Food Consumption and Food Efficiency - P1 males

There were no test substance-related effects on food consumption or food efficiency in P1 males during premating at any dose level. Occasional findings of statistically significant increases in food consumption were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship. The statistically significant decreases in food efficiency in P1 males on days 0-7 and for the entire premating period (days 0-70) at 1500 ppm were considered spurious and due to a slightly higher food consumption on days 0-7 in this group.

Sperm Parameters - P1 males

There were no test substance-related effects on sperm motility, morphology, epididymal sperm or testicular spermatid numbers at any dose level.

Reproductive Indices and Precoital Interval

The following parameters (where relevant) were not affected by test substance administration: precoital interval length, mating, fertility, gestation length, number of implantation sites, or implantation efficiency at any dose level.

Cause of Death P1 adult rats

There were no test substance-related deaths in the study. Of the 120 P1 adult males, one animal was sacrificed in extremis on day 14 because of a fractured nose.

Organ Weight Data - P1 adult rats

There were no treatment related organ weight changes. Gross Findings - P1 Adult Rats

At the terminal sacrifice, there were no test substance-related gross observations. All gross observations were consistent with normal background lesions in rats of this age and stock.

Microscopic Findings - P1 Adult Rats

In P1 adult rats, there were no test substance-related microscopic findings in the liver, brain or reproductive organs. All microscopic findings were considered to be incidental lesions commonly found in rats of this age and stock.

P1 Adult Reproductive Failures

The failure of 18 P1 adult pairs to produce litters was not related to test substance exposure. Gross and microscopic evaluation revealed a morphological explanation of their infertility in 3 P1 individuals. (absence of recent corpora lutea). The cause of the reproductive failure in the remaining pairs was not determined.

239.1.2 Parent females

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

Intake of Test Substance - P1 females (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

1.92, 9.6, 19.1 and 29.5 mg/kg/day for P1 females during premating;

1.67, 8.6, 17.0 and 26.2 mg/kg/day for P1 females during gestation;

3.39, 17.7, 33.8 and 55.7 mg/kg/day for P1 females during the first 2 weeks of lactation.

Clinical Observations and Mortality - P1 females

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed during premating, gestation, or lactation at any dose level.

Mean Body Weights and Body Weight Gains - P1 females

There were no test substance-related effects on body weight or body weight gain during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant decreases in body weight gain were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship

Food Consumption and Food Efficiency - P1 females

There were no test substance-related effects on food consumption or food efficiency in P1 females during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant increases in food consumption or decreases in food efficiency were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Estrous Cycle Parameters - P1 females

There were no test substance-related effects on the mean percent days in estrus, diestrus, or proestrus, or mean cycle length at any dose level.

In P1 females, the mean percent days in estrus at 1000 and 1500 ppm were slightly higher than the control value (47 and 40%, respectively, vs. 30% for the control group). Since the increase was greater at 1000 than 1500 ppm, was not associated with any change in mean estrous cycle length or adverse reproductive outcome, and was not observed in F1 females, it was not considered test substance-related.

The distribution of estrous cycle stages at sacrifice was similar across groups.

Reproductive Indices and Precoital Interval

See 4.1.1.

Cause of Death - P1 Adult Rats

There were no deaths amongst P1 adult females.

Organ Weight Data P1 adult rats (see Table A6.8.2-2)

In P1 female adult rats, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group. Mean absolute and relative (organ wt./body wt.) values were both decreased 9%, as compared to control values. Only the decrease in mean

relative spleen weight was statistically significant. No differences were observed in P1 male spleen weight values.

All other individual and mean organ weight differences in P1 rats were considered to be spurious and unrelated to test substance administration.

Gross Findings -

P1 Adult Rats

See 4.1.2.

	Microscopic Findings - P1 Adult Rats	
	See 4.1.2.	X
	P1 Adult Reproductive Failures	
	See 4.1.2.	X
239.1.3 F1 males	*No effects / describe significant effects referring to data in results table*	
	Intake of Test Substance - F1 males (see 4.2)	
	The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:	
	2.25, 11.5, 23.5 and 36.1 mg/kg/day for F1 males during	
	premating. Clinical Observations and Mortality - F1 males	
	There was no test substance-related mortality during the study. No test substance-related clinical observations were observed at any dose level.	
	Mean Body Weights and Body Weight Gains - F1 males	
	No test substance-related effects on body weight or body weight gain were observed at any dose level.	
	Food Consumption and Food Efficiency - F1 males	
	There were no test substance-related effects on food consumption or food efficiency in F1 males during premating. Occasional findings of statistically significant increases in food consumption and decreases in food efficiency (due to slightly higher food consumption) were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.	
	Sperm Parameters - F1 males	
	There were no test substance-related effects on sperm motility, morphology, epididymal sperm or testicular spermatid numbers at any dose level.	
	Reproductive Indices and Precoital Interval	
	The following parameters (where relevant) were not affected by test substance administration: precoital interval length, mating, fertility, gestation length, number of implantation sites, or implantation efficiency at any dose level.	
	F1 Offspring Data	
	Litter Size, Sex Ratio and Pup Survival	
	There were no test substance-related effects on the number of pups born, born alive, alive on day 4, 7, 14, or 21, nor were there any effects on sex ratio, or survival indices during the lactation period at any concentration level.	
	Clinical Observations in pups	
	There were no test substance-related clinical observations in F1 litters at any concentration level. Clinical observations observed in pups in this study occurred at low incidences and/or were not dose-related.	
	Pup Weights	
	No test substance-related effects were observed on F1 pup weights a	

