.2.2	Purity	*Give purity in % of active substance* [redacted]
181.1.2.3	Stability	*Describe stability of test material* Not stated.
181.1.2.4	Radiolabelling	*give structural location of radio labelling, give reason if not labelled* Not deemed necessary for the purposes of this study.
181.2 Test Animals		Non-entry field
181.2.1 Species		Rat.
181.2.2 Strain		Charles Sprague-Dawley (CD)
181.2.3 Source		Charles River Breeding Laboratories, Wilmington, Massachusetts.
181.2.4 Sex		Male.
181.2.5 Age/weight/height at study initiation		*Young adults recommended* Age: not stated. Weight: 71.5 ± 7.3 g (mean ± SEM).
181.2.6 Number of animals		Sixty three.
181.2.7 Controls		Yes
181.3 Administration/ Exposure		*(fill in respective route in the following, delete other routes)* Oral administration of the test substances in the diet.
181.3.1 Duration of treatment		21 days.
181.4 Procedures		Non-entry field
181.4.1 Experimental		Test animals were individually housed in suspended, stainless steel cages in an environmentally controlled room with a 12-hour light-dark cycle. Rats were individually fed a diet containing 0.34 and 0.71 mg/kg of Zn and Cu, respectively. Deionized water was supplied *ad libitum*. Different Zn and Cu sources were added to the basal diet at 30 mg/kg of Zn and 6 mg/kg of Cu to create a 3x3 factorial experiment. Seven rats were randomly assigned to each of these treatments. Supplemented diets were fed for four weeks, at which point four randomly selected rats from each treatment were sacrificed (Phase 1). The rest of the animals were fed the unsupplemented basal diet for an additional week (Phase 2) and then sacrificed. The protocol for animal care was approved by the University of Florida's Institutional Animal Care and Use Committee. All rats were anesthetized by inhaling methoxyflurane and bled by cardiac puncture. To obtain heparinized plasma, blood was centrifuged at 700 g for 25 minutes, supernatant decanted and frozen until analyzed for Zn and Cu. Tissues were immediately excised. The liver and both kidneys were frozen at - 80°C, and the rear leg muscles (biceps femoris, vastus lateralis and gluteous, combined) and bones (femur, tibia and fibula, combined) were frozen at -20°C.

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181.4.2 Analytical methods

Total metallothionine (MT) was measured in kidney and liver by the $^{109}Cd^{2+}$-binding method. The Zn and Cu concentrations in plasma, liver, kidney, muscle and bone were measured bv flame atomic absorption spectrophotometry on a Perkin-Elmer Model 5000 with AS-50. Standard Reference Material 1577a was used to evaluate the reliability of analytical methods.

181.4.3 Statistics

All data was analyzed using SAS. Tissue and plasma Zn, Cu and MT data were analyzed using General Linerar Model procedure, and in case of significance ($p<0.05$) Waller-Duncan's K-ratio T test was used for multiple comparisons.

182 RESULTS AND DISCUSSION

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

182.1 Results

Weight gains were not different ($p>0.05$) among treatments or experimental phases, but there was a tendency for CuLys to have a higher average daily gain than $CuSO_4$ for Phase l. Diet intakes were similar for all groups for both phases.

Phase l: Plasma Zn concentrations of rats were not affected ($p>0.05$) by Zn or Cu source (**Table A6.2(35)-1**). However, plasma Cu concentrations were lower ($p<0.05$) for CuO than $CuSO_4$ or CuLys-supplemented rats.

There were no effects (Zn or Cu: $p>0.05$) on the Zn concentrations of most tissues (**Table A6.2(35)-2**). Mean Zn concentrations were relatively constant for all tissues across treatments. Bone Zn concentrations, however, were higher ($p<0.05$) for CuLys- than for CuO-supplemented rats.

There was an interaction effect for bone Zn concentrations. Bone Zn concentrations were higher ($p<0.05$) for CuLys than $CuSO_4$ rats that were supplemented with $ZnSO_4$, and CuLys supplementation resulted in higher ($p<0.05$) bone Zn concentrations than did CuO for rats receiving ZnMet (**Figure A6.2(35)-1**). There were no bone Zn differences ($p>0.05$) from Cu source for the ZnLys source. Bone Zn concentrations were higher ($p<0.05$) for ZnLys than $ZnSO_4$ rats that received $CuSO_4$ supplementation. However, ZnLys had the lowest ($p<0.05$) bone Zn concentrations when CuLys was the Cu-supplementation source (**Figure A6.2(35)-2**). There were no differences ($p>0.05$) in Zn source for Zn tissue concentrations when CuO was the supplemental Cu source.

All tissue Cu concentrations were affected ($p<0.05$) by supplemental Cu source (**Table A6.2(35)-3**). In all tissues where Cu was measured, CuO was the lowest ($p<0.05$) available source of Cu. Furthermore, $CuSO_4$-supplemented rats had higher ($p<0.05$) Cu concentrations in muscle than from CuLys supplementation. Different Zn sources did not affect ($p>0.05$) tissue Cu.

Kidney MT concentrations followed the same pattern as Cu concentrations, with CuO being the lowest ($p<0.05$) MT inducer (**Table A6.2(35)-4**). There was no effect ($p>0.05$) of Zn source on tissue MT concentrations for kidney or liver and no Cu effect ($p>0.05$) for liver MT.

Phase 2: Plasma Zn concentrations of depleted rats were not affected ($p>0.05$) by Zn or Cu source (**Table A6.2(35)-5**). However, plasma Cu concentrations of depleted rats were lower ($p<0.05$) for CuO- than CuLys-supplemented rats. There were no main effects (Zn or Cu; $p>0.05$) for the Zn concentrations for most tissues of depleted rats (**Table A6.2(35)-6**). Kidney Zn concentrations were lower ($p<0.05$) resulting from $CuSO_4$ supplementation than for CuO-supplemented rats.

There was an interaction effect for kidney Zn concentrations after depletion. Kidney Zn concentrations after depletion were highest ($p<0.05$) for CuO and lowest ($p<0.05$) for $CuSO_4$ supplementation in the rats also receiving ZnLys supplementation (**Figure A6.2(35)-3**). There were no kidney Zn differences ($p>0.05$) from different Cu source for the ZnMet- or $ZnSO_4$ supplemented rats.

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Kidney Zn concentrations were higher ($p<0.05$) for ZnLys than $ZnSO_4$ supplemented rats that were also given CuO supplementation. However, ZnLys had the lowest ($p<0.05$) kidney Zn concentrations when $CuSO_4$ was the Cu source (**Figure A6.2(35)-4**). There were no differences ($p>0.05$) in Zn source when CuLys was the Cu source.

Most tissue Cu concentrations after depletion were not affected ($p<0.05$) by supplemental Cu source (**Table A6.2(35)-7**). In liver, however, CuO-supplemented rats had the lowest ($p<0.05$) Cu concentration. Different Zn sources did not affect ($p>0.05$) tissue Cu. There was no effect ($p>0.05$) of Zn or Cu source on tissue MT concentrations for kidney or liver after one week of depletion (**Table A6.2(35)-8**).

182.2 Discussion

The lower plasma Cu concentration in Phase 1 for animals supplemented with CuO confirms the poor bioavailability of this source.

Plasma Zn and Cu concentrations decreased during the week of depletion (Phase 2). CuLys-supplemented rats had higher plasma Cu concentration than CuO-supplemented rats, but this time CuO was not different than $CuSO_4$, which could suggest a higher retention for CuLys.

Since tissue Zn concentrations were not affected by Zn source, this may suggest equal availability of all Zn sources at this level of supplementation. Copper, however, may be involved in bone Zn deposition, since CuO-treated rats (a less available form) had lower bone Zn than CuLys-treated rats. Most tissue Zn concentrations decreased during depletion, but bone showed no change. Kidney Zn concentrations of CuO-supplemented rats were inexplicably higher than those of $CuSO_4$.

Mean tissue Cu concentrations reflected the same trends as plasma concentrations, indicating that CuO was less bioavailable than CuLys or $CuSO_4$. Furthermore, $CuSO_4$ supplemented rats had the highest muscle Cu concentrations, which suggests that Cu from this source is taken up by muscle cells more readily. The only tissue to retain the same proportions of Cu following depletion to those before the beginning of depletion was the liver, as liver Cu concentrations of CuOsupplemented rats were lower than other treatments. Kidney and muscle Cu concentrations, however, stabilized and there was no difference for the different sources, suggesting a lower retention for CuLys and $CuSO_4$.

The interaction effects shown in bone following supplementation indicate increased bone Zn deposition by the ZnLys and CuLys treatments except when combined, in which case bone Zn concentrations drop. This observation might support the theory that when complexed, the mineral is "smuggled" across the membrane by the other molecule's (in this case lysine) transport mechanism. This also seemed to be the case when the sulphate forms were administered together. Following, depletion, there was also an interaction effect, this time in kidney Zn concentrations.

Mean MT concentrations were not affected by Zn source, suggesting equal biological values. They were, however, influenced by Cu source, as CuO-supplemented rats had lower MT concentrations. This suggests that Cu influences MT expression when the available dietary Cu is very low. Following the week of depletion, all MT levels stabilized and no differences were observed for different treatments. These results indicate that, at adequate supplemental levels, organic sources of Zn and Cu are metabolized similarly in most aspects to the best inorganic sources ($CuSO_4$ and $ZnSO_4$).

182.3 Toxic effects, clinical signs

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

No effects.

5.13 Recovery of labelled compound

state percentage

Not applicable.

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Annex Point IIA6.2	*Specify section no., heading and species as appropriate*
IUCLID: 5.0/35	**A6.2(35), Bioavailability of copper**

183 APPLICANT'S SUMMARY AND CONCLUSION

183.1 Materials and methods

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

A study was carried out to compare bioavailability, interactions and retention of complexed and inorganic sources of Zn and Cu fed to rats. The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP.

Sixty three male Charles Sprague-Dawley (CD) strain rats weighing 71.5 ± 7.3 g (mean ± SEM) were individually housed in an environmentally controlled room with a 12-hour light-dark cycle. Rats were fed a diet containing 0.34 and 0.71 mg/kg of Zn and Cu, respectively. Water was supplied *ad libitum*. Different Zn (Zn methionine, ZnMet; Zn lysine, ZnLys; Zn sulphate, $ZnSO_4$) and Cu (Cu lysine, CuLys; Cu sulphate, $CuSO_4$; Cu oxide, CuO) sources were added to the basal diet at 30 mg/kg of Zn and 6 mg/kg of Cu to create a 3x3 factorial experiment. Seven rats were randomly assigned to each of these treatments. Supplemented diets were fed for 4 weeks, at which point 4 randomly selected rats from each treatment were sacrificed (Phase 1). The rest of the animals were fed the unsupplemented basal diet for an additional week (Phase 2) and sacrificed.

Rats were anesthetized by inhaling methoxyflurane and bled by cardiac puncture. To obtain heparinized plasma, blood was centrifuged at 700 g for 25 minutes. The supernatant was decanted and frozen until analyzed for Zn and Cu. Liver, kidney, muscle and bone were immediately excised and frozen.

Analytical methods: Total metallothionine (MT) was measured in kidney and liver by the $^{109}Cd^{2+}$-binding method. Zn and Cu concentrations in plasma, liver, kidney, muscle and bone were measured by flame atomic absorption spectrophotometry. A standard reference material was used to confirm reliability of analytical methods.

Statistics: All data were analyzed by SAS. Tissue and plasma Zn, Cu and MT data were analyzed using General Linerar Model procedure. In case of significance ($p<0.05$) Waller-Duncan's K-ratio T test was used for multiple comparisons.

183.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

For the purposes of this summary, the results reported below exclude those relating to effects on plasma and tissue Zn concentrations. For completeness, however, these results have been addressed in section 4.1.

Weight gains seen in this study were not different among treatments or experimental phases, but there was a slight tendency in Phase 1 for CuLys to have a higher average daily gain than $CuSO_4$. Diet intakes were similar for all groups for both phases.

Phase 1: Plasma Cu concentrations were lower for CuO than $CuSO_4$ or CuLys-supplemented rats. All tissue Cu concentrations were affected by supplemental Cu source. In all tissues where Cu was measured, CuO was the lowest available source of Cu. Furthermore, $CuSO_4$-supplemented rats had higher Cu concentrations in muscle than from CuLys supplementation. Different Zn sources did not affect tissue Cu. Kidney MT concentrations followed the same pattern as Cu concentrations, with CuO being the lowest MT inducer. There was no effect of Zn source on tissue MT concentrations for kidney or liver and no Cu effect for liver MT.

Phase 2: Plasma Cu concentrations of depleted rats were lower for CuO- than CuLys-supplemented rats. Most tissue Cu concentrations after depletion were not affected by supplemental Cu source. In liver, however, CuO-supplemented rats had the lowest Cu concentration. Different Zn sources did not affect tissue Cu. There was no effect of Zn or Cu source on tissue MT concentrations for kidney or liver

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	after one week of depletion.
183.3 Conclusion	Plasma Cu concentrations were lower for CuO- than $CuSO_4$ and CuLys-supplemented rats. In all tissues where Cu was measured, CuO was the least available source of Cu. Furthermore, in muscle, $CuSO_4$-supplemented rats had higher Cu concentrations than CuLys-supplemented rats. Kidney MT concentrations followed the same pattern as Cu concentrations, with CuO-fed rats having the lowest MT concentrations. Plasma Cu concentrations of depleted rats were lower for CuO- than CuLys-supplemented rats. In liver, CuO-supplemented rats had the lowest Cu concentration. Copper oxide was less available than CuLys and $CuSO_4$ when added in adequate dietary levels.
183.3.1 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
183.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the relative bioavailability of copper. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is a well-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper bioavailability. A reliability indicator of 2 has been assigned on this basis. *(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)*

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

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Table A6.2(35)-4. Mean tissue metallothionein concentrations for rats supplemented with different sources of Zn and Cu (tag MT/g[a])[b]

Sources	Kidney	Liver
$ZnSO_4$	77	40
ZnMet	76	42
ZnLys	80	38
Cu0	68[c]	46
$CuSO_4$	82[d]	38
CuLys	84[d]	37

[a] jtg MT/g = mg of metallothionein per gram of wet tissue.
[b] SEM are as follows: kidney = 10, liver =12.
[c,d] Means with different superscripts within column and mineral differ ($p<0.05$).

Table A6.2(35)-6. Mean tissue Zn concentrations for rats supplemented with different sources of Zn and Cu for four weeks and depleted for one week (mg/kg, DMB[a])[b]

Sources	Bone	Kidney	Liver	Muscle
$ZnSO_4$	153	68	66	53
ZnMet	155	68	69	55
ZnLys	157	62	70	57
Cu0	153	71[c]	67	56
$CuSO_4$	158	59[d]	71	55
CuLys	154	67c,d	67	54

[a] DMB = dry matter basis; bone also fat free.
[b] SEM are as follows: bone = 5, kidney = 9, liver = 5, muscle = 8.
[c,d] *Means with different superscripts within column and mineral differ ($p<0.05$).*

Table A6.2(35)-7. Mean tissue Cu concentrations for rats supplemented with different sources of Zn and Cu for four weeks and then depleted for one week (mg/kg, DMB[a])[b].

Sources	Kidney	Liver	Muscle
$ZnSO_4$	23	8	6
ZnMet	26	9	5
ZnLys	20	10	7
Cu0	22	7[c]	5
$CuSO_4$	24	10[d]	6
CuLys	23	11[d]	7

[a] DMB = dry matter basis
[b] SEM are as follows: kidney = 8, liver = 2, muscle = 2.
[c,d] Means with different superscripts within column and mineral differ ($p<0.05$)

COMMENTS FROM ...

Date *Give date of comments submitted*

Table A6.2(35)-1. Mean plasma Zn and Cu concentrations for rats supplemented with different sources of Zn and Cu (~g/ml)[a]

Sources	Zn	Cu
$ZnSO_4$	2.5	1.0
ZnMet	2.2	0.8
ZnLys	2.6	0.9
CuO	2.2	0.2[b]
$CuSO_4$	2.5	1.2[c]
CuLys	2.6	1.3[c]

[a] SEM are as follows: Zn = 0.75, Cu = 0.27

[b,c] Means with different superscripts within column and mineral differ ($p<0.05$)

Table A6.2(35)-2. Mean tissue Zn concentrations for rats supplemented with different sources of Zn and Cu (mg/kg, DMB[a])[b].

Sources	Bone	Kidney	Liver	Muscle
$ZnSO_4$	149	84	76	58
ZnMet	150	85	77	57
ZnLys	150	84	76	58
CuO	147[c]	85	75	56
$CuSO_4$	150[c,d]	85	77	58
CuLys	153[d]	83	77	58

[a] DMB = dry matter basis; bone also fat free.

[b] SEM are as follows: bone = 6, kidney = 14, liver = 10, muscle = 8.

[c,d] Means with different superscripts within column and mineral differ ($p<0.05$).

Table A6.2(35)-3. Mean tissue Cu concentrations for rats supplemented with different sources of Zn and Cu (mg/kg, DMB[a])[b]

Sources	Kidney	Liver	Muscle
$ZnSO_4$	27	11	7
ZnMet	29	12	6
ZnLys	30	12	6
CuO	22c	9c	4c
$CuSO_4$	32[d]	13d	8d
CuLys	32[d]	14d	6e

[a] DMB = dry matter basis.

[b] SEM are as follows: kidney = 6, liver = 2, muscle = 2.

c,d,e Means with different superscripts within column and mineral differ ($p<0.05$)

Table A6.2(35)-4. Mean tissue metallothionein concentrations for rats supplemented with different sources of Zn and Cu (tag MT/g[a])[b]

Sources	Kidney	Liver
ZnSO	77	40
ZnMet	76	42
ZnLys	80	38
CuO	68[c]	46
CuSO	82[d]	38
CuLys	84[d]	37

[a] jtg MT/g = mg of metallothionein per gram of wet tissue.

[b] SEM are as follows: kidney = 10, liver =12.

[c,d] Means with different superscripts within column and mineral differ ($p<0.05$).

Table A6.2(35)-5. Mean plasma Zn and Cu concentrations for rats supplemented with different sources of Zn and Cu for four weeks and then depleted for one week (~g/ml)[a]

Sources	Zn	Cu
$ZnSO_4$	1.7	0.2
ZnMet	2.0	0.4
ZnLys	1.6	0.3
Cu0	1.8	0.1[b]
$CuSO_4$	1.7	0.2[b,c]
CuLys	1.8	0.5[c]

[a] SEM are as follows: Zn = 0.34, Cu = 0.22.
[b,c] Means with different superscripts within column and mineral differ ($p<0.05$)

Table A6.2(35)-6. Mean tissue Zn concentrations for rats supplemented with different sources of Zn and Cu for four weeks and depleted for one week (mg/kg, DMB[a])[b]

Sources	Bone	Kidney	Liver	Muscle
ZnS0	153	68	66	53
ZnMet	155	68	69	55
ZnLys	157	62	70	57
Cu0	153	71[c]	67	56
$CuSO_4$	158	59[d]	71	55
CuLys	154	67c,d	67	54

[a] DMB = dry matter basis; bone also fat free.
[b] SEM are as follows: bone = 5, kidney = 9, liver = 5, muscle = 8.
[c,d] Means with different superscripts within column and mineral differ ($p<0.05$).

Table A6.2(35)-7. Mean tissue Cu concentrations for rats supplemented with different sources of Zn and Cu for four weeks and then depleted for one week (mg/kg, DMB[a])[b].

Sources	Kidney	Liver	Muscle
ZnS0	23	8	6
ZnMet	26	9	5
ZnLys	20	10	7
Cu0	22	7[c]	5
$CuSO_4$	24	10[d]	6
CuLys	23	11[d]	7

[a] DMB = dry matter basis
[b] SEM are as follows: kidney = 8, liver = 2, muscle = 2.
[c,d] Means with different superscripts within column and mineral differ ($p<0.05$)

Table A6.2(35)-8. Mean tissue metallothionein concentrations for rats supplemented with different sources of Zn and Cu for four weeks and then depleted for one week (Eag **MT/g[a])[b]**

Sources	Kidney	Liver
$ZnSO_4$	44	30
ZnMet	37	32
ZnLys	42	30
Cu0	43	32
$CuSO_4$	40	33
CuLys	39	27

[a] **jtg** MT/g = mg of metallothionein per gram of wet tissue.
[b] SEM are as follows: kidney = 9, liver = 6, no differences ($p<0.05$)

Figure A6.2(35)-1

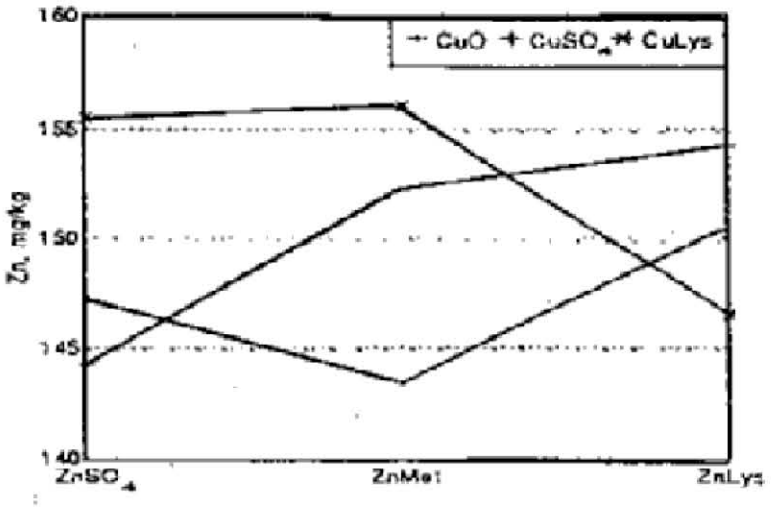

Figure 1. Mean bone (dry, fat-free basis) Zn concentrations for rats supplemented with different Cu sources when supplementing different Zn sources.

Figure A6.2(35)-2

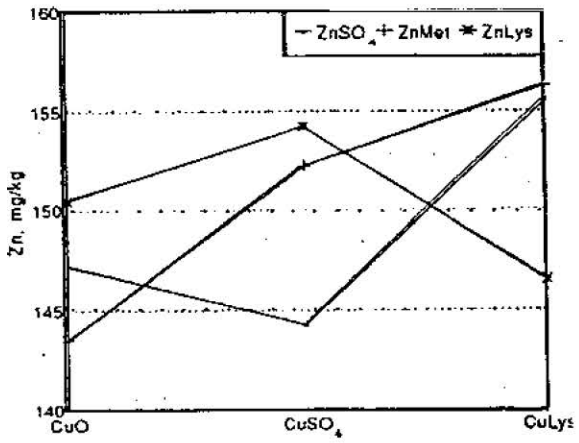

Figure 2. Mean bone (dry, fat-free basis) Zn concentrations for rats supplemented with different Zn sources when supplementing different Cu sources. SEM (mg/kg) 6.0.

Figure A6.2(35)-3

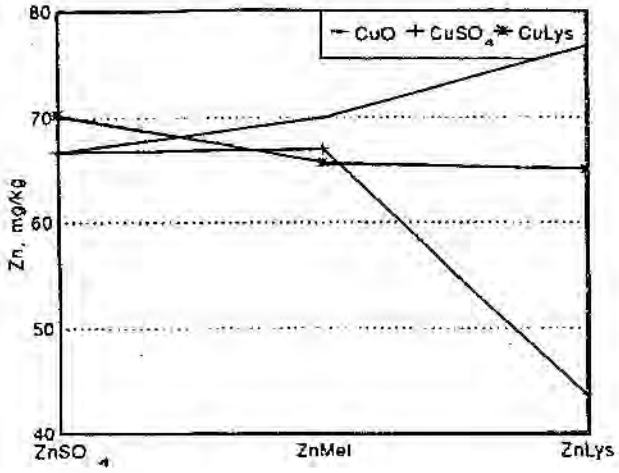

Figure 3. Mean kidney (dry basis) Zn concentrations for rats supplemented with different Cu sources when supplementing different Zn sources.

Figure A6.2(35)-4

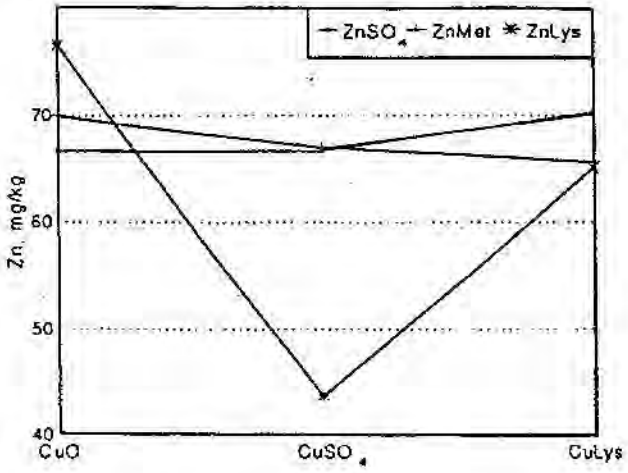

Figure 4. Mean kidney (dry basis) Zn concentrations for rats supplemented with different Zn sources when supplementing different Cu sources. SEM (mg/kg) 6.0.

Section A6.2

Annex Point IIA6.2 Metabolism in mammals

IUCLID: 5.0/01

Copper Compound Dossier – Dossier Preparation and Choice of Studies

Official use only

This document describes the strategy employed by the Wood Preservatives Copper Task Force in preparing the Toxicokinetics Section (Section 6.2) of the Biocidal Product Dossier for Copper Compounds.

As copper is a widely researched compound, unlike the majority of biocidal active substances, there is a substantial amount of data available in the public domain and it is considered that these data should be utilised in order to minimise unnecessary animal testing.

There is a huge collection of public domain references available on the toxicokinetics of copper in animals and humans. These studies have been conducted with copper salts or radioactive copper. Since copper and its salts have been used widely for many years, these studies have been well documented and reviewed by several authors and organisations (e.g. WHO, 1998).

The thirty five studies chosen for the Adsorption, Distribution, Metabolism and Excretion section and the bioavailability section were chosen to represent a weight of evidence approach with the most relevant studies found in the public domain.

The task force has decided to divide the Toxicokinetic Section of the dossier into three sections:

1. The essentiality of copper.
2. The Adsorption, Distribution, Metabolism and Excretion of copper.

3. The comparative bioavailability of copper sulphate, copper carbonate and copper oxide.

The pivotal studies in each section have been chosen and summarised according to the Technical Notes for Guidance on the Preparation of Dossiers and Study Evaluation. The reviews on the toxicokinetics will also be included in the dossier as supporting documentation (Ralph & McArdle, 2001; WHO, 1998). In addition, detailed literature survey will be included as required.

This strategy ensures that the authorities will be able to

Section A6.2

Annex Point IIA6.2 **Metabolism in mammals**

IUCLID: 5.0/01

determine the toxicokinetic profile of copper for risk assessment purposes and the section will fulfil the BPD requirements for this particular section of the overall dossier.

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

[redacted]

Section A6.2

Annex Point IIA6.2 **Metabolism in mammals**

IUCLID: 5.0/01

[redacted]

COMMENTS FROM ...

Date *Give date of comments submitted*

Section A 6.4.1		**Repeated dose toxicity in the Rat**	
Annex Point 6.4 .1		*Specify section no. and heading, route and species*	
IUCLID: 5.4/04		**A6.4.1(01), Subchronic Oral Toxicity Test**	
		1 REFERENCE	**Official use only**
1.1	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Hébert, C.D., 1993. NTP Technical Report on toxicity studies of cupric sulphate (CAS No. 7758-99-8) administered in drinking water and feed to F344/N rats and B6C3F1 mice. National Toxicology Program, Toxicity Report Series No. 29, United States Department of Health and Human Services (NIH Publication 93-3) (published)	X
1.2	**Data protection**	No *(indicate if data protection is claimed)* 1.2.1 Data owner *Give name of company* – Not applicable	
1.2.2	Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* Not applicable	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No - The method was developed by the US NTP specifically for the purposes of this study *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
2.2	**GLP**	Yes *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
2.3	**Deviations**	See Section 5.5.5 *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
		3 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values depending on the true methodological parameters.	
3.1	**Test material**	Copper sulphate *or give name used in study report*	X
3.1.1	Lot/Batch number	*List lot/batch number if available* 533344	
3.1.2	Specification	Not reported *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	
3.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Blue, crystalline solid	
3.1.2.2	Purity	*Give purity in % of active substance* [redacted]	X

Section A 6.4.1	**Repeated dose toxicity in the Rat**
Annex Point 6.4 .1	*Specify section no. and heading, route and species*
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**

3.1.2.3 Stability	*Describe stability of test material* Stable at room temperature	
3.2 Test Animals	Non-entry field	
3.2.1 Species	Rat	
3.2.2 Strain	F344/N	
3.2.3 Source	Simonsen Laboratories, Gilroy, California, USA	
3.2.4 S ex	Male and Female	
3.2.5 Age/weight at study initiation	Test animals were approximately 6 weeks old at study initiation. Male mean bodyweights ranged from 119-120 g, mean female bodyweights ranged from 105-107 g	
3.2.6 Number of animals per group	*Give number* *specify, if there are differences for example for treatment and recovery groups* In the base study, groups of 10 animals per sex were tested at each dose level. A supplementary study was carried out on 10 males and females per sex per dose for haematology and clinical chemistry evaluations on Days 5 and 21 (all surviving base-study rats were also subject to the same examinations on test termination – Day 92).	
3.2.7 Control animals	Yes	X
3.3 Administration/ Exposure	Oral *(fill in respective route in the following, delete other routes)*	
3.3.1 Duration of treatment	92 Days	
3.3.2 Frequency of exposure	*ad libitum* for 7-days a week	
3.3.3 Postexposure period		
3.3.4 Oral		
3.3.4.1 Preparation of active ingredient in feed	Copper sulphate was mixed with NIH-07 Open Formula Diet in meal form. Homogeneity analysis were conducted on the copper sulphate feed mixture using inductively coupled plasma-atomic emission spectroscopy. Samples taken prior to study initiation and twice during the study, confirmed homogeneity between feed mixtures.	
3.3.4.2 Concentration in vehicle	Feed mix was available *ad libitum* throughout the study period. 0 (control), 500, 1000, 2000, 4000 or 8000 ppm were administered to the test organisms in feed. Doses were based on a preliminary 2-week feed study.	X
3.3.4.3 Duration of exposure	92-Days	
3.3.4.4 Controls	Yes –vehicle only	
3.4 Examinations	*Non entry field*	
3.4.1 Observations	*Non entry field*	
3.4.1.1 Clinical signs	*yes/no (give time periods for observation)* Yes – test animals were observed weekly for clinical signs	

Section A 6.4.1	**Repeated dose toxicity in the Rat**
Annex Point 6.4 .1	*Specify section no. and heading, route and species*
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**

3.4.1.2 Mortality	*yes/no (give time periods for observation)* Yes – test animals were observed twice daily for mortality/morbidity.	
3.4.2 Body weight	*yes/no (give time periods for determinations)* Yes - Individual bodyweights were recorded prior to the start of the study, on Day 1 and weekly thereafter.	
3.4.3 Food consumption	*yes/no (give time periods for determinations)* Yes – test animals were observed once weekly for food consumption.	
3.4.4 Water consumption	*yes/no (give time periods for determinations)* Not reported	
3.4.5 Ophthalmoscopic examination	*yes/no (give time periods for examinations)* See histological examinations	
3.4.6 Haematology	Yes number of animals: taken from all supplementary animals and base-study rats. Blood samples were collected from the retroorbital sinus time points: Supplementary rats - Day 5 and 21, Base study rats – Day 92 and test termination Parameters: hematocrit , haemoglobin concentration, erythrocyte count, reticulocytes, nucleated erythrocytes, mean cell volume and haemoglobin, concentration, platelets and leukocyte count and differential.	
3.4.7 Clinical Chemistry	Yes number of animals: taken from all supplementary animals and base-study rats time points: Supplementary rats - Day 5 and 21, Base study rats – Day 92 and test termination Parameters: alanine aminotransferase , alkaline phosphatase, 5'-nucleotidase, sorbitol dehydrogenase, bile salts, total protein, albumin, creatinine and urea nitrogen.	
3.4.8 Urinalysis	Yes number of animals: taken from all supplementary animals and base-study rats time points: Supplementary rats - Day 5 and 21, Base study rats – Day 92 and test termination Parameters: creatinine, glucose, protein, asparate aminotransferase, N-acetyl-**β**-D-glucosaminidase, volume and specific gravity.	X
3.4.9 Tissue Metal Level Analysis	Yes Number of animals: Plasma and tissue samples (liver, kidney and testis) were collected from all surviving male base-study rats Time Points: Day 92 - copper, zinc, magnesium and calcium analysis. Blood samples (2 ml) were collected from the retroorbital sinus and placed into 3 ml Vacutainer® tubes containing EDTA. The samples were centrifuged and the separated plasma collected. To prepare for analysis, samples were weighed to the nearest 0.1 mg, digested in a nitric acid-perchloric acid mixture and heated until evolution of nitric acid was complete. The residue was then dissolved in 10% perchloric acid solution and an aliquot removed for analysis by ICP-AES. Metal concentrations were determined by comparing the instrument response to the digested tissues to spiked tissue standards.	
3.5 Sacrifice and pathology	Non entry field	

Section A 6.4.1	**Repeated dose toxicity in the Rat**	
Annex Point 6.4 .1	*Specify section no. and heading, route and species*	
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**	
3.5.1 Organ Weights	Yes organs: liver, kidneys, adrenals, testes, uterus, ovaries, thymus, spleen, brain, heart	X
3.5.2 Gross and histopathology	Yes Number of animals: Complete necropsies were performed on all animals in the control and high dose groups and on all other animals that died early Time point: See above Parameters: adrenal glands, brain (three sections), esophagus, eyes (if grossly abnormal) femur with marrow, gross lesions, heart, intestines (large: cecum, colon, rectum: small: duodenum, jejunum. Ileum), kidneys, liver, lung/mainstream bronchi, lymph nodes (mandibular, mesenteric) mammary gland, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pharynx (if grossly abnormal), pituitary gland, preputial or clitoral glands, prostate gland, salivary glands, spinal cord/sciatic nerve (if neurological signs were present), spleen, stomach (forestomach, glandular stomach), testes (with epididymis) thymus, thyroid gland, trachea, urinary bladder and uterus	
3.5.3 Other examinations	Non entry field	
3.5.3.1 Supplemental histological examination	To characterise the distribution of copper in the liver and kidney, section of both organs from selected male and females were stained for copper using the rhodanine method. In order to determine the nature of the proteinaceous droplets (see in previous study on rats) sections from selected animals were stained for carbohydrate (PAS method), protein (Mallory-Heidenhain method), lipofuscin (AFIP method) and **α**-2-microglobulin (immunochemistry). Liver sections from the same rats were stained for lipofuscin, and kidney and liver sections from rats of both sections were examined by transmission electron microscopy. Perl's stain for iron was used to stain sections of spleen from rats in all groups.	
3.5.3.2 Sperm morphology and vaginal cytology	Sperm morphology and vaginal cytology evaluations were performed on rats from the 0, 500, 200 and 4000 ppm groups (10 animals per sex and dose group). The method employed was as follows: National Toxicology Program (NTP) 1987. Technical Protocol for Sperm Morphology and Vaginal Cytology Evaluations in Toxicity Testing for Rats and Mice, 10/31/82 version. Research Triangle Park, N.C. Females: 12 days prior to sacrifice, the vaginal vaults of 10 individuals per dose group were lavaged and the aspirated lavage fluid and cells stained with Toluidine Blue. Relative numbers of leukocytes, nucleated epithelial cells and large squamous epithelial cells were determined and used to ascertain estrous cycle stage. Males: Sperm motility was evaluated at necropsy. The left testis and epididymis were weighed, the tail of the epididymis was removed from the epididymis body and weighed. Test yolk was applied to slides and a small incision made in the cauda. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the number of motile and non-motile spermatozoa counted for five microscopic fields per slide. Following motility determination, each left cauda were placed in phosphate buffered saline solution for sperm density determination with a hemacytometer	