Annex Point IIA6.8.2	*Two Generation Reproduction Study in Rats by Oral Administration*

any dose level. The increase in pup weight in F1 litters at 100 ppm on lactation day 7 was considered spurious since it was not dose-related.

Developmental Landmarks

There were no test substance-related effects on the age at preputial separation in F1 males at any dose level.

Cause of Death - F1 Adult Rats

There were no deaths amongst F1 adult males.

Organ Weight Data

F1 Adult Rats

In F1 male (and female) adult rats, there were no test substance-related organ weight effects. All individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

F_1 Weanlings (see Table A6.8.2-3)

In F1 male (and female) weanlings, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group, that was not statistically significant. Mean absolute spleen weights were decreased 9% in both sexes, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were decreased 10% and 11% in male and female F1 weanlings, respectively, as compared to controls.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross Findings

F1 Adult Rats

At the terminal sacrifice, there were no test substance-related gross observations. All gross observations were consistent with normal background lesions in rats of this age and stock.

F1 Weanlings

There were no test substance-related gross observations in F1 weanlings. Gross observations occurred at low incidences, were randomly distributed across control and treatment groups, and/or were lesions common to rats of this stock and age.

F1 Pups

There were no test substance-related gross observations in F1 pups. Observations in pups of lungs not expanded and no milk spot in the stomach are nonspecific lesions that are commonly seen in all pups that are born dead, and thus are

not considered to be test substance related.

Microscopic Findings

F1 Adult Rats

In F1 adult rats, there were no test substance-related microscopic findings in the liver, brain or reproductive organs. All microscopic findings were considered to be incidental lesions commonly found in rats of this age and stock.

F1 Weanlings

There were no test substance-related microscopic findings in the liver and brain of the F1 weanlings. The few lesions present (amongst F1 and F2 weanlings) are common spontaneous lesions in this age and stock of rat.

F1 Adult Reproductive Failures

The failure of 9 F1 adult pairs to produce litters was not related to test substance exposure. The cause of reproductive failure in one F1 female was dystocia. The cause of the reproductive failure in the remaining pairs was not determined.

239.1.4 F1 females

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

Intake of Test Substance - F1 females (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

2.65, 13.3, 26.7 and 43.8 mg/kg/day for F1 females during premating;

1.69, 8.5, 17.1 and 26.5 mg/kg/day for F1 females during gestation;

3.27, 17.6, 35.2 and 55.4 mg/kg/day for F1 females during the first 2 weeks of lactation.

Clinical Observations and Mortality - F1 females

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed during premating, gestation, or lactation at any dose level.

Mean Body Weights and Body Weight Gains - F1 females

There were no test substance-related effects on body weight gain during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant increases in body weight or body weight gain were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Food Consumption and Food Efficiency - F1 females

There were no test substance-related effects on food consumption during premating, gestation or lactation at any dose level. Occasional findings of statistically significant increases in food consumption and decreases in food efficiency (due to slightly higher food consumption) were considered spurious due to their small magnitude, sporadic nature

and/or lack of dose-response relationship.

Estrous Cycle Parameters - F1 females

There were no test substance-related effects on the mean percent days in estrus, diestrus, or proestrus, or mean cycle length any dose level. The distribution of estrous cycle stages at sacrifice was similar across groups.

Reproductive Indices and Precoital Interval

See 4.1.3.

F1 Offspring Data

See 4.1.3.

Developmental Landmarks

There were no test substance-related effects on the age at vaginal opening in F1 females at any dose level. In F1 females, the mean age at vaginal opening at 1500 ppm was significantly increased (33.6 vs. 32.1 days for the control group) but the delay was small (1.5 days) and was within the laboratory's historical control range (see below). Therefore, the apparent delay in vaginal opening was not considered test substance-related.