Section A 6.4.1	**Repeated dose toxicity in the Rat**
Annex Point 6.4 .1	*Specify section no. and heading, route and species*
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**

3.6	**Statistics**	The following statistical procedures were followed; Dunnet, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1095-1121 Williams, D. A. 1971. Biometrics, 27, 103-117 Williams, D.A. 1972. The comparison of several dose levels with a zero dose control. Biometrics 28, 519-531 Shirley, E. 1977. A nonparametric equivalent of William's test for contrasting increasing dose levels of a treatment. Biometrics 33, 386-389 Dun, O.J. 1964. Multiple comparisons using rank sums. Technometrics 6, 241-252 Jonckheere, A.R. 1954. A distribution free k-sample test against ordered alternatives. Biometrika, 41, 133-145 Dixon & Massay 1951 Introduction to Statistical Analysis, McGraw-Hill Book Co.	X

4 RESULTS AND DISCUSSION

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

4.1	**Observations**	Non entry field
4.1.1	Clinical signs	*no effects / describe effects* No clinical signs of toxicity could be directly attributed to cupric sulphate consumption in any male or female group. For further details please refer to Table A6_4-5
4.1.2	Mortality	*no mortalities at any dose/concentration level / describe significant effects referring to data given in results table* Except for one female that was accidentally killed, all rats survived to the end of the study. For further details please refer to TableA6_4.5
4.2	**Body weight gain**	*no effects / describe significant effects referring to data given in results table* Final mean bodyweights of test organisms were lower than those of the controls for male rats in the 500, 4000 and 8000 ppm groups and for female rats in the 8000 ppm group. These differences were most pronounced in males in the high dose (8000 ppm). For further details please refer to Table A6_4.5
4.3	**Food consumption and compound intake**	*no effects / describe significant effects referring to data given in results table* For male and female rats in the 500, 1000, 2000 and 4000 ppm groups, average daily food consumption was similar to that of the controls. However, food consumption by both sexes in the 8000 ppm dose groups was below that of the controls. Despite this, the average daily compound consumption increased proportionally with increasing concentrations of copper sulphate in the feed. For further details please refer to Table A6_4.5

Section A 6.4.1	**Repeated dose toxicity in the Rat**
Annex Point 6.4 .1	*Specify section no. and heading, route and species*
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**

4.4	**Neurotoxicity**	Determination of neurotoxicity was not part of this study. Information on neurotoxicity is presented in TNG Summary 6.9 and IULICD 5.9 (Murthy, R.C., Lal, S., Saxena, D.K., Shukla, G.S., Mohd Ali, M and Chandra, S.V. (1981). Effect of Manganese and Copper Interaction on Behaviour and Biogenic Amines in Rats Fed a 10% Casein Diet. Chem. Biol. Interactions, 37: 299 – 308).
4.5	**Ophtalmoscopic examination**	Not reported. See Section 3.5.2
4.6	**Blood analysis**	*Non entry field*
4.6.1 Haematology		*no effects / describe significant effects referring to data given in results table* Significant changes in haematology parameters were noted in both sexes at all time points. At Day 5, significant increases in hematocrit (HCT) and hemoglobin (HGB) concentrations were noted in high dose male and female rats. By Day 21, these parameters were significantly decreased for male rats in the two highest dose groups (4000 and 8000 ppm) and female rats in the three highest dose groups. At Day 92, HCT and HGB concentrations were significantly decreased in males in the two highest dose groups and in females in the highest dose group. At Day 5, significant increases in erythrocyte (RBC) counts were noted in males in the two highest dose groups and in the high dose females; on Day 92, the only significant increase in RBC count was noted in the high-dose males. In both sexes, in the two highest dose groups, significant decreases in reticulocytes counts were noted on Day 5. By Day 21, reticulocyte counts in males and females in the same dose groups were significantly greater than those of the controls; at Day 92, this parameter was significantly increased in high dosed males. The only significant change noted 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 males in the two highest dose groups and in females in the highest dose group; mean cell hemoglobin (MCH) values were also significantly decreased for males in the two highest dose groups. At Days 21 and 92, decreases in MCV and MCH were noted in both sexes in the three highest dose groups, and all decreases were significant with the exception of the Day 92 MCH values for females receiving 4000 ppm. The only significant changes in mean cell hemoglobin concentrations were increases noted on Day 21 in high dose females and in males in the two highest dose groups. At Days 5 and 21, significant increases in platelet counts were noted in males and females in the three highest dose groups; the Day 5 platelet count for males in the 1000 ppm group was also significantly increased compare to the controls. At Day 92, increases in platelet counts were noted for both sexes in the two highest dose groups, but this was only significant for males. Leukocyte counts were increased at all time points in both sexes in the two highest dose groups, with significant increases occurring at Day 5 in high-dose males, at Day 21 in males in the 4000 ppm dose group, and at Day 92 in high-dose males and females: leukocyte count was also significantly increased at Day 21 in males receiving 2000 ppm copper sulphate. Significant increases in lymphocytes were noted at Day 5 in high dose males, at Day 21 in males receiving 2000 or 4000 ppm copper

sulphate, and at Day 92 in high dose females. The only other significant change in haematology parameters was an increase in segmented neutrophils at Day 92 in high dose male rats.

For further details please refer to Table A6_4-1

4.6.2 Clinical chemistry

no effects / describe significant effects referring to data given in results table

Significant changes in serum chemistry parameters occurred in male and female rats at all time point in the two highest groups. Alanine aminotransferase activities were significantly increased at all time points in both sexes in the two highest dose groups; and was significantly increased at Day 92 in males receiving 1000 or 2000 ppm. At Days 5 and 21, decreases in alkaline phosphate activities were noted in both sexes in the two highest dose groups; except for Day 21 in males in the 4000 ppm group, all these decreases were significant. Changes in sorbitol dehydrogenase (SDH) were limited to Days 21 and 92. At both of these time points, SDH activates 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 the control values, 5'necleotidase was significantly decrease in highdose females at Days 5 and 21 and in high dose males at Day 5; at Day 92, however, this parameter was significantly increased in males receiving 4000 and 8000 ppm cupric sulphate.

At Day 5, slight increases in bile salts were noted in males in the three highest dose groups; however, female bile salts were decreased for all treated groups, with significant decreases in the 1000 and 8000 ppm groups. By Day 21, no significant changes 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 copper sulphate.

At all time points, total protein was significantly decreased in high dose males and in females in the 4000 and 8000 ppm dose groups; at Days 5 and 21, total protein was also significantly decreased in males and females receiving 4000 and 2000 ppm copper sulphate respectively. At Days 5 and 21, decreases in albumin concentrations were noted in both sexes at the three highest doses, all of these were significant, excluding the Day 21 for males receiving 2000 ppm. At Day 92, this parameter was significantly decreased in high dose males and females in the two highest groups.

Urea nitrogen (UN) was significantly increased for both sexes in the two highest groups at Day 5, and by Day 21, this was significantly increased in males in the three highest dose groups and females in the highest dose group. At Day 92, UN was significantly elevated in the high-dose males and females as well as females receiving 1000, 2000 or 4000 ppm copper sulphate. The only significant change in creatinine was an increased noted in high dose females on Day 92.

For further information please refer to Table A6_4-2

4.6.3 Urinalysis

no effects / describe significant effects referring to data given in results table

Significant changes in urinalysis parameters were noted in supplemental study rats at Days 19 and in base study Day 90. Significant increases in urinary aspirate aminotransferase (AST) activities, occurred at Days 19

and 90 in both sexes in the highest dose groups. Increases in this parameter also occurred at both time points in male and female rats in the 4000 ppm groups. 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 were noted in both sexes in the highest dose group on Day 90; at this time point, increases also occurred in males and females in the 4000 ppm groups. Glucose output 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 was noted in the high dose males at Day 19, however, the Day 90 elevation in base study rats, this parameter was significantly increased relative to the controls in males in the two highest dose groups. No significant changes in glucose or protein output were noted in females at either time point.

Please refer to Table A6_4-3 for further information.

4.7 Sacrifice and pathology

Non entry field

4.7.1 Organ weights

no effects / describe significant effects referring to data given in results table

Significant changes in absolute organ weights were limited to males and females in the high dose groups and included decreases in absolute brain, heart, kidney, liver, lung and thymus weights in males and absolute kidney weight in females. Generally, relative organ weights for treated groups were similar to those of the controls or increased with decreasing mean body weights in the two highest dose groups (4000 and 8000 ppm).

For further information please refer to Table A6_4-5

4.7.2 Gross and histopathology

no effects / describe significant effects referring to data given in results table

Gross lesions were present in the forestomach of both sexes receiving copper sulphate at concentrations of 2000 ppm or greater. The limiting ridge that forms the junction of the forestomach squamous mucosa with the glandular gastic mucosa appeared enlarged in all rats in the 4000 and 8000 ppm dose groups.

Histopathological findings that correspond to the gross lesions consisted of minimal to moderate hyperplasia of the squamous mucosa at the site of the limiting ridge. This lesion was characterised by a thickening and increased folding of the squamous mucosa; hyperkeratosis was also a component of the squamous cell hyperplasia. The increased incidence and severity of this lesion were dose related. When this lesion was more severe, 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 or erosion/ulceration and no lesions were present in other areas of the squamous mucosa.

Other histopathological findings were present in the liver and kidney in both sexes. There was a dose related increase in the incidence and severity of chronic-active inflammation in the liver of male and female rats. This lesion was present in most rats in the 4000 and 8000 ppm

groups and in one male in the 2000 ppm group and was characterised by multiple foci of a mixture of mononuclear inflammatory cells, primarily macrophages. These foci of inflammation occurred primarily in the periportal portion of the hepatic lobules. Necrosis of one to several hepatocytes was often observed adjacent to or within the foci of inflammation.

Chemical related cytoplasmic alteration was present in the kidneys of male and female rats at doses of 2000 ppm and greater. This lesion was morphologically similar in both sexes but was less severe in females. A few droplets were also present in the tubule lumina of female rats. In treated male rats, the protein droplets were much larger and more numerous than those in the control males or in the treated females, and many large droplets were present in the tubule lumina. These droplets stained strongly positive for protein but were negative by iron, PAS and acid-fast staining methods. Results of **α**-2-microglobulin staining of kidney sections from male and female control and high dose rats were inconclusive. While the kidneys of male rats stained positive for **α**-2-microglobulin, there were no clear qualitative differences in staining between treated and control rats. Also present in the kidneys of rats in the high dose groups was minimal nuclear enlargement in renal tubule cells. Degeneration of the renal tubule epithelium was present in three females in the 8000 ppm group.

4.8 Other

The results of the analysis indicated that copper accumulated in the liver and kidney in a dose related manner and was accompanied by an accumulation of zinc in these tissues. Copper concentrations were significantly increased in the kidney and liver of rats in all treated groups. Copper levels were also significantly elevated in the plasma and testis of rats in the three highest dose groups. Significant increases in zinc concentration in the kidney and liver were noted in animals in the three highest dose groups, and concentrations of calcium in plasma were significantly decreased in the 4000 and 8000 ppm groups. Significant increases in magnesium were noted in the kidney and plasma of rats receiving 2000 ppm copper sulphate as well as in the plasma of rats receiving 8000 ppm copper sulphate.

4.9 Tissue Metal Level Analysis

For further information please refer to Table A6_4-4

4.10 onneoploastic lesions

A summary of nonneoplastic lesions is presented in the attached document Table A6_4-6

4.11 Supplemental histological examination

Liver and kidneys of rats were stained for the presence of copper. Positive staining in liver sections was limited to 4000 and 8000 ppm. At 8000 ppm, staining in the liver had a clear periportal to midzonal distribution and consisted of a few to numerous (10-20) red granules of 1-2 mm in the cytoplasm of hepatocytes. In addition there was minimal staining of the cytoplasm in some of the cells in the inflammatory foci. At 4000 ppm, staining of the hepatocytes was limited to the periportal area and there was a marked reduction in the number of cells stained and the number of granules per cell.

Kidney sections also stained positive for copper only in the two highest dose groups. Staining consisted of red granules in the cytoplasm of the renal tubule epithelium and a diffuse or stippled red staining of the

		protein droplets in the cytoplasm and the tubule lumen. However, many of these (especially in the 4000 ppm group) did not stain positive for copper. Positive staining of the kidney tubule cells was limited to the cortex; there was not staining in the medullary rays outer and inner medulla. Sections of hear and spleen showed no positive stained in any dose group. Sections of spleen from 4 rats per dose group were evaluated for iron. In the 8000 ppm groups there was only a few iron-positive granules in the cytoplasm of macrophages in the red pulp. The reduction in ironpositive material in the spleens from the 2000 and 4000 ppm groups was much less prominent than the 8000 ppm group, but a minimal decrease was evident compared to the controls. Transmission electron microscopy of the livers of both sexes showed that within the cytoplasm of hepatocytes in the periportal area, there was degenerative changes consisting of increased numbers of secondary lysosomes, many of which were enlarged and contained clear, non-staining crystalline structures and electron-dense material. Kidneys had mild to marked increases in the number and size of electron dense protein droplets in the cytoplasm of the proximal convoluted tubule epithelium. In addition to changes in the size and number, many droplets in the kidneys of male rats had irregular crystalline shapes	
4.12	Sperm Morphology and Vaginal Cytology	There were no significant findings in males or females. See attached Table A6_4-7	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	*Give guidelines and describe/discuss deviations from test guidelines or, in case of non-guideline study, briefly describe method* The aim of the study was to examine the effect of copper sulphate (0, 500, 1000, 2000, 4000 or 8000 ppm) administered to male and female B6C3F1 mice in feed for 13 weeks. The test organisms were observed throughout the study for signs of clinical toxicity, mortality, bodyweight changes and food consumption. Throughout the study blood and urine samples were collected to determine haematology, clinical chemistry and urinalysis parameters and tissue metal level, At the end of the study period all animals were sacrificed and subject to pathological examinations to determine any histological, sperm morphology or vaginal cytology abnormalities. The study was conducted to a methodology developed by the US National Toxicology Programme specifically for the test. The study was conducted in accordance with GLP.	X X
5.2	**Results and discussion**	*Summarize relevant results; discuss dose-response relationship.* Hematological, clinical chemistry and urinalysis evaluations of rats revealed variable chemical-related changes that were, for the most part, restricted to the 4000 and 8000 ppm groups. Increases in serum alanine aminotransferase and sorbitol dehydrogenase activities in both sexes were indicative of hepatocellular damage, as were increases in 5'-nucleotidase and bile salts in males. Decreases in mean cell volume, hematocrit and haemoglobin indicated the development of a microcytic anaemia, while increases in reticulocyte numbers at the same time points suggested a compensatory response to the anaemia by the bone marrow. Increases in urinary glucose and N-acetyl-β-Dglucosaminidase (a lysosome enzyme) and asparate aminotransferase (a	

cytosolic enzyme) were suggestive of renal tubule epithelial damage.

Dose related increases in copper occurred in all male rat tissues examined. These increases were accompanied by increases in zinc in the liver and kidney. Plasma calcium was significantly reduced in the 4000 and 8000 ppm groups, and there was a trend towards reduction in calcium in the kidney and testis as well. In the 8000 ppm group, plasma magnesium was significantly increased relative to the controls.

Rats in the three highest dose groups had hyperplasia and hyperkeratosis of the forestomach, inflammation of the liver and increases in the number and size of protein droplets in the epithelial cytoplasm and the lumina of the proximal convoluted tubules. Many of the droplets in the male kidneys were large and had irregular crystalline shapes. These droplets stained strongly positive for protein but were negative for iron, PAS, and acid-fast (lipofuscin) staining methods. **A**-2-microglobulin was present in the droplets of male rats, but there was no dose-related qualitative difference in the content of this protein. In the 4000 and 8000 ppm groups, copper was distributed in a periportal to midzonal pattern in the liver and was restricted to the cytoplasm of the proximal convoluted tubule epithelium in the kidney. Copper was present in some, but not all, of the protein droplets. Transmission electron microscopy of the livers of rats of each sex revealed increases in the number of secondary lysosomes in hepatocytes in the periportal area.

5.3 Conclusion *Non entry field*

5.3.1 LO(A)EL *Give critical effect and dose/concentration, if necessary separately for males and females*

The LOAEL for forestomach lesions was 2000 ppm for both males and females.

The LO(A)EL for liver damage was 2000 ppm for males and 4000 ppm for females.

The LO(A)EL for kidney damage was 2000 ppm for males and 1000 ppm for females.

5.3.2 NO(A)EL *Give dose/concentration, if necessary separately for males and females*

The NO(A)EL for forestomach lesions was 1000 ppm for both males and females.

The NO(A)EL for liver damage was 1000 ppm for males and 2000 ppm for females.

The NO(A)EL for kidney damage was 1000 ppm for males and 500 ppm for females.

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

5.3.4 Deficiencies Yes

The study deviated from 'Directive 88/302/EEC B.26 Subchronic 90-Day Oral Toxicity Study in Rodents' as follows;

- No additional top dose group or control animals group were

included in the study for observation of recovery from toxic effects after the treatment period.

- Ophthalomological examinations were only carried out where the eyes showed clinical signs of gross abnormalities. General eye examinations of the control and high dose group were not carried out.
- Sensory activity and signs of neurotoxicity were not determined towards the end of the study. The study was conducted prior to this requirement being included in the guidelines. However, signs of reproductive toxicity were included in the test methodology. See Section 6.4.14.
- Heamatological examinations did not include a measure of blood clotting time/potential.
- It was not reported if animals were fasted overnight prior to blood sampling.
- Determinations of plasma or serum did not include sodium, potassium or total cholesterol analysis.
- Histopathological examinations did not include the aorta.

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Reference • [redacted]

Section A 6.4.1	**Repeated dose toxicity in the Rat**
Annex Point 6.4 .1	*Specify section no. and heading, route and species*
IUCLID: 5.4/04	**A6.4.1(01), Subchronic Oral Toxicity Test**

Materials and Methods

[redacted]

Results and discussion

[redacted]

Conclusion

[redacted]

Reliability

[redacted]

Acceptability

[redacted]

Remarks

- [redacted]

- [redacted].

Table A6_4-2. Results of Significant Clinical Chemistry Effects

(Use this or similar table, if relevant effects occur and if time sequence is important. Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

Parameter	Control 0 ppm			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92
Males																		
Alanine aminotransferase	-	-	-	-	-	-	-	-	↑	-	-	↑	↑	↑	↑	↑	↑	↑
Alkaline phosphate	-	-	-	-	-	-	-	-	-	-	-	-	↓	-	-	↓	↓	-
Sorbital dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑	-	↑	↑
5'nucleotidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↓	-	↑
Bile salts	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	↑	↑
Total protein	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓
Albumin concentrations	-	-	-	-	-	-	-	-	-	↓	-	-	↓	↓	-	↓	↓	↓
Urea nitrogen	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑	-	↑	↑	↑
Females																		

Alanine aminotransferase	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	↑	↑	↑	↑
Alkaline phosphate	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	-
Sorbital dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑
5'nucleotidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-
Bile salts	-	-	-	-	-	-	↓	-	-	-	-	↑	-	-	↑	↓	-	-
Total protein	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓	↓	↓	↓
Albumin concentrations	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓	↓	↓	↓
Urea nitrogen	-	-	-	-	-	-	-	-	↑	-	-	↑	↑	-	↑	↑	↑	↑
Creatinine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑

Table A6_4-5. Results of repeated dose toxicity study

Parameter	Control 0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	dose-response +/-

Copper carbonate

	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	1'[a]
number of animals examined	10	10	10	10	10	10	10	10	10	10	10	10		
Mortality	0	0	0	0	0	1	0	0	0	0	0	0	-	-
clinical signs*	0	0	0	0	0	1	0	0	0	0	0	0	-	-
body weight (grams) (initial: final)	119 : 362	106 : 193	120 : 335	106 : 196	119 : 360	105 : 199	119 : 354	107 : 196	120 : 338	107 : 188	119 : 275	106 : 179	+	+
Final weight relative to controls (%)	-	-	92	101	99	103	98	101	93	97	76	93	+	+
food consumption (g/day)	16.3	11.1	16.6	11.0	17.0	11.3	16.5	11.3	16.5	10.8	14.4	10.1	+	+
Compound consumption (mg/kg/day)	-	-	32	34	64	68	129	135	259	267	551	528	+	+
Organ weight														
Brain	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Heart	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right kidney	-	-	-	-	-	-	-	-	-	-	↓	↓	+	
Liver	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Lungs	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right testis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	↓	-	+	-

* *specify effects; for different organs give special findings in the order organ weight, gross pathology and microscopic pathology if there are effects*

a *give number of animals affected/total number of animals, percentage, or just ↑ or ↓ for increased or decreased*

organ weights reported in absolute weight

	COMMENTS FROM ... *(specif)*
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_4-1. Results of Significant Haematology Effects

(Use this or similar table, if relevant effects occur and if time sequence is important. Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

Parameter	Control 0 ppm			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92
Males																		
Hematocrit concentrations	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	↑	↓	↓
Haemoglobin concentrations	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	↑	↓	↓
Erythrocyte count	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	↑	-	↑
Reticulocyte count	-	-	-	-	-	-	-	-	-	-	-	-	↓	↑	-	↓	↑	↑
Nucleated erythrocytes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	-	-
Leukocyte counts	-	-	-	-	-	-	-	-	-	-	↑	-	-	↑	-	↑	-	↑
Segmented neutrophils	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑

Parameter	Control 0 ppm			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92
Females																		
Hematocrit concentrations	-	-	-	-	-	-	-	-	-	-	↓	-	-	↓	-	↑	↓	↓
Haemoglobin concentrations	-	-	-	-	-	-	-	-	-	-	↓	-	-	↓	-	↑	↓	↓
Erythrocyte count	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-
Reticulocyte count													↓	↑		↓	↑	
Leukocyte count																		↑

Copper carbonate

* $p < 0,05$

Give only those parameters which are changed in at least one dose group compared to control. Usually only statistically significant effects

Depending on number of parameters changed one table each for Haematology, Clinical Chemistry, Urinalysis

Table A6_4-2. Results of Significant Clinical Chemistry Effects

(Use this or similar table, if relevant effects occur and if time sequence is important. Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

Parameter	Control 0 ppm			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92
Males																		
Alanine aminotransferase	-	-	-	-	-	-	-	-	↑	-	-	↑	↑	↑	↑	↑	↑	↑
Alkaline phosphate	-	-	-	-	-	-	-	-	-	-	-	-	↓	-	-	↓	↓	-
Sorbital dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑	-	↑	↑
5'nucleotidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↓	-	↑
Bile salts	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	↑	↑
Total protein	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓
Albumin concentrations	-	-	-	-	-	-	-	-	-	↓	-	-	↓	↓	-	↓	↓	↓
Urea nitrogen	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑	-	↑	↑	↑
Females																		

Copper carbonate

Parameter	Control 0 ppm			500 ppm			1000 ppm			2000 ppm			4000 ppm			8000 ppm		
	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92	Day 5	Day 21	Day 92
Alanine aminotransferase	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	↑	↑	↑	↑
Alkaline phosphate	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	-
Sorbital dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	-	↑	↑
5'nucleotidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓
Bile salts	-	-	-	-	-	-	↓	-	-	-	-	-	-	-	↑	↓	-	-
Total protein	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓	↓	↓	↓
Albumin concentrations	-	-	-	-	-	-	-	-	-	↓	↓	-	↓	↓	↓	↓	↓	↓
Urea nitrogen	-	-	-	-	-	-	-	-	↑	-	-	↑	↑	-	↑	↑	↑	↑
Creatinine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑

Table A6_4-3. Results of Significant Urinalysis Effects

(Use this or similar table, if relevant effects occur and if time sequence is important. Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

Parameter	Control 0 ppm		500 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm	
Males	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**
Urinary aspartate aminotransferase	-	-	-	-	-	-	-	-	↑	↑	↑	↑
N-acetyl-β-D-glucosaminidase	-	-	-	-	-	-	-	-	-	↑	-	↑
Glucose output	-	-	-	-	-	-	↑	-	-	↑	-	↑
Protein output	-	-	-	-	-	-	-	-	-	↑	↓	↑
Females			-	-	-	-	-	-	-	-	-	-

Copper carbonate

Parameter	Control 0 ppm		500 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm	
Males	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**	**Day 19**	**Day 90**
Urinary aspartate aminotransferase	-	-	↑	-	-	↑	-	↑	↑	↑	↑	↑
N-acetyl-β-D-glucosaminidase	-	-	-	-	-	-	-	↑	-	↑	-	↑
Glucose output	-	-	-	-	-	-	-	-	-	-	-	-
Protein output	-	-	-	-	-	-	-	-	-	-	-	-

* $p < 0,05$

Give only those parameters which are changed in at least one dose group compared to control. Usually only statistically significant effects
Depending on number of parameters changed one table each for Haematology, Clinical Chemistry, Urinalysis

Table A6_4-4. Results of Significant Tissue Metal Concentrations Effects from Male Rats

(Use this or similar table, if relevant effects occur and if time sequence is important. Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

Parameter	Control 0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm
Copper						
Kidney	-	↑	↑	↑	↑	↑
Liver	-	↑	↑	↑	↑	↑
Plasma	-	-	-	↑	↑	↑
Testis	-	-	-	↑	↑	↑
Calcium						
Kidney	-	-	-	-	-	-
Liver	-	-	-	-	-	-
Plasma	-	-	-	-	↓	↓
Testis	-	-	-	-	-	-
Magnesium						

Copper carbonate

Parameter	Control 0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm
Kidney	-	-	-	↑	-	-
Liver	-	-	-	-	-	-
Plasma	-	-	-	↑	-	↑
Testis	-	-	-	-	-	-
Zinc						
Kidney	-	-	-	↑	↑	↑
Liver	-	-	-	↑	↑	↑
Plasma	-	-	-	-	-	-
Testis	-	-	-	-	-	-

* $p < 0,05$

Give only those parameters which are changed in at least one dose group compared to control. Usually only statistically significant effects

Depending on number of parameters changed one table each for Haematology, Clinical Chemistry, Urinalysis

Table A6_4-5. Results of repeated dose toxicity study

Parameter	Control 0 ppm		500 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm		dose-response +/-	
	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]
number of animals examined	10	10	10	10	10	10	10	10	10	10	10	10		
Mortality	0	0	0	0	0	1	0	0	0	0	0	0	-	-
clinical signs*	0	0	0	0	0	1	0	0	0	0	0	0	-	-
body weight (grams) (initial: final)	119 : 362	106 : 193	120 : 335	106 : 196	119 : 360	105 : 199	119 : 354	109 : 196	120 : 338	107 : 188	119 : 275	106 : 179	+	+
Final weight relative to controls (%)	-	-	92	101	99	103	98	101	93	97	76	93	+	+
food consumption (g/day)	16.3	11.1	16.6	11.0	17.0	11.3	16.5	11.3	16.5	10.8	14.4	10.1	+	+
Compound consumption (mg/kg/day)	-	-	32	34	64	68	129	135	259	267	551	528	+	+
Organ weight														
Brain	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Heart	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right kidney	-	-	-	-	-	-	-	-	-	-	↓	↓	+	
Liver	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Lungs	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right testis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	↓	-	+	-

** specify effects; for different organs give special findings in the order organ weight, gross pathology and microscopic pathology if there are effects*

[a] *give number of animals affected/total number of animals, percentage, or just ↑ or ↓ for increased or decreased*

organ weights reported in absolute weight

Table A6_4-6a Summary of the Incidence of Nonneoplastic Lesions in Male Rats.

	0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)		(10)	(10)	(10)	(10)
Hepatodiaphragmatic nodule	1 (10%)		1 (10%)	1 (10%)		
Inflammation, chronic active				1 (10%)	10 (100%)	10 (100%)
Pancreas	(10)					(10)
Atrophy	2 (20%)					1 (10%)
Stomach, forestomach	(10)	(1)	(10)	(10)	(10)	(10)
Hyperplasia				10 (100%)	10 (100%)	10 (100%)
Stomach, glandular	(10)		(10)	(10)	(10)	(10)
Mineralization			1 (10%)			
Cardiovascular System						
Heart	(10)					(10)
Inflammation, chronic active	10 (100%)					5 (50%)
Endocrine System						
Pituitary gland	(10)					(10)
Cyst						1 (10%)
General Body System						
None						
Genital System						
Epididymis	(10)					(10)
Inflammation, chronic active	1 (10%)					
Preputial gland	(10)					(10)
Inflammation, chronic active	7 (70%)					8 (80%)
Prostate	(10)					(10)
Inflammation, chronic active	1 (10%)					1 (10%)
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)					(10)
Inflammation, chronic active	1 (10%)					
Special Senses System						
None						
Urinary System						
Kidney	(10)	(9)	(10)	(10)	(10)	(10)
Cytoplasmic alteration				3 (30%)	10 (100%)	10 (100%)
Nephropathy	10 (100%)	9 (100%)	10 (100%)	8 (80%)	9 (90%)	5 (50%)
Proximal convoluted renal tubule, karyomegaly						10 (100%)

[1] Number of animals examined microscopically at site and number of animals with lesion

Table A6_4-6b Summary of the Incidence of Nonneoplastic Lesions in Female Rats.

	0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Accidently killed			1			
Survivors						
Terminal sacrifice	10	10	9	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Intestine small, jejunum	(10)		(1)			(10)
Inflammation, acute	1 (10%)					
Liver	(10)	(1)	(2)	(10)	(10)	(10)
Hepatodiaphragmatic nodule	2 (20%)	1 (100%)	2 (100%)	2 (20%)		
Inflammation, chronic active					6 (60%)	10 (100%)
Inflammation, focal	2 (20%)					
Mesentery		(1)				
Fat, necrosis		1 (100%)				
Pancreas	(10)		(1)			(10)
Atrophy	1 (10%)					1 (10%)
Stomach, forestomach	(10)		(10)	(10)	(10)	(10)
Cyst epithelial inclusion						1 (10%)
Hyperplasia				7 (70%)	10 (100%)	10 (100%)
Cardiovascular System						
Heart	(10)					(10)
Inflammation, chronic active						1 (10%)
Endocrine System						
None						
General Body System						
None						
Genital System						
Clitoral gland	(10)		(1)			(10)
Inflammation, chronic active	9 (90%)					10 (100%)
Ovary	(10)	(1)	(1)			(10)
Cyst		1 (100%)				
Hematopoietic System						
None						
Integumentary System						
None						

Table A6_4-6b Summary of the Incidence of Nonneoplastic Lesions in Female Rats (cont.).

	0 ppm	500 ppm	1000 ppm	2000 ppm	4000 ppm	6000 ppm
Musculoskeletal System						
None						
Nervous System						
Brain	(10)		(1)			(10)
Gliosis	1 (10%)					
Respiratory System						
Lung	(10)					(10)
Inflammation, chronic active	1 (10%)					1 (10%)
Special Senses System						
None						
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Cyst	1 (10%)					
Cytoplasmic alteration			1 (10%)	9 (90%)	10 (100%)	10 (100%)
Mineralization		1 (10%)				
Nephropathy			1 (10%)	1 (10%)		2 (20%)
Pigmentation						2 (20%)
Proximal convoluted renal tubule, karyomegaly						10 (100%)
Renal tubule, degeneration						3 (30%)

[a] Number of animals examined microscopically at site and number of animals with lesion.

Table A6_4-7 Summary of the Reproductive Evaluations in Male and Female Rats

TABLE D1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Feed Study of Cupric Sulfate[1]

Study Parameters	0 ppm	500 ppm	2000 ppm	4000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	361 ± 5	345 ± 9	352 ± 7	339 ± 5*
Left epididymis	0.440 ± 0.009	0.428 ± 0.004	0.444 ± 0.013	0.432 ± 0.007
Left cauda epididymis	0.145 ± 0.005	0.139 ± 0.005	0.146 ± 0.004	0.135 ± 0.004
Left testis	1.51 ± 0.02	1.49 ± 0.03	1.52 ± 0.04	1.50 ± 0.03
Spermatid measurements				
Spermatic heads (10^7/g testis)	10.93 ± 0.43	11.35 ± 0.53	12.56 ± 0.48	10.75 ± 0.57
Spermatic 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 ± 3.18	92.03 ± 3.89	81.03 ± 3.60
Spermatozoal measurements				
Motility (%)	71.44 ± 1.85	72.98 ± 1.60	67.14 ± 2.16	70.09 ± 2.02
Concentration (10^7/g cauda epididymal tissue)	385.9 ± 56.5	510.7 ± 46.2	773.3 ± 97.3	782.6 ± 25.0

[1] Data presented as mean ± standard error. Differences from the control group for testis, epididymal, and cauda epididymal weights, spermatid measurements, and spermatozoal measurements are not significant by Dunn's test.
* Significantly different (P≤0.05) from the control group by Williams' test

TABLE D2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Feed Study of Cupric Sulfate[1]

Study Parameters	0 ppm	500 ppm	2000 ppm	4000 ppm
n	10	10	10	10
Necropsy body weight (g)	196 ± 2	184 ± 3	196 ± 3	186 ± 3**
Estrous cycle length (days)	4.85 ± 0.11	4.75 ± 0.11	4.93 ± 0.09	5.20 ± 0.13
Estrous stages (% of cycle)				
Diestrus	33.3	37.5	36.2	42.5
Proestrus	10.6	11.7	10.0	10.8
Estrus	33.3	31.7	31.7	25.8
Metestrus	22.5	19.2	22.1	20.0
Uncertain diagnoses (%)	0.0	0.0	0.0	0.8

[1] Data presented as mean ± standard error. Estrous cycle lengths are not significant by Shirley's test. By multivariate analysis of variance (MANOVA), dosed groups do not differ significantly from controls in cycle length or in the relative length of time spent in the estrous stages.
** Significantly different (P≤0.01) from the control group by Williams' test.