Historical Control Data for Haskell Laboratory 1999-2003:

Study Start Date	Mean Day of Vaginal Opening
24-Sep-03	32.3
26-Sep-02	31.3
24-Apr-02	33.9
15-Oct-02	31.3
15-Mar-02	31.7
20-Feb-02	33.0
15-Mar-01	31.4
12-Dec-00	32.1
18-Sep-00	32.3
29-Jul-99	32.8
17-Jun-99	32.1
8-Apr-99	33.1
15-Mar-99	32.5
Mean	32.3
Standard	0.8
Minimum	31.3
Maximum	33.9

Ovarian Follicle Counts - F1 females

There was no significant difference in the total number of primordial and pre-antral follicles between the control and 1500 ppm F1 adult females.

Cause of Death - F1 females

There were no test substance-related deaths in the study. Of the 120 F1 females the following premature deaths

occurred:

- one animal from the 500 ppm group was sacrificed in extremis on day 109 due to dystocia,
- one animal from the 500 ppm group was found dead on day 17 due to pyelonephritis,
- one animal from the 1000 ppm group was sacrificed in extremis on day 119 for morbidity of undetermined cause.

Organ Weight Data

F1 Adult Rats

In F1 female (and male) adult rats, there were no test substance-related organ weight effects. All individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

F1 Weanlings (see Table A6.8.2-3)

In F1 female (and male) weanlings, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group, that was not statistically significant. Mean absolute spleen weights were decreased 9% in both sexes, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were decreased 10% and 11% in male and female F1 weanlings, respectively, as compared to controls.
All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

X
X
X

Gross Findings

X

F1 Adult Rats

See 4.1.4.

X

F1 Weanlings

See 4.1.4.

X

F1 Pups

See 4.1.4.

Microscopic Findings

F1 Adult Rats See 4.1.4.

F1 Weanlings

See 4.1.4.

F1 Adult Reproductive Failures

See 4.1.4.

239.1.5 F2 males

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

F2 Offspring Data

Litter Size, Sex Ratio and Pup Survival

There were no test substance-related effects on the number of pups born, born alive, alive on day 4, 7, 14, or 21, nor were there any effects on sex ratio, or survival indices during the lactation period at any concentration level.

Clinical Observations in pups

There were no test substance-related clinical observations in F1 litters at any concentration level. Clinical observations observed in pups in this study occurred at low incidences and/or were not dose-related. X

Pup Weights

No test substance-related effects were observed on F2 pup weights at any dose level.

Organ Weight Data - F2 Weanlings (see Table A6.8.2-4)

The F2 weanlings had a decrease in mean spleen weight parameters, at the high dose (1500 ppm), that was similar to that observed in the F_1 weanlings. Mean absolute spleen weights were decreased 10% and 15% in males and females, respectively, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were also decreased 10% and 15% in males and females, respectively, as compared to controls. Except for the male mean absolute spleen weight decrease (10%), these differences were statistically significant.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross Findings

F2 Weanlings

There were no test substance-related gross observations in F2 weanlings. Gross observations occurred at low incidences, were randomly distributed across control and treatment groups, and/or were lesions common to rats of this stock and age.

F2 Pups

There were no test substance-related gross observations in F2 pups. Observations in pups of lungs not expanded and no milk spot in the stomach are nonspecific lesions that are commonly seen in all pups that are born dead, and thus are not considered to be test substance related.

Microscopic Findings - F2 Weanlings

There were no test substance-related microscopic findings in the liver and brain of the 21 weanlings. The few lesions present (amongst F1 and F2 weanlings) are common spontaneous lesions in this age and stock of rat. X

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

239.1.6 F2 females

F1 Offspring Data

See 4.1.5.

Organ Weight Data – F2 Weanlings (see Table A6.8.2-4)

The F2 weanlings had a decrease in mean spleen weight parameters, at the high dose (1500 ppm), that was similar to that observed in the F1 weanlings. Mean absolute spleen weights were decreased 10% and 15% in males and females, respectively, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were also decreased 10% and 15% in males and females, respectively, as compared to controls. Except for the male mean absolute spleen weight decrease (10%), these differences were statistically significant.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross Findings

F2 Weanlings

See 4.1.5.

F2 Pups

See 4.1.5.

Microscopic Findings – F2 Weanlings

See 4.1.5.