Section A6.4.1		**Repeated dose toxicity in the Mouse**	
Annex Point 6.4.1		*Specify section no. and heading, route and species*	
IUCLID: 5.4/05		**A6.4.1(02), Subchronic Oral Toxicity Test**	
		1 REFERENCE	**Official use only**
1.3	**Reference**	*Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* Hébert, C.D., 1993. NTP Technical Report on toxicity studies of cupric sulphate (CAS No. 7758-99-8) administered in drinking water and feed to F344/N rats and B6C3F1 mice. National Toxicology Program, Toxicity Report Series No. 29, United States Department of Health and Human Services (NIH Publication 93-3) (published)	X
1.4	**Data protection**	No *(indicate if data protection is claimed)* 1.4.1 Data owner *Give name of company* - Not applicable	
1.4.2	Criteria for data protection	*Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others:* Not applicable	
		6 GUIDELINES AND QUALITY ASSURANCE	
6.3	**Guideline study**	No - The method was developed by the US National Toxicology Programme specifically for the purposes of this study *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*	
6.4	**GLP**	Yes *(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)*	
6.5	**Deviations**	See Section 5.3.5 *(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")*	X
		7 MATERIALS AND METHODS	
		In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values depending on the true methodological parameters.	
7.1	**Test material**	Copper sulphate *or give name used in study report*	X
7.1.1	Lot/Batch number	*List lot/batch number if available* 533344	
7.1.2	Specification	Not reported *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*	

Section A6.4.1 Annex Point 6.4.1 IUCLID: 5.4/05		Repeated dose toxicity in the Mouse *Specify section no. and heading, route and species* A6.4.1(02), Subchronic Oral Toxicity Test	
7.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Blue, crystalline solid	
7.1.2.2	Purity	*Give purity in % of active substance* [redacted]	X
7.1.2.3	Stability	*Describe stability of test material* Stable at room temperature	
7.2	**Test Animals**	Non-entry field	
7.2.1	Species	Mouse	
7.2.2	Strain	$B6C3F_1$	
7.2.3	Source	Simonsen Laboratories, Gilroy, California, USA	
7.2.4	Sex	Male and Female	
7.2.5	Age/weight at study initiation	Test animals were approximately 6 weeks old at study initiation. Male mean bodyweights ranged from 20.9-21.6 g, mean female bodyweights ranged from 17.1-18.6 g	
7.2.6	Number of animals per group	*Give number* *specify, if there are differences for example for treatment and recovery groups* In the study, groups of 10 animals per sex were tested at each dose level.	
7.2.7	Control animals	Yes (10 males and 10 females)	
7.3	**Administration/ Exposure**	Oral *(fill in respective route in the following, delete other routes)*	
7.3.1	Duration of treatment	92 Days	
7.3.2	Frequency of exposure	*ad libitum* for 7-days a week	
7.3.3	Postexposure period	None	
7.3.4	**Oral**	*Non entry field*	
7.3.4.1	Preparation of active ingredient in feed	Copper sulphate was mixed with NIH-07 Open Formula Diet in meal form. Homogeneity analysis were conducted on the copper sulphate feed mixture using inductively coupled plasma-atomic emission spectroscopy. Samples taken prior to study initiation and twice during the study, confirmed homogeneity between feed mixtures.	
7.3.4.2	Concentration in vehicle	Feed mix was available *ad libitum* throughout the study period. 0 (control), 1000, 2000, 4000, 8000 or 16,000 ppm were administered to the test organisms in feed. Doses were based on a preliminary 2-week feed study.	X
7.3.4.3	Duration of exposure	92-Days	
7.3.4.4	Controls	Yes –vehicle only	
7.4	**Examinations**	*Non entry field*	
7.4.1	Observations	*Non entry field*	
7.4.1.1	Clinical signs	*yes/no (give time periods for observation)*	

Section A6.4.1	**Repeated dose toxicity in the Mouse**
Annex Point 6.4.1	*Specify section no. and heading, route and species*
IUCLID: 5.4/05	**A6.4.1(02), Subchronic Oral Toxicity Test**

	Yes – test animals were observed weekly for clinical signs	
7.4.1.2 Mortality	*yes/no (give time periods for observation)*	
	Yes – test animals were observed twice daily for mortality/morbidity.	
7.4.2 Body weight	*yes/no (give time periods for determinations)*	
	Yes - Individual bodyweights were recorded prior to the start of the study, on Day 1 and weekly thereafter.	
7.4.3 Food consumption	*yes/no (give time periods for determinations)*	
	Yes – test animals were observed once weekly for food consumption.	
7.4.4 Water consumption	*yes/no (give time periods for determinations)*	
	Not reported	
7.4.5 Ophthalmoscopic examination	*yes/no (give time periods for examinations)*	
	See histological examinations Section 3.5.2	
7.4.6 Haematology	No haematology parameters were investigated	
7.4.7 Clinical Chemisty	No clinical chemistry parameters were investigated	
7.4.8 Urinalysis	No urinalysis investigation was carried out	
7.5 Sacrifice and pathology	*Non entry field*	
7.5.1 Organ Weights	Yes organs: liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain, heart	X
7.5.2 Gross and histopathology	Yes Number of animals: Complete necropsies were performed on all animals in the control and high dose groups and on all other animals that died early Time point: See above Parameters: adrenal glands, brain (three sections), esophagus, eyes (if grossly abnormal) femur with marrow, gross lesions, heart, intestines (large: cecum, colon, rectum: small: duodenum, jejunum. Ileum), kidneys, liver, lung/mainstream bronchi, lymph nodes (mandibular, mesenteric) mammary gland, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pharynx (if grossly abnormal), pituitary gland, preputial or clitoral glands, prostate gland, salivary glands, spinal cord/sciatic nerve (if neurological signs were present), spleen, stomach (forestomach, glandular stomach), testes (with epididymis) thymus, thyroid gland, trachea, urinary bladder and uterus.	X
7.5.3 Other examinations	*Non entry field*	
7.5.4 Supplemental histological examination	To characterise the distribution of copper in the liver and kidney, sections of both organs from selected males and females were stained for copper using the rhodanine method. In order to determine the nature of the proteinaceous droplets (see in previous study on rats) sections from selected animals were stained for carbohydrate (PAS method), protein (Mallory-Heidenhain method), lipofuscin (AFIP method) and **α**-2-microglobulin (immunochemistry). Perl's stain for iron was used to stain sections of spleen from mice in all groups	
7.5.5 Sperm morphology and vaginal cytology	Sperm morphology and vaginal cytology evaluations were performed on rats from the 0, 500, 200 and 4000 ppm groups (10 animals per sex and dose group). The method employed was as follows:	X

Section A6.4.1	**Repeated dose toxicity in the Mouse**
Annex Point 6.4.1	*Specify section no. and heading, route and species*
IUCLID: 5.4/05	**A6.4.1(02), Subchronic Oral Toxicity Test**

National Toxicology Program (NTP) 1987. Technical Protocol for Sperm Morphology and Vaginal Cytology Evaluations in Toxicity Testing for Rats and Mice, 10/31/82 version. Research Triangle Park, N.C.

Females: 12 days prior to sacrifice, the vaginal vaults of 10 individuals per dose group were lavaged and the aspirated lavage fluid and cells stained with Toluidine Blue. Relative numbers of leukocytes, nucleated epithelial cells and large squamous epithelial cells were determined and used to ascertain estrous cycle stage.

Males: Sperm motility was evaluated at necropsy. The left testis and epididymis were weighed, the tail of the epididymis was removed from the epididymis body and weighed. Test yolk was applied to slides and a small incision made in the cauda. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the number of motile and non-motile spermatozoa counted for five microscopic fields per slide. Following motility determination, each left cauda were placed in phosphate buffered saline solution for sperm density determination with a hemacytometer. X

7.6 Statistics

The following statistical procedures were followed; X

Dunnet, C.W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1095-1121

Williams, D. A. 1971. Biometrics, 27, 103-117

Williams, D.A. 1972. The comparison of several dose levels with a zero dose control. Biometrics 28, 519-531

Shirley, E. 1977. A nonparametric equivalent of William's test for contrasting increasing dose levels of a treatment. Biometrics 33, 386- 389

Dun, O.J. 1964. Multiple comparisons using rank sums. Technometrics 6, 241-252

Jonckheere, A.R. 1954. A distribution free k-sample test against ordered alternatives. Biometrika, 41, 133-145

Dixon & Massay 1951 Introduction to Statistical Analysis, McGrawHill Book Co.

8 RESULTS AND DISCUSSION

(Describe findings. If appropriate, include table. Sample tables are given below.)
Non entry field

8.1 Observations

8.1.1 Clinical signs

no effects / describe effects

No clinical signs of toxicity, considered to be substance related, were observed in male or female mice during the course of the study.

8.1.2 Mortality

no mortalities at any dose/concentration level / describe significant effects referring to data given in results table

No mice in any of the dose groups died or were killed before the end of

Section A6.4.1	**Repeated dose toxicity in the Mouse**	
Annex Point 6.4.1	*Specify section no. and heading, route and species*	
IUCLID: 5.4/05	**A6.4.1(02), Subchronic Oral Toxicity Test**	
	the 13-week study.	
8.2	**Body weight gain**	*no effects / describe significant effects referring to data given in results table* Mice exhibited a dose-related growth depression which resulted in more severe body weight depression at higher dose levels. Final mean bodyweights and bodyweight gains were slightly lower than those of the control group for males in the 4000 ppm group and were significantly lower for males and females in the 8000 and 16,000 ppm groups. See Table A6_4-1
8.3	**Food consumption and compound intake**	*no effects / describe significant effects referring to data given in results table* For both sexes in all dose groups, the average daily feed consumption was similar to, or exceeded that of the controls. The average daily compound consumption increased proportionally with increasing concentrations of copper sulphate pentahydrate in the feed. See Table A6_4-1
8.4	**Opthalmoscopic examination**	No effects X
8.5	**Neurotoxicity**	Determination of neurotoxicity was not part of this study. Information on neurotoxicity is presented in TNG Summary 6.9 and IULICD 5.9 (Murthy, R.C., Lal, S., Saxena, D.K., Shukla, G.S., Mohd Ali, M and Chandra, S.V. (1981). Effect of Manganese and Copper Interaction on Behaviour and Biogenic Amines in Rats Fed a 10% Casein Diet. Chem. Biol. Interactions, 37: 299 – 308).
8.6	**Blood analysis**	*Non entry field*
8.6.1 Haematology		*no effects / describe significant effects referring to data given in results table* Not applicable
8.6.2 Clinical chemistry		*no effects / describe significant effects referring to data given in results table* Not applicable
8.6.3 Urinalysis		*no effects / describe significant effects referring to data given in results table* Not applicable
8.7	**Sacrifice and pathology**	*Non entry field*
8.7.1 Organ weights		*no effects / describe significant effects referring to data given in results table* Significant decreases in organ weights were noted for the heart and kidney of high dose (16,000 ppm) male mice and the thymus and kidney of the high dose females. In addition, dose related decreases in absolute liver weights were noted for males and females, with significant decreases occurring in both sexes in the 8000 and 16,000 ppm groups and the 4000 ppm group in males. Generally relative organ weights for males and females in all dosed groups were greater than that of the controls, and many of these increases were significant for the higher dose groups. These changes in absolute and relative organ weights could be attributed to the lower final mean weights of mice in the higher

Section A6.4.1	**Repeated dose toxicity in the Mouse**
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doses. See Table A6_4-1.

8.7.2 Gross and histopathology

no effects / describe significant effects referring to data given in results table

Chemical related gross lesions were limited to the forestomach of seven male and four female mice in the 16,000 ppm groups. This lesion was characterised as a focal white discolouration of the squamous mucosa in the area of the limiting ridge where it forms a junction with the glandular gastric mucosa. Histopathological findings included minimal to mild squamous cell hyperplasia with hyperkeratosis of the forestomach mucosa at the site of the limiting ridge. This lesion was present in male and female mice receiving 4000 ppm test substance or greater. There was no evidence of inflammation or erosion/ulceration in the forestomach, and there was no increase in hyperplasia or hyperkeratosis in other portions of the forestomach mucosa. See attached Table A6_4-2.

8.8 Other

Non entry field

8.8.1 Supplemental histological examination

The livers and kidneys of male mice in all groups and female mice in the control group and 16,000 ppm were stained for the presence of copper. Positive staining was limited to the livers of high-dose male and female mice. Staining was extremely minimal and consisted of only a few positive staining hepatocytes in the entire liver section. Hepatocytes staining positive for copper contained a maximum of approximately 10 red granules per cell. Due to limited number of cells stained, no distribution of copper was apparent. There was no staining of livers in the lower doses or in the controls, and no staining was present in the kidneys of any mice.

Because of the reduction in iron in the spleen of rats (see Rat Repeat Dose Toxicity), additional sections of spleen from four mice in each dosed and control group were stained for iron. There was no difference between dosed and control mice in the amount of iron-positive granules in the spleen.

8.8.2 Sperm Morphology and Vaginal Cytology

No significant findings were noted in males or females in any dose group. See attached Table A6_4-3

9 APPLICANT'S SUMMARY AND CONCLUSION

9.1 Materials and methods

Give guidelines and describe/discuss deviations from test guidelines or, in case of non-guideline study, briefly describe method

The aim of the study was to examine the effect of copper sulphate (0, 1000, 2000, 4000, 8000 or 16,000 ppm) administered to male and female B6C3F1 mice in feed for 13 weeks. The test organisms were observed throughout the study for signs of clinical toxicity, mortality, bodyweight changes and food consumption. At the end of the study period all animals were sacrificed and subject to pathological examinations, sperm morphology and vaginal cytology.

The study was conducted to a methodology developed by the US National Toxicology Programme specifically for the test. The study was conducted in accordance with GLP.

9.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

There were no mortalities or signs of clinical toxicity observed in any of

Section A6.4.1	**Repeated dose toxicity in the Mouse**	
Annex Point 6.4.1	*Specify section no. and heading, route and species*	
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	the test species during the duration of the study. Opthalmoscopic examinations revealed no abnormalities at any dose level tested. At gross pathology, significant decreases in heart and kidney weight were noted in the high dose males in the thymus and kidneys of high dose females. There was also a significant decrease in liver weights in both sexes in the 8000 and 16000 ppm dose groups. Chemical related gross lesions were limited to the forestomach of 7 male and 4 females in the 16000 ppm dose group. Histopathological findings included minimal to mild squamous cell hyperplasia with hyperkeratosis of the forestomach mucosa at the site of the limiting ridge. Minimal positive staining for copper was present in the liver and was limited to the high-dose male and female mice. No significant findings were noted following examination of the sperm morphology and vaginal cytology.	
9.3 Conclusion	*Non entry field*	
9.3.1 LO(A)EL	*Give critical effect and dose/concentration, if necessary separately for males and females* 2000 ppm for males and females	X
9.3.2 NO(A)EL	*Give dose/concentration, if necessary separately for males and females* 1000 ppm for males and females	X
9.3.3 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 1	X
9.3.4 Deficiencies	*No/Yes (If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)* Yes The study deviated from 'Directive 88/302/EEC B.26 Subchronic 90-Day Oral Toxicity Study in Rodents' as follows; • No additional top dose group or control animals group were included in the study for observation of recovery from toxic effects after the treatment period. • Ophthalomological examinations were only carried out where the eyes showed clinical signs of gross abnormalities. General eye examinations of the control and high dose group were not carried out. • Sensory activity and signs of neurotoxicity were not determined towards the end of the study. The study was conducted prior to this requirement being included in the guidelines. However, signs of reproductive toxicity were included in the test methodology. See Section 6.4.14. • Haematological, clinical chemistry and urinalysis parameters were not investigated. • Histopathological examinations did not include the aorta.	

Evaluation by Competent Authorities

Section A6.4.1	**Repeated dose toxicity in the Mouse**
Annex Point 6.4.1	*Specify section no. and heading, route and species*
IUCLID: 5.4/05	**A6.4.1(02), Subchronic Oral Toxicity Test**

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Reference • [redacted]

Materials and Methods • [redacted]

Results and discussion [redacted]

Conclusion • [redacted]

Reliability [redacted]

Section A6.4.1	**Repeated dose toxicity in the Mouse**
Annex Point 6.4.1	*Specify section no. and heading, route and species*
IUCLID: 5.4/05	**A6.4.1(02), Subchronic Oral Toxicity Test**
Acceptability	[redacted]
Remarks	• [redacted] • [redacted]

Table A6_4-1. Results of repeated dose toxicity study

Parameter	Control 0 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm		16,000 ppm		dose-response +/-	
	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]
number of animals examined	10	10	10	10	10	10	10	10	10	10	10	10	-	-
Mortality	0	0	0	0	0	0	0	0	0	0	0	0	-	-
clinical signs	0	0	0	0	0	0	0	0	0	0	0	0	-	-
body weight (grams) (initial: final)	21.6:32.9	17.7:28.5	20.9:32.6	18.6:28.5	21.0:31.1	17.2:27.8	20.9:29.5	17.1:26.9	21.1:29.0	18.3:25	21.6:26.8	17.8:21.7	+	+
Final weight relative to controls (%)	-	-	99	100	95	97	90	94	88	88	82	76	+	+
food consumption (g/day)	4.2	5.0	4.8	5.0	5.1	5.9	4.8	6.2	5.1	5.9	5.0	5.4	-	-
Compound consumption (mg/kg/day)	-	-	173	205	382	494	736	1048	1563	2106	3201	4157		
Organ weight														
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right kidney	-	-	-	-	-	-	-	-	-	-	↓	↓	+	-
Liver	-	-	-	-	-	-	↓	-	↓	↓	↓	↓	+	+
Lungs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Right testis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	↓	-	+

	COMMENTS FROM ... *(specif)*
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_4-1. Results of repeated dose toxicity study

Parameter	Control 0 ppm		1000 ppm		2000 ppm		4000 ppm		8000 ppm		16,000 ppm		dose-response +/-	
	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]	m[a]	f[a]
number of animals examined	10	10	10	10	10	10	10	10	10	10	10	10	-	-
Mortality	0	0	0	0	0	0	0	0	0	0	0	0	-	-
clinical signs	0	0	0	0	0	0	0	0	0	0	0	0	-	-
body weight (grams) (initial: final)	21.6:32.9	17.7:28.5	20.9:32.6	18.6:28.5	21.0:31.1	17.2:27.8	20.9:29.5	17.1:26.9	21.1:29.0	18.3:25	21.6:26.8	17.8:21.7	+	+
Final weight relative to controls (%)	-	-	99	100	95	97	90	94	88	88	82	76	+	+
food consumption (g/day)	4.2	5.0	4.8	5.0	5.1	5.9	4.8	6.2	5.1	5.9	5.0	5.4	-	-
Compound consumption (mg/kg/day)	-	-	17.	205	382	494	736	1048	1563	2106	3201	4157		
Organ weight														
Brain	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Right kidney	-	-	-	-	-	-	-	-	-	-	↓	-	+	-
Liver	-	-	-	-	-	-	↓	-	↓	↓	↓	↓	+	+
Lungs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Right testis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	↓	-	+

Table A6_4-2a Summary of the Incidence of Non-Neoplastic Lesions in Male Mice

	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(1)				(10)
Infarct		1 (100%)				
Stomach, forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia				2 (20%)	6 (60%)	10 (100%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						

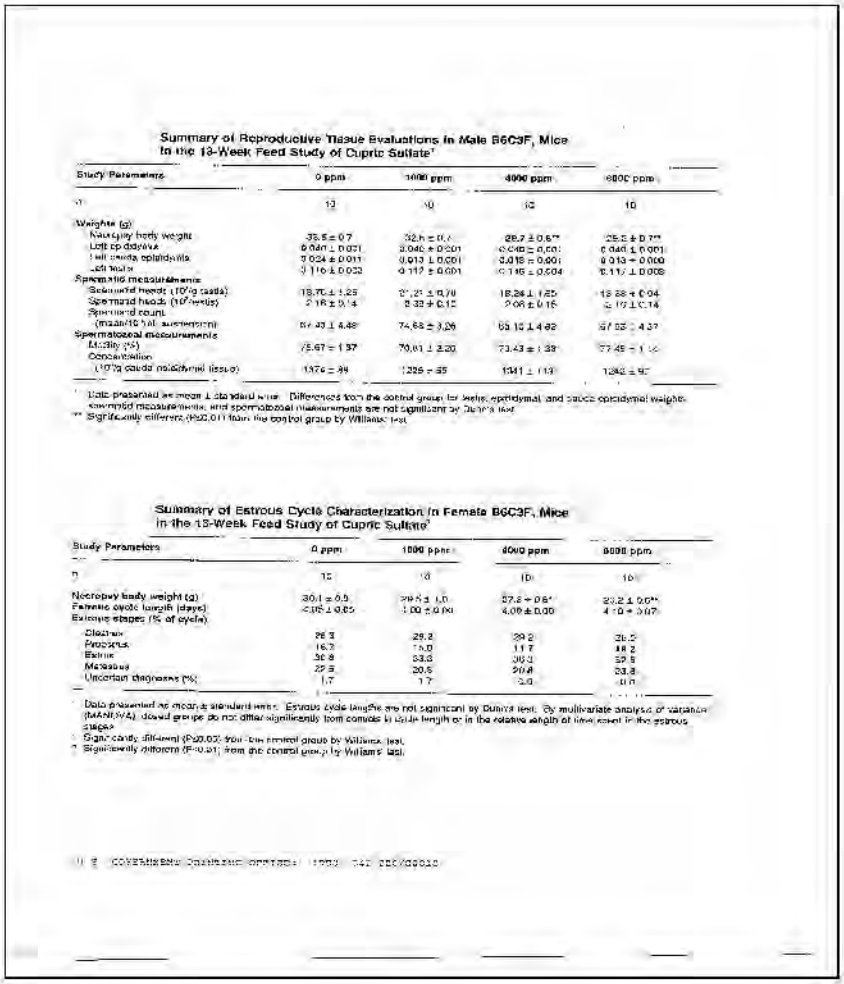
TABLE A6_4-2B SUMMARY OF THE INCIDENCE OF NON-NEOPLASTIC LESIONS IN FEMALE MICE

In Female B6C3F1 Mice in the 13-Week Feed Study of Cupric Sulfate

	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10		10	10	10	10
Alimentary System						
Stomach, forestomach	(10)		(10)	(10)	(10)	(10)
Hyperplasia				5 (50%)	8 (80%)	10 (100%)
Cardiovascular System						
Heart	(10)					(10)
Myocardium, mineralization	1 (10%)					
Endocrine System						
None						
General Body System						
None						
Genital System						
Clitoral gland	(10)					(8)
Cyst	1 (10%)					
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Urinary bladder	(10)					(10)
Transitional epithelium, mineralization	3 (30%)					

[1] Number of animals examined microscopically at site and number of animals with lesion.

TABLE A6_4-3 SUMMARY OF REPRODUCTIVE TISSUE EVALUATIONS IN MALE AND FEMALE MICE



Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Feed Study of Cupric Sulfate[1]

Study Parameters	0 ppm	1000 ppm	4000 ppm	8000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	35.5 ± 0.7	32.5 ± 0.7	28.7 ± 0.6**	25.5 ± 0.7**
Left cauda epididymis	0.041 ± 0.001	0.040 ± 0.001	0.040 ± 0.001	0.041 ± 0.001
Left epididymis	0.024 ± 0.001	0.023 ± 0.001	0.018 ± 0.001	0.013 ± 0.000
Left testis	0.116 ± 0.002	0.117 ± 0.001	0.116 ± 0.004	0.112 ± 0.008
Spermatid measurements				
Spermatid heads (10^6/g cauda)	18.70 ± 1.25	21.21 ± 0.70	18.28 ± 1.25	18.68 ± 0.94
Spermatid heads (10^6/testis)	2.16 ± 0.14	2.32 ± 0.12	2.06 ± 0.16	2.07 ± 0.14
Spermatid count (mean/10 fields, subsample)	67.40 ± 4.48	74.68 ± 3.26	69.10 ± 4.82	67.05 ± 4.37
Spermatozoal measurements				
Motility (%)	75.67 ± 1.37	70.61 ± 2.20	73.43 ± 1.39	77.45 ± 1.16
Concentration (10^6/g caudal epididymal tissue)	1376 ± 89	1225 ± 65	1341 ± 113	1243 ± 95

[1] Data presented as mean ± standard error. Differences from the control group for testis, epididymal, and cauda epididymal weights; examined reproductive events; and spermatozoal measurements are not significant by Dunn's test.
** Significantly different (P≤0.01) from the control group by Williams' test

Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Feed Study of Cupric Sulfate[1]

Study Parameters	0 ppm	1000 ppm	4000 ppm	8000 ppm
n	10	10	10	10
Necropsy body weight (g)	30.1 ± 0.5	29.5 ± 1.0	27.2 ± 0.6*	23.2 ± 0.6**
Female cycle length (days)	4.05 ± 0.05	4.00 ± 0.00	4.00 ± 0.00	4.10 ± 0.07
Estrous stages (% of cycle)				
Diestrus	26.3	28.2	29.2	26.5
Proestrus	16.3	15.0	11.7	18.2
Estrus	36.8	33.8	30.1	29.5
Metestrus	22.5	20.5	29.0	23.8
Uncertain diagnoses (%)	1.7	1.7	0.0	0.0

[1] Data presented as mean ± standard error. Estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance (MANOVA), dosed groups do not differ significantly from controls in cycle length or in the relative length of time spent in the estrous stages
* Significantly different (P≤0.05) from the control group by Williams' test.
** Significantly different (P≤0.01) from the control group by Williams' test.

Table 6.4_1. Copper Concentrations in Tissues of Adult Humans and Animals: Major Organs[a]

	Copper Concentration[b] (μg/g)				
Tissue/organ	Human	Rat	Pig	Mouse	Other
Kidney	12 ± 7 (19)	7.9 ± 5.5 (14)	7.3 ± 4.5 (4)	4.4 ± 1.1 (3)	5.8, 7.9 (2) (chick) 6.9, 10 (2) (dog)
Liver	6.2 ± 0.8 (9)	4.6 ± 1.1 (23)	5.2 ± 0.7 (5)	4.1[c], 4.7[d] (2)	3.0, 2.9 (2) (chicken) 67 ± 23 (5) (dog)[e]
Brain	5.2 ± 1.1 (10)	3.1 ± 1.2 (10)	3.9 ± 1.5 (4)	4.0 ± 2.1 (4)	
Heart	4.8 ± 1.9 (14)	4.8[f], 6.2 (2)	4.6 (1)		4.6 (1) (cow)
Bone	4.1 ± 1.3 (8)	2.5 ± 0.6 (3)	1.4, 2.4 (2)		4.4 (1) (sheep)
Stomach	2.2 ± 0.7 (7)		1.6 (1)		
Intestine	1.0, 3.0 (2)	1.7, 2.1 (2)		1.7 (1)	
Spleen	1.5 ± 0.4 (14)	2.3 ± 2.2 (8)	1.4 ± 0.3 (4)	1.2[c], 4.2 (2)	2.3, 4.2 (2) (dog)
Lung	1.3 ± 0.4 (11)	1.8 ± 0.6 (5)	1.2, 1.4 (2)	3.9 (1)	2.6 (1) dog
Blood	1.11 ± 0.13 (5)				
Plasma	1.13 ± 0.15 (70)	1.28 ± 0.26 (12)	1.75 ± 0.43 (11)	0.38[c] (1)	0.42 ± 0.20 (9) (chicken)
Muscle	0.9 ± 0.3 (7)	1.0 ± 0.4 (5)	1.0 ± 0.6 (3)		3.7 (1) (cow) 0.5-0.9 (3) (beef)[g]
Skin	0.8 ± 0.4 (9)	1.7 ± 0.8 (4)	1.0, 1.5 (2)		0.4 (1) (cow)
Adipose	0.2, 0.3 (2)	0.35 (1)	0.8, 0.7 (2)	2.4 (1)	1.2 (1) (whale)

[a] Owen (1982a).
[b] Mean ± SD of reported mean values for number of reports in parentheses.
[c] Keen and Hurley (1980).
[d] M.C. Linder, L. Rigby and C. Murillo, unpublished results.
[e] Based on recent data. Owen believes that the high values reflect the high Cu content of dog food, which contains a great deal of liver (Owen, 1982a). However, other factors, such as albumim, may be involved. See Chapter 4, Section 4.1.2.
[f] Linder and Munro (1973).
[g] Souci *et al.* (1981).

Sections A6.5 & A6.7	**Combined Chronic toxicity/Carcinogenicity**
Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

184 REFERENCE

Official use only

1.1 Reference

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

Carlton, W.W. and Price, P.S., (1973). Dietary Copper and the Induction of Neoplasms in the Rat by Acetylaminofluorene and Dimethylnitrosamine. Fd Cosmet. Toxicol. **11:** 827-840 (published).

1.2 Data protection

No

(indicate if data protection is claimed)

1.2.1 Data owner

Give name of company

Public domain.

184.1.1 Companies with letter of access

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

Letter of access not required.

184.1.2 Criteria for data protection

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

No data protection claimed.

185 GUIDELINES AND QUALITY ASSURANCE

185.1 Guideline study

No. This was a non-regulatory study to determine whether a high level of Cu would have an inhibitory effect on the induction of neoplasia by acetylaminofluorene (AAF) or dimethylnitrosamine (DMN) and to determine whether the incidence of neoplasia would be increased or whether neoplasms would appear earlier in rats fed a diet low in Cu.

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

185.2 GLP

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

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

185.3 Deviations

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

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

186 MATERIALS AND METHODS

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

Sections A6.5 & A6.7 **Annex Points IIA6.5 & IIA6.7** **IUCLID 5.4/11 & 5.7/01**	**Combined Chronic toxicity/Carcinogenicity** *Specify section no., heading, route and species as appropriate* **A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**
186.1 Test material	Cu^{2+} as copper sulphate ($CuSO_4$) Acetylaminofluorene (AAF) Dimethylnitrosamine (DMN) *or give name used in study report*
186.1.1 Lot/Batch number	Not stated. *List lot/batch number if available*
186.1.2 Specification	Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
186.1.3 Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Refer to section 2.1.
186.1.4 Purity	*Give purity in % active substance* [redacted]
186.1.5 Stability	*Describe stability of test material* Not stated.
186.2 Test Animals	Non-entry field
186.2.2 Species	Rat
186.2.3 Strain	Sprague-Dawley
186.2.4 Source	An un-named commercial supplier.
186.2.5 Sex	Male. Not stated.
186.2.6 Age/weight at study initiation	
186.2.7 Number of animals per group	

Diet	Other treatment	No. of rats
Copper-deficient (1 ppm Cu).	Control	50
	DMN	74
	AAF	55
Excess copper (800 ppm Cu).	Control	58
	DMN	102
	AAF	65

X

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7 IUCLID 5.4/11 & 5.7/01	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate* **A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**	
186.2.7.1 at interim sacrifice	After 90 days of feeding, 5 rats from each diet group were killed. Thereafter, each 30 days and additional 5 animals from each group were killed.	
186.2.7.2 at terminal sacrifice	All surviving animals were killed at study termination.	
186.2.8 Control animals	Refer to section 2.2.6. Control animals received the basal diet containing the appropriate amount of $CuSO_4$.	X
186.3 Administration/ Exposure	Oral in the diet. *Fill in respective route in the following, delete other routes*	
186.3.1 Duration of treatment	9 months.	
186.3.2 Interim sacrifice(s)	Refer to section 2.2.6.1.	X
186.3.3 Final sacrifice	Refer to section 2.2.6.2.	X
186.3.4 Frequency of exposure	7 days a week.	
186.3.5 Postexposure period	None.	
	Oral	
186.3.6 Type	$CuSO_4$ was administered in the diet. DMN was administered in the drinking water. AAF was administered in the diet.	
186.3.7 Concentration	The purified basal diet (Cu-deficient) contained 1 ppm Cu. The excess Cu diet contained 800 ppm Cu as $CuSO_4$. DMN was added to drinking water at a concentration of 50 ppm. AAF was added to the diets at a concentration of 0.06%.	
186.3.8 Vehicle	Basal diet.	
186.3.9 Concentration in vehicle	Refer to section 3.3.7.	
186.3.10 Total volume applied	Not applicable.	
186.3.11 Controls	Controls received either basal diet only (Cu deficient) or 800 ppm Cu as $CuSO_4$.	
186.4 Examinations		
186.4.2 Body weight	Yes.	
186.4.3 Food consumption	No.	
186.4.4 Water consumption	No.	
186.4.5 Clinical signs	Yes.	
186.4.6 Macroscopic investigations	Yes.	

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

186.4.7 Ophthalmoscopic examination	No.	
186.4.8 Haematology	No.	
186.4.9 Clinical Chemistry	No.	
186.4.10 Urinalysis	No.	
186.4.11 Pathology	Yes.	
186.4.11.1 Organ Weights	Yes	
	from:	at interim sacrifice (3, 5 and 7 months), at terminal sacrifice.
	Organs:	Liver; enlarged neoplastic kidneys.
	Other:	None.
186.4.11.2 Histopathology	Yes	
	From:	All dose groups
	From:	at interim sacrifice (at 90 days and each 30 days thereafter), at terminal sacrifice.
	Organs:	Spleen, kidneys, lungs, heart, thyroid gland, adrenal gland, duodenum and pancreas.
	Other:	None.

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

Concentrations of Cu in non-neoplastic and neoplastic hepatic and renal tissues were determined.

186.5 Statistics

Simple statistical methods were applied, as appropriate.

186.6 Further remarks

Liver and kidney Cu concentrations were determined by atomic absorption spectrophotometry. Copper analyses were carried out on 5 g pooled samples of liver. The analyses were run in triplicate and precautions were taken to prevent Cu-contamination of the tissues.

187 RESULTS AND DISCUSSION

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

187.1 Results

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

Non-entry field.

187.1.2 Body weight

Mean bodyweight data are summarised in **Figure A6.5(01) & A6.7(01)-1**. Rats fed the Cu-deficient control diet had the highest mean bodyweights. The mean weights of the Cu-deficient-DMN group were well below those of Cu-deficient control rats. The excess-Cu control and excess-Cu DMN groups had similar mean weights, approximately 120 g below the mean weight of the Cu-deficient control group after 6 months. AAF was markedly toxic and the mean weights of rats fed either of the AAF diets were markedly below those of the Cu-deficient

control, with the excess-Cu-AAF having the lowest mean weights of the various groups.

187.1.3 Mortality

After 3 months, the mortality rate in the six groups varied from 2% to 69% (**Table A6.5(01) & A6.7(01)-1**). Deaths were lowest in the Cu-deficient control and greatest in the excess-Cu-DMN group. These data indicate that excess Cu, DMN and AAF were all toxic and increased the number of deaths over that in the Cu-deficient control group. At the termination of the experiment, the lowest mortality (16%) was observed in the Cu-deficient control group and the highest in the excess-CuDMN group. This group, which had the highest mortality during the first 3 months (69%) had the fewest deaths later.

187.1.4 Organ weights

Liver weights expressed as a percentage of body weight were similar for all groups except those receiving AAF (**Table A6.5(01) & A6.7(01)-2**). Of these, Cu-deficient-AAF animals generally had higher liver weights than those in the excess-Cu-AAF group. Some of this increase in size was due to the presence of neoplasms.

187.1.5 Copper determinations

The Cu content of grossly non-neoplastic hepatic tissue from rats fed Cu-deficient diets did not vary greatly, although values for the group fed AAF were generally lower than for other groups (**Table A6.5(01) & A6.7(01)-3**). The Cu content of livers from the rats fed excess-Cu diets with carcinogens was greater than that found in the excess-Cu control rats.

The Cu content of neoplastic hepatic tissue from rats receiving Cu-deficient carcinogenic diets was similar to that in grossly normal tissue (**Table A6.5(01) & A6.7(01)-4**). In the two groups of rats which were fed the excess-Cu-AAF diet and had grossly separable neoplasms, the neoplastic tissue contained less Cu than the non-neoplastic tissue from the same animal.

Renal neoplasms were observed grossly only in the Cu-deficient-DMN rats and the concentrations of Cu were lower in these neoplasms than in the non-neoplastic renal tissue. The Cu concentration of this latter tissue was somewhat lower than that found in the kidneys of the Cu-deficient control rats (**Table A6.5(01) & A6.7(01)-5**). The Cu concentration of large neoplasms was lower than that found in small neoplasms (under 22 g).

187.1.6 Macroscopic investigations

Liver: Livers of rats fed the Cu-deficient-DMN diet for 3 or 4 months varied in appearance from those that were grossly normal to those with severe macroscopic changes and fusion of lobes. Some were tan-coloured and slightly swollen. Abnormal features of livers from rats fed this diet for 5-8 months varied in severity and included: swelling, colour variation, presence of clear cysts, haematocysts and/or neoplasms.

Grossly visible lesions of the livers were observed at the monthly samplings in Cu-deficient-AAF rats. Abnormalities observed after 3 months included discoloration, enlargement and presence of focal pale areas. After 4 months, a few clear cysts were also present. Later, livers

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7 IUCLID 5.4/11 & 5.7/01	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate* **A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

were pale, cystic and markedly enlarged, and neoplasms ranging in size from pin-point nodules to 3 cm in diameter were observed in all lobes.

Livers from Cu-deficient and excess-Cu control rats were grossly normal. Livers from excess-Cu-DMN rats were either normal or slightly off-colour after 3 and 4 months. Few further changes were observed after 5 and 6 months, except for prominent capsular vessels. Cysts, swollen lobes and haematocysts occurred in livers of rats fed for 7 months. Livers from the 4 rats killed after 8 months were more severely affected; haematocysts were observed in 2 livers and a neoplasm was found in one other.

Livers of excess-Cu-AAF rats had a striking appearance in one rat at month 3 that was consistently present in one or more livers at the other autopsy periods; the hepatic surface was converted into a mass of small nodules. This was more marked on the visceral surface. In addition, clear cysts were present peripherally after 5 months. Increased hepatic size, cysts and small white foci also appeared after 6 months. Neoplasms were larger after 7 months, and all livers from rats fed for 8 months had clear cysts, neoplasms and capsular nodularity, although there still was some variation in the severity of gross alterations.

Kidney: Grossly enlarged kidneys with neoplasms were seen after 5 months in Cu-deficient-DMN rats. The kidneys of 4 of the 5 rats had neoplasms of various sizes. After 6 months, neoplasms were present in all 5 rats. Grossly apparent neoplasms were present in 3 of the 5 rats examined after 7 months. Only one renal neoplasm was obvious at autopsy in 5 rats killed after 8 months. Three of the 13 rats on this treatment which died during the study had grossly apparent renal neoplasms.

Other organs: Abnormalities other than alterations of the liver and kidneys observed at autopsy in Cu-deficient-DMN rats included pale, expanding masses in the lungs of 2 rats. Grossly detectable neoplasms were observed in the lungs of excess-Cu-DMN rats after 7 and 8 months.

Neoplasms at locations other than the liver were most numerous in Cu-deficient-AAF rats. After 5 months, 3 rats had grossly obvious neoplasms in one or more of the following locations: ventral throat area, middle of side, groin area and base of ear. After 6 months, neoplasms were noted in the lungs of 2 rats and in the spleen of another. At month 7, neoplasms were present in the ventral thorax, spleen, abdomen, perianal region, base of ear, right rear leg and small intestine.

Fewer extrahepatic neoplasms were found in excess-Cu-AAF rats. Those that occurred werelocated at the base of the ear, along the lateral abdomen and in the lungs.

No gross abnormalities were observed in the urinary bladder of animals in any group.

187.1.7 Histopathology

Liver: Commonly occurring non-neoplastic lesions in the livers of carcinogen-treated rats included biliary-ductule cell hyperplasia, proliferation of biliary ducts and the presence of haematocysts. Many

Sections A6.5 & A6.7	**Combined Chronic toxicity/Carcinogenicity**
Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

of the proliferated biliary ducts were dilated and some were markedly enlarged, accounting for the clear cysts noted at autopsy. The cystic ducts had an epithelial lining of simple squamous to low cuboidal cells and when multiple were separated by a fine connective-tissue stroma.

The incidence rates of hepatic neoplasms and other lesions observed in the rats fed the various diets are summarized in **Table A6.6Ø-6**. Lesions listed as transitional nodules were localized groups of hepatocytes differing in staining intensity from the surrounding parenchyma but showing only minimal deviation of nuclear morphology and no compression of the surrounding parenchyma, those classed as hepatomas were larger foci of hepatocytes showing changes in nuclear morphology and causing compression of the surrounding parenchyma, and the hepatocellular carcinomas were large, highly cellular neoplasms showing marked alterations in nuclear and cytoplasmic morphology, containing areas of necrosis and blood cysts and invading blood and lymph vessels. In addition to hepatomas and hepatocellular carcinomas, a fibrosarcoma and cholangiocarcinoma of the liver were observed in rats of the Cu-deficient-DMN group. Hepatomas, hepatocellular carcinomas, cholangiomas and one cholangiocarcinoma were observed in the livers of rats fed the Cudeficient-AAF diet. Lung metastases of hepatocellular carcinomas were observed in the AAF-fed groups. The Cu level of the diet appeared to have little or no effect on the incidence rate of hepatic neoplasms.

Kidney: Fibrosarcomas, adenomas and adenocarcinomas were observed in kidneys of Cu-deficient-DMN rats. Emboli of tumour cells from a renal fibrosarcoma were observed in the lung. One fibrosarcoma was found in a kidney from a rat fed the Cu-deficient control diet. No renal neoplasms were observed either grossly or microscopically in the rats from other groups killed for autopsy. One renal adenoma was observed in a rat that died after 7 months on the excess-Cu-DMN treatment.

Other organs: Neoplasms in locations other than liver and kidneys included those of the lung, spleen, skin and -intestine. The neoplasms observed included adnexal gland adenocarcinomas, keratoacanthomas, splenic lymphoma, alveolar-cell adenomas and adenocarcinomas, adenocarcinoma arising from the epithelium of the intestinal mucosa, squamous cell carcinomas of the skin and lungs, fibrosarcoma of the dermis and a rhabdomyosarcoma. Incidences of these neoplasms were less in rats receiving excess Cu and a carcinogen (**Table A6.5(01) & A6.7(01)-6**).

187.2 Discussion

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

Body weight & mortality: The Cu-deficient diet (1 ppm) was adequate to sustain normal growth. However, the excess-Cu diet was toxic.

DMN was toxic at the level given, mean body weights being reduced and mortality increased compared with the Cu-deficient control group. The combination of carcinogen and excess Cu appeared not to be additive, although total mortality was slightly greater in the excess-Cu

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

group. The mortality rate after 3 months was less in both the DMN groups and it appeared that rats surviving the early toxic effects developed some tolerance.

AAF produced a greater reduction in weight than DMN. The administration of AAF and excess Cu had an additive toxic effect, as this diet markedly decreased body-weight gains, producing the lowest means in the experimental groups.

Mortality over the experimental period did not vary greatly between the Cu-deficient-AAF and the excess-Cu-AAF groups. This illustrates one of the differences in the response to the two carcinogens. Excess Cu in the diet markedly increased the mortality rate after DMN administration but appeared to have little effect when AAF was fed.

Organ weights: Enlargement of the liver was restricted, with few exceptions, to the AAF-fed rats. DMN administration slightly increased the mean liver weight expressed as a percentage of body weight. Livers from AAF-fed rats were generally enlarged, some greatly. The rats receiving AAF had the lowest body weights and little body fat. These factors, combined with the presence of many cysts and neoplasms, account for the high liver-weight values observed in the AAF-fed groups.

Cu determinations: The Cu content of livers from rats fed the Cu-deficient diets did not vary greatly. However, the hepatic Cu concentration of excess-Cu control rats was less than the mean concentrations for the two carcinogen-treated groups. Thus, the carcinogens appeared to increase the retention of Cu by the liver in animals receiving the excess Cu diet. The Cu levels of non-neoplastic and grossly neoplastic hepatic tissue from rats fed the Cu-deficient diet and treated with DMN or AAF were similar, but the Cu content of non-neoplastic hepatic tissue from rats fed the excess-Cu diet with AAF was greater than that of neoplastic tissue.

The Cu content of the renal tissues decreased in the following order: normal tissue in Cu-deficient control rats, non-neoplastic tissue in Cu-deficient-DMN rats, DMNinduced small neoplasms and DMN-induced large neoplasms. Part of the lower Cu content may be due to the tissue composition of the neoplasms; fibrosarcomas are composed of connective tissue known to have a low Cu content.

Macroscopic and microscopic investigations: The incidence of hepatic neoplasms in DMN-treated rats was similar for the Cu-deficient and excess-Cu groups. DMN-induced renal neoplasms were found to originate from the epithelium of the renal tubules and the connective tissue of the interstitium. Those originating in the interstitial cells were classified as fibrosarcomas on the basis of morphology and staining reactions. Seventeen rats killed for autopsy had one or more neoplasms, including 12 fibrosarcomas, seven adenomas and two adenocarcinomas.

Organs with neoplasms induced by AAF included the liver, spleen, lung, skin, muscle, pancreas and intestine, although neoplasms were uncommon in the last three organs. The numbers of hepatic neoplasms

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7 IUCLID 5.4/11 & 5.7/01	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate* **A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**
	in AAF-treated rats on the Cu-deficient and excess-Cu diets were similar and it appeared that the concentration of Cu had no effect upon the incidence of hepatic neoplasms. However, the latency period may have been slightly increased, as hepatocellular carcinomas and metastases occurred 1 month later in the excess-Cu group. The incidence of extrahepatic neoplasms in rats killed for autopsy was 40% in the Cu-deficient-AAF group, but only 17% of the rats fed the excess-Cu-AAF diet had neoplasms outside the liver. When the extrahepatic tumours from rats found dead after receiving the AAF treatment for at least 3 months are combined with those from rats killed for autopsy, the difference in incidence of neoplasms between Cu-deficient and excess-Cu groups was decreased (31% vs. 23%) but the data suggest that the Cu supplement acted to reduce the number of extrahepatic neoplasms.
187.3 Time to tumours	*For dermal route and skin tumours: give mean time until appearance of tumour or time until appearance of first tumour or other measure* Tumours were evident in rats treated with the carcinogens AAF and DMN from the time of the first interim sacrifice at 3 months.
187.4 Other	*Describe any other significant effects* None.
	188 APPLICANT'S SUMMARY AND CONCLUSION
188.1 Materials and methods	*Give concise description of method; give test guidelines no. and discuss relevant deviations from test guidelines* A study was carried out to determine whether a high level of Cu would have an inhibitory effect on the induction of neoplasia by acetylaminofluorene (AAF) or dimethylnitrosamine (DMN) and to determine whether the incidence of neoplasia would be increased, or whether neoplasms would appear earlier in rats fed a diet low in Cu. Six experimental groups of Sprague-Dawley rats were included in this study. Three groups were fed a basal diet containing 1 ppm Cu ("Cu-deficient diet") and a further 3 groups received the basal diet supplemented with $CuSO_4$ to give a Cu concentration of 800 ppm ("excess-Cu diet"). Within each of these two dietary regimens, one group received DMN in the drinking water and the other received AAF in the diet. Groups without these carcinogens served as controls. The initial number of animals used in each group was as follows: Cudeficient control, 50 rats; Cu-deficient-DMN, 74 rats; Cu-deficientAAF, 55 rats; excess-Cu-control, 58 rats; excess-Cu-DMN, 102 rats; excess-Cu-AAF, 65 rats. The numbers in each group varied because preliminary studies showed that higher DMN concentrations were toxic. DMN was added to the drinking water for 6 months at a concentration of 50 ppm for 4 days out of every 8. Similarly, AAF was added to the diets for 6 months at a concentration of 0.06% for 4 days out of every 8. After 90 days, 5 rats from each diet group were killed. Each 30 days thereafter, an additional 5 animals from each group were killed. Spleen,

Sections A6.5 & A6.7

Annex Points IIA6.5 & IIA6.7

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A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper

kidneys, lungs, heart, thyroid gland, adrenals, duodenum and pancreas were taken from each animal and fixed in 10% formalin. The liver was divided into 2 portions; one of which was retained for analysis of Cu content; the other was fixed in formalin. Liver and enlarged neoplastic kidneys were weighed prior to fixation. Fixed tissues were processed, sectioned and stained with H&E for histological examination.

Liver and kidney Cu concentrations were determined by atomic absorption spectrophotometry. The analyses were run in triplicate and precautions were taken to prevent Cu-contamination of the tissues.

188.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Growth response: Rats fed the Cu-deficient control diet consistently had the highest mean bodyweights. Mean weights of other groups decreased in the following sequence: Cu-deficient-DMN; excess-Cu control and excess-Cu DMN had similar mean weights; Cu-deficient AAF; excess-Cu-AAF. AAF was considered to be markedly toxic.

Mortality: After 3 months, mortality in the 6 groups was as follows: Cu-deficient control, 2%; Cu-deficient-DMN, 38%; Cu-deficient-AAF, 15%; excess-Cu control, 33%; excess-Cu-DMN, 69%; excess-Cu-AAF, 39%. At study termination, the lowest mortality (16%) was seen in Cu-deficient controls and the highest in the excess-Cu-DMN group (57%). Excess Cu, DMN and AAF were all toxic at the levels administered. Excess Cu in the diet markedly increased the mortality rate after DMN administration but appeared to have little effect when AAF was fed.

Organ weights: Liver weights expressed as percentage of body weight were similar for all groups except those receiving AAF, for which elevated values were obtained. Of these, Cu-deficient-AAF animals generally had higher liver weights than those in the excess-Cu-AAF group. Some of this increase was due to the presence of neoplasms.

Copper determinations:

Liver: The Cu content of non-neoplastic hepatic tissue from rats fed Cu-deficient diets did not vary greatly, although values for the group fed AAF were generally lower than for the other groups (mean Cu contents at study termination were 4.5, 3.9 and 2.8 ppm for the control, DMN and AAF groups, respectively). The Cu content of livers from rats fed excess-Cu diets with DMN and AAF was, however, greater than that found in the excess-Cu control rats (mean Cu contents at study termination were 244, 394 and 354 ppm for the control, DMN and AAF groups, respectively). The carcinogens therefore appeared to increase retention of Cu by the liver in animals receiving the excess-Cu diet.

The Cu content of neoplastic hepatic tissue from rats receiving Cudeficient carcinogenic diets was similar to that in grossly normal tissue. In rats fed the excess-Cu-AAF diet and that had grossly separable neoplasms, neoplastic tissue contained less Cu (347 and 163 ppm at 5 and 8 months, respectively) than non-neoplastic tissue (418 and 294 ppm, respectively) from the same animal.

Kidney: Gross renal neoplasms were observed only in the Cu-deficient-DMN rats and the concentrations of Cu were lower in these neoplasms

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate*
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than in non-neoplastic tissue. The Cu concentration of this latter tissue was somewhat lower than that in the kidneys of Cu-deficient control rats. The Cu concentration of large neoplasms was lower than that of small neoplasms. This was attributed in part to the composition of neoplasms containing connective tissue known to have low Cu content.

Macroscopic investigations:

Liver: Livers from control rats fed both Cu-deficient and excess-Cu diets were grossly normal.

The incidence of hepatic neoplasms in DMN-treated rats was similar for the Cu-deficient and excess-Cu groups. Livers of rats fed the Cu-deficient-DMN diet for 3 or 4 months varied in appearance from those that were grossly normal to those with severe macroscopic changes. Some were tan-coloured and slightly swollen. Features of livers from rats fed this diet for 5-8 months included: swelling, colour variation, presence of clear cysts, haematocysts and/or neoplasms. Livers from excess-Cu-DMN rats were either normal or slightly off-colour after 3 and 4 months. Few further changes were observed after 5 and 6 months, except for prominent capsular vessels. Cysts, swollen lobes and haematocysts occurred in livers of rats fed for 7 months. Livers from 4 rats killed after 8 months were more severely affected; haematocysts were observed in 2 livers and a neoplasm in one other.

Gross hepatic lesions were observed at monthly samplings in Cu-deficient-AAF rats. At 3 months, these included discoloration, enlargement and presence of focal pale areas. After 4 months, a few clear cysts were also present. Later, livers were pale, cystic and enlarged. Neoplasms of varying size were found in all lobes. At 3 months, the surface of the liver of one rat fed the excess-Cu-AAF diet was converted into a mass of nodules. This was also seen in one or more livers at the other autopsy periods, and was more marked on the visceral surface. Clear cysts were also present peripherally after 5 months. Increased hepatic size, cysts and small white foci appeared after 6 months. Neoplasms were larger after 7 months, and all livers at 8 months had clear cysts, neoplasms and capsular nodularity.

The numbers of hepatic neoplasms in AAF-treated rats on the Cu-deficient and excess-Cu diets were similar and it appeared that the concentration of Cu had no effect upon the incidence of hepatic neoplasms. However, the latency period may have been slightly increased, as hepatocellular carcinomas and metastases occurred 1 month later in the excess-Cu group.

Kidney: Grossly enlarged kidneys with neoplasms were seen after 5 months in Cu-deficient-DMN rats. The kidneys of 4/5 rats had neoplasms of various sizes. After 6 months, neoplasms were present in all 5 rats. Grossly apparent neoplasms were present in 3/5 rats examined after 7 months. Only one renal neoplasm was obvious at autopsy in 5 rats killed after 8 months. 3/13 rats on this treatment which died during the study had grossly apparent renal neoplasms.

Other organs: Abnormalities observed at autopsy in Cu-deficient-

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate*
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DMN rats included pale, expanding masses in the lungs of 2 rats. Grossly detectable neoplasms were observed in the lungs of excess-CuDMN rats after 7 and 8 months.

Neoplasms at locations other than the liver were most numerous in Cu-deficient-AAF rats. After 5 months, 3 rats had grossly obvious neoplasms in one or more of the following locations: ventral throat area, middle of side, groin area and base of ear. After 6 months, neoplasms were noted in the lungs of 2 rats and in the spleen of another. At month 7, neoplasms were present in the ventral thorax, spleen, abdomen, perianal region, base of ear, right rear leg and small intestine.

Fewer extrahepatic neoplasms were found in excess-Cu-AAF rats (17% compared with 40% in the excess-Cu-AAF). Those that occurred werelocated at the base of the ear, along the lateral abdomen and in the lungs. It was considered that the Cu supplement acted to reduce the number of extrahepatic neoplasms.

No gross abnormalities were observed in the urinary bladder of animals in any group.

Histopathology:

Liver: Commonly occurring non-neoplastic lesions in the livers of carcinogen-treated rats included biliary-ductule cell hyperplasia, proliferation of biliary ducts and the presence of haematocysts.

Transitional nodules were localized groups of hepatocytes showing only minimal deviation of nuclear morphology and no compression of the surrounding parenchyma. Hepatomas were larger foci of hepatocytes showing changes in nuclear morphology and causing compression of the surrounding parenchyma. Hepatocellular carcinomas were large, highly cellular neoplasms showing marked alterations in nuclear and cytoplasmic morphology, containing areas of necrosis and blood cysts and invading blood and lymph vessels. In addition to hepatomas and hepatocellular carcinomas, a fibrosarcoma and cholangiocarcinoma were observed in Cu-deficient-DMN rats. Hepatomas, hepatocellular carcinomas, cholangiomas and one cholangiocarcinoma were observed in livers of Cu-deficient-AAF rats. The Cu level of the diet appeared to have no effect on the incidence rate of hepatic neoplasms.

Kidney: Fibrosarcomas, adenomas and adenocarcinomas were seen in kidneys of Cu-deficient-DMN rats. One fibrosarcoma was found in a kidney from a rat fed the Cu-deficient control diet. No renal neoplasms were observed either grossly or microscopically in the rats from other groups killed for autopsy. One renal adenoma was observed in a rat that died after 7 months on the excess-Cu-DMN treatment.

Other organs: Neoplasms in locations other than liver and kidneys included those of the lung, spleen, skin and -intestine. The neoplasms observed included adnexal gland adenocarcinomas, keratoacanthomas, splenic lymphoma, alveolar-cell adenomas and adenocarcinomas, adenocarcinoma arising from the epithelium of the intestinal mucosa, squamous cell carcinomas of the skin and lungs, fibrosarcoma of the dermis and a rhabdomyosarcoma. The incidences of these neoplasms

Sections A6.5 & A6.7 Annex Points IIA6.5 & IIA6.7 IUCLID 5.4/11 & 5.7/01	Combined Chronic toxicity/Carcinogenicity *Specify section no., heading, route and species as appropriate* **A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**
	were less in rats receiving excess Cu and a carcinogen.
188.3 Conclusion	Microscopic examination of tissue samples confirmed the following: *Liver:* livers from excess-Cu control rats confirmed the occurrence of liver necrosis and transitional nodules in 3/32 and 1/32 animals, respectively. Neither of these lesions was found in the livers of animals fed a Cu-deficient diet. Exposure to DMN and AAF increased the incidence of liver necrosis and transitional nodules, and each induced a similar incidence of liver tumours in rats fed both the Cu-deficient and excess-Cu diets. It was concluded that the Cu level of the diet had no effect on the incidence of hepatic neoplasms. *Kidney:* In the DMN group, 17/30 rats on the Cu-deficient diet had kidney tumours compared with 0/29 given excess Cu. There were no kidney tumours in the AAF-treated groups. *Other organs:* The incidence of AAF-induced extra-hepatic tumours was apparently reduced by the excess-Cu diet (5/30, compared with 11/27 in the Cu-deficient group).
188.3.2 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4* 2
188.3.3 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP. There were also a number of deficiencies in the methodology used, when compared with the requirements of currently accepted guidelines for the conduct of carcinogenicity studies (e.g. OECD 451), including the following: • The test substance was not characterised in detail; • Only males were used, rather than both sexes; • Only two $CuSO_4$ test concentrations were used; • No blood sampling was reported for adversely affected animals; • The range of tissues examined macroscopically was limited; • The range of organ weights reported was limited; • The range of organs examined microscopically was limited; • The duration of the study was 9 months (24 months recommended). However, these deficiencies do not necessarily compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. It has also been referred to in reviews of the carcinogenicity of copper. Overall, this is an adequately-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of

Sections A6.5 & A6.7	Combined Chronic toxicity/Carcinogenicity
Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

copper carcinogenicity. A reliability indicator of 2 has been assigned on this basis.

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date [redacted]

Guidelines and quality [redacted]

Materials and Methods [redacted]

[redacted]

Results and discussion [redacted]

Conclusion [redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks [redacted]

Sections A6.5 & A6.7	**Combined Chronic toxicity/Carcinogenicity**
Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate*
IUCLID 5.4/11 & 5.7/01	**A6.5(01) & A6.7(01), Combined Chronic toxicity/Carcinogenicity of copper**

Table 3. Table A6.5(01) & A6.7(01)-3. Copper content of non-neoplastic liver tissue from rats fed copper-deficient and excess-copper diets with DMN or AAF treatment for 3-8 months.

Duration of treatment (months)	Copper content (ppm)*						
	Diet...	Copper-deficient			Excess-copper		
	Other treatment...	Control	DMN	AAF	Control	DMN	AAF
3		4.6	3.2	3.1	314	380	412
4		4.2	3.5	2.9	234	460	372
5		4.3	4.5	2.0	236	432	418
6		4.1	4.4	2.2	200	270	312
7		5.0	4.2	2.6	236	383	314
8		4.8	3.8	3.9	---	438	294
	Mean	4.5	3.9	2.8	244	394	354

DIVIN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.

** One pooled sample was analysed for each group at each time.*

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

Table A6.5(01) & A6.7(01)-1. Mortality in groups of rats fed copper-deficient or excess-copper diets with DMN or AAF treatment for 3-9 months.

Mortality (%)							
	Diet	**Copper-deficient**			**Excess-copper**		
Duration of treatment (months)	**OTHER TREATMENT...**	**Control**	**DMN**	**AAF**	**Control**	**DMN**	**AAF**
	No of rats*.....	50	74	55	58	102	65
3		2	38	15	33	69	39
4		2	41	15	40	71	39
5		8	45	20	43	71	40
6		10	49	35	43	71	40
7		16	53	48	45	72	43
8		16	53	51	45	72	51
9		16	57	--	45	--	54
	Change in % mortality over 6-month period	14	19	36	12	3	15

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water. AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet. * Total no. of rats started on diet.

Table A6.5(01) & A6.7(01)-2. Liver weight of rats fed copper-deficient or excess-copper diet with DMN or AAF treatment and killed for autopsy after treatment for 3-9 months.

Mean liver weight (% of body weight)*			
Experimental group	**Month 3**	**5**	**7**
Copper-deficient diet:			
Control	3.2 (3.0-3.6)	2.9 (2.7-3.0)	3.1 (2.8-3.4)
+ DMN	3.6 (3.2-4.2)	3.6 (1.3-3.8)	3.2 (2.7-3.1)
+ AAF	6.3 (4.8-8.5)	16.2 (13.4-18.8)**	12.8 (9.4-16.1)**
Excess-copper diet:			
Control	4.0 (3.5-4.9)	3.3 (2.9-3.8)	3.3 (3.0-3.5)
+ DMN	4.1 (2.9-4.7)	3.7 (2.7-5.1)	3.7 (3.1-4.3
+ AAF	5.9 (5.2-7.0)	6.4 (5.5-7.2)**	9.3 (6.4-12.1)**

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water. AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet. * With ranges of value in parentheses.
** Large liver weights were due to the presence of hepatomas or hepatocellular carcinomas.

Table 3. Copper content of non-neoplastic liver tissue from rats fed copper-deficient and excess-copper diets with DMN or AAF treatment for 3-8 months.

Duration of treatment (months)	Diet...	Copper content (ppm)*					
		Copper-deficient			Excess-copper		
	Other treatment...	Control	DMN	AAF	Control	DMN	AAF
3		4.6	3.2	3.1	314	380	412
4		4.2	3.5	2.9	234	460	372
5		4.3	4.5	2.0	236	432	418
6		4.1	4.4	2.2	200	270	312
7		5.0	4.2	2.6	236	383	314
8		4.8	3.8	3.9	---	438	294
Mean		4.5	3.9	2.8	244	394	354

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water. AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet. * One pooled sample was analysed for each group at each time.

Table A6.5(01) & A6.7(01)-4. Copper content of selected hepatic neoplasms compared with that of non-neoplastic hepatic tissue from rats fed copper-deficient and excess-copper diets with DMN and AAF treatment

Experimental group	Duration of treatment (months)	Copper content (ppm) of	
		Non-neoplastic tissue	Neoplastic tissue
Copper-deficient diet: + DMN	4	3.5	4.2
	6	4.4	4.4
+ AAF	5	2.0	1.9
	6	2.2	2.6
	7	2.6	2.6
	8	3.9	2.7
Excess-copper diet: + AAF	5	418	347
	8	294	163

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water. AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet.

Table A6.5(01) & A6.7(01)-5. Copper content of selected renal samples from rats fed a copper-deficient diet (1 ppm) with or without DMN treatment.

Experimental group	Tissue	Copper content (ppm) of renal tissues at month			
		5	6	7	8
Copper-deficient diet:					
Control	Normal	8.1	7.6	9.3	9.4
+ DMN	Non-neoplastic	7.3	7.0	7.2	7.1
	Small neoplasms	5.5	2.6	2.7	---
	Large (>22g) neoplasms	2.4	2.0	1.8	2.0

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water

TableA6.5(01) & A6.7(01)-6. Incidence of hepatic lesions and neoplasms in rats fed copper-deficient and excess-copper diets with DMN or AAF treatment

and killed at monthly intervals for autopsy.

		Incidence (%)* of						
Experimental group	**Total no. of rats killed**	**Liver necrosis**	**Transitional nodules**	**Hepatomas**	**Hepatocellular Carcinomas**	**Metastases**	**Kidney neoplasms**	**Other neoplasms**
Copper deficient:								
Control	42	0.0	0.0	0.0	0.0	0.0	2.4	0.0
+ DMN	30	30.8	76.7	23.3	10.0	0.0	56.7	30.0
+ AAF	27	22.2	100.0	92.6	40.7	3.7	0.0	40.0
Excess-copper diet:								
Control	32	9.4	3.1	0.0	0.0	0.0	0.0	0.0
+ DMN	29	55.2	82.8	27.6	13.8	0.0	0.0	24.1
+ AAF	30	30.0	100.0	90.0	30.0	10.0	0.0	16.7

DMN – 50 ppm dimethylnitrosamine in drinking-water for 4-day periods alternating with distilled water.
AAF – 0.06% acetylaminofluorene in the diet for 4-day periods alternating with control diet. *
Percentage of rats affected

Figure A6.5(01) & A6.7(01)-1

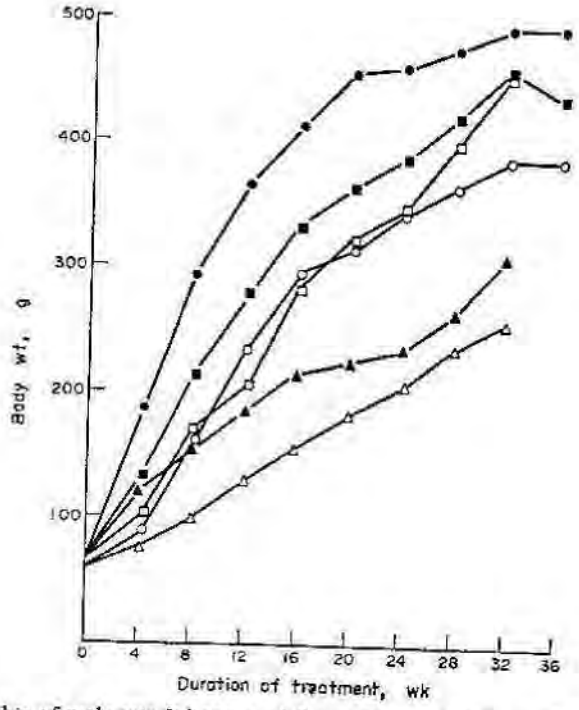

FIG. 1. Mean body weights of male rats fed copper-deficient (●——●; ■——■; ▲——▲) and excess-copper (○——○; □——□; △——△) diets and treated with DMN (■; □), AAF (▲; △) or no carcinogen (controls: ●; ○).

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

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

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

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

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

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

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

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

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

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

	first application of DMBA. Feeding of $CuSO_4$ continued throughout the entire experimental period. These animals had access to the $CuSO_4$ solution *ad libitum*.		
191.3.5 Postexposure period	None.		
	Oral		
191.3.6 Type	$CuSO_4$ was administered in drinking water.		
191.3.7 Concentration	$CuSO_4$ was dissolved in water to a concentration of 198 mg/l (approximately 50 mg Cu^{2+}/l). Treatment water was supplied *ad libitum*.		
191.3.8 Vehicle	Tap water.		
191.3.9 Concentration in vehicle	$CuSO_4$ was dissolved in water to a concentration of 198 mg/l (approximately 50 mg Cu^{2+}/l).		
191.3.10 Total volume applied	Not stated.		
191.3.11 Controls	Vehicle (water).		
	Dermal		
191.3.12 Area covered	Not stated.		
191.3.13 Occlusion	Not stated.		
191.3.14 Vehicle	Olive oil.		
191.3.15 Concentration in vehicle	5 mg/ml (0.5%).		
191.3.16 Total volume applied	0.5 ml.		
191.3.17 Duration of exposure	Not stated.		
191.3.18 Removal of test substance	Not stated. *give solvent, detergent*		
191.3.19 Controls	Untreated.		
	Intraperitoneal/Intravenous/Intratracheal instillation		
191.3.20 Vehicle	For parenteral administration, a fatty emulsion of DMBA was produced in 1.2% w/w lecithin, 0.3% w/v poloxalkol, 15% cottonseed oil and water.		
191.3.21 Concentration in vehicle	0.5% w/v DMBA.	X	X
191.3.22 Total volume applied	0.1 or 0.15 ml.		
191.3.23 Controls	Untreated.		

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

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

191.4 Examinations			
191.4.1 Body weight	No.		
191.4.2 Food consumption	No.		
191.4.3 Water consumption	No.		
191.4.4 Clinical signs	No.		
191.4.5 Macroscopic investigations	Not reported.		
191.4.6 Ophthalmoscopic examination	No.		
191.4.7 Haematology	No.		
191.4.8 Clinical Chemistry	No.		
191.4.9 Urinalysis	No.		
191.4.10 Pathology	No.		
191.4.10.1 Organ Weights	No.		
191.4.11 Histopathology	Yes.		
	from:	all dose groups	
	from:	all surviving animals and all animals that died during the study.	
	Organs:	Thymus, liver, kidneys, spleen, ovaries.	X
Other examinations	*E.g. enzyme induction, cell proliferation, reversibility of effects*		
	Vaginal smears for investigation of effects on the incidence of oestrus.		
191.5 Statistics	Chi-square test and Wilcoxon ranking test were applied as appropriate.		
191.6 Further remarks	Pseudopregnant females refers to virgin mice housed together with vasectomised males. Vasectomy was performed under pentobarbital anaesthesia (70 mg/kg). Each group consisted of 3-4 virgins and 1-2 vasectomised males per cage.		
	192 RESULTS AND DISCUSSION		
	Describe findings. If appropriate, include table. Sample tables are given below.		
192.1 Results	*No effects / describe significant effects referring to data in results table*		
	Non-entry field.		
192.1.1 Experiments A & B	The results of Experiments A and B are shown in **Table A6.5(02) & A6.7(02)-1**.		
	Histopathology:		
	A single application of 0.75 mg DMBA caused a high incidence of ovarian tumours in C75BL/6J virgin mice. These tumours varied in size from 8 – 15 mm in diameter, and were classified histologically as granulosa cell tumours. Mice receiving the combination of DMBA and		

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

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

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

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

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

effect on the initiation step of DMBA oncogenesis. Instead, it appeared that the greater availability of copper in the body delayed the full expression of the carcinogenic lesions induced by DMBA.

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

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

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

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

192.3 Time to tumours

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

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

192.4 Other

Describe any other significant effects

None.

193 APPLICANT'S SUMMARY AND CONCLUSION

193.1 Materials and methods

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

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

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

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

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

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

74 weeks after DMBA treatment.

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

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

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

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

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

Annex Points IIA6.5 & IIA6.7	*Specify section no., heading, route and species as appropriate* **A6.5(02) & A6.7(02), Combined Chronic toxicity/Carcinogenicity of copper**
IUCLID: 5.4/12 & 5.7/02	
193.2 Results and discussion	*Summarize relevant results; discuss dose-response relationship.*

Experiments A and B: The incidences of ovarian tumours in Experiment A after 76 weeks were 0/5, 4/5, and 0/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu-treated mice, respectively. The incidences of these tumours in Experiment B after 46 weeks were 0/10, 0/12, 11/11 and 6/11 in the untreated controls, copper-treated mice, DMBA-treated mice and DMBA/copper treated mice respectively. The results of these two experiments suggested that $CuSO_4$ may inhibit DMBA-induced tumour development.

The incidences of lymphomas in Experiment A were 0/5, 1/5, and 5/5 in the untreated controls, DMBA-treated mice and DMBA plus Cu treated mice respectively. Although these results implied that incidence of lymphomas were greater in DMBA plus $CuSO_4$-treated mice than in those receiving DMBA only, this finding could not be repeated in Experiment B (incidences of lymphoma 1/10, 2/12, 3/11 and 3/11 in the untreated controls, Cu-treated mice, DMBA-treated mice and DMBA plus Cu-treated mice, respectively). It was therefore concluded that $CuSO_4$ had no effect on the induction of lymphomas by DMBA.

Experiment C: Tumour incidence in the 12 mice given $CuSO_4$ alone (1 breast tumour, 2 lymphomas and no lung or ovarian tumours) was similar to that in the 19 untreated controls (2 lymphomas, no breast, lung or ovarian tumours). $CuSO_4$ had no effect on the incidence of DMBA-induced lung adenomas (incidence 4/9 in DMBA plus Cutreated mice and 4/10 in mice treated with DMBA only), although it appeared to prolong the survival of DMBA-treated mice (mean survival 28 weeks compared with 19 weeks in mice treated with DMBA only), and to slightly reduce the total number of tumours seen, as compared with mice given DMBA only.

Experiment D: No information was given on the tumour incidence in mice given $CuSO_4$ alone. However, mice given DMBA plus $CuSO_4$ had a greater number of mammary tumours (9 tumours amongst an original group of 18) than those given DMBA alone (5 tumours amongst an original group of 19). This increase was attributed to the greater longevity of Cu-treated mice.

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

193.3 Conclusion

DMBA was injected or administered by skin paintings to C57BL/6J and to strain A female mice kept on a diet supplemented with $CuSO_4$. It was found that $CuSO_4$ had no effect on the incidence of DMBAinduced adenomas of the lung, lymphomas and breast tumours. $CuSO_4$ did not prevent the induction of pre-cancerous lesions in the ovary, but may have delayed the development of granulosa cell tumours.

193.3.1 Reliability

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