239.2 Other

Intake of Test Substance

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

- 1.53, 7.7, 15.2 and 23.6 mg/kg/day for P1 males during premating;
- 1.92, 9.6, 19.1 and 29.5 mg/kg/day for P1 females during premating;
- 1.67, 8.6, 17.0 and 26.2 mg/kg/day for P1 females during gestation;
- 3.39, 17.7, 33.8 and 55.7 mg/kg/day for P1 females during the first 2 weeks of lactation;
- 2.25, 11.5, 23.5 and 36.1 mg/kg/day for F1 males during premating;
- 2.65, 13.3, 26.7 and 43.8 mg/kg/day for F1 females during premating;
- 1.69, 8.5, 17.1 and 26.5 mg/kg/day for F1 females during gestation; and
- 3.27, 17.6, 35.2 and 55.4 mg/kg/day for F1 females during the first 2 weeks of lactation

Tissue Metal Concentrations

P1 Male Rats

No test substance-related changes in the concentration of copper, iron, manganese or zinc were observed in the liver or brain at any dose level. Plasma concentration data was not available for P1 males; however, this had no impact on the study since plasma data was available for P1 females, F1 males and females, and F2 male and female weanlings. The statistically significant decrease in liver iron concentration at 1500 ppm was considered spurious since there was high interindividual animal variability across groups for this parameter and because the change did not correlate, as in P1 females, with increased liver copper

concentration.

P1 Female Rats

A test substance-related increase in the concentration of copper and a decrease in the concentration of iron were observed in the liver at 1500 ppm. No change in the concentration of copper or iron was observed in the plasma or brain at any dose level.

No test substance-related changes in the concentration of manganese or zinc were observed in the liver, plasma or brain.

F1 Male Rats

The concentration of copper was increased in the liver at 1000 and 1500 ppm. Although of small magnitude compared to the increase observed in P1 and F1 females, the increase was considered possibly test substance-related since a few animals in these groups had concentrations that were 2-3 fold higher than the control group mean. However, this is an expected physiological response of homeostatic control and the absence of liver pathology (histological data) confirms that this is not an adverse effect. No test substance-related change in the concentration of copper was observed in the plasma or brain at any dose level. The statistically significant increase in the concentration of copper in the plasma at 1500 ppm was considered spurious due to the small magnitude of the change and considering the variability of the plasma values across groups.

No test substance-related changes in the concentration of iron, manganese or zinc were observed in the liver, plasma or brain. The statistically significant increases in liver zinc concentration at 500 ppm and in copper concentration in the brain at 1000 ppm were considered spurious since they were not dose-related.

F1 Female Rats

A test substance-related increase in the concentration of copper was observed in the liver and brain at 1500 ppm. No change in the concentration of copper was observed in the plasma at any dose level.

No test substance-related changes in the concentration of iron, manganese or zinc were observed in the liver, plasma or brain. The statistically significant increase in manganese concentration in the brain at 1500 ppm was considered spurious due to the small magnitude of the change and considering the inter-animal variability of across groups. The statistically significant decrease in plasma zinc concentration at 1000 ppm was considered spurious since it was not dose-related.

F1 and F2 Weanlings

A test substance-related increase in the concentration of copper was observed in the liver of F1 and F2 male and female weanlings at 1000 and 1500 ppm. The concentration of copper was slightly increased in the brain of F1 and F2 male (but not female) weanlings at 1500 ppm, and was considered possibly test substance-related. No change in the concentration of copper was observed in the plasma of F2 weanling at any dose level. Plasma concentration data was not available for the F1 weanlings; however, this had no impact on the study since plasma data was available for P1 females, F1 males and females, and F2 male and female weanlings.

The concentration of iron in the plasma was decreased in F2 male and

female weanlings at 1500 ppm and was considered possibly test substance-related.

The statistically significant increase in manganese concentration in the brain of F2 male and female weanlings at 1500 ppm was considered spurious due to the small magnitude of the change and considering the inter-animal variability across groups. The statistically significant increase in zinc concentration in the liver in F1 male and female weanlings at 1000 and 1500 ppm was considered spurious since it was small and the magnitude of the change was independent of dose. The statistically significant increase in zinc concentration in the brain of F2 male weanlings at 1500 ppm was considered spurious since it was small and was not observed in F2 females or F1 males or females at this dose level. The statistically significant decrease in zinc concentration in the liver of F2 female weanlings at 100 ppm was considered spurious since it was not dose-related.

240 APPLICANT'S SUMMARY AND CONCLUSION

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

A two-generation reproduction study, which involved the production of one set of litters in each generation, was conducted with copper sulphate pentahydrate. Throughout the study, Crl:CD®(SD)IGS BR rats (30/sex/concentration) were fed diets containing 0, 100, 500, 1000 or 1500 ppm copper sulfate. Following at least 70 days of diet administration (premating), the P1 and F1 generation males and females were co-housed within their respective treatment groups to produce F1 and F2 litters, respectively. Dams were allowed to deliver and rear their offspring until weaning (postpartum day 21). At weaning, 30 F1 rats/sex/group were randomly selected to comprise the F1 generation and were given the same dietary concentration level as their respective P1 generation sires and dams. F1 and F2 litters were culled to 4 pups/sex/litter (litter size permitting) on postnatal day 4; all remaining pups were discarded without further evaluation. Brain and liver samples were collected from culled pups on postnatal day 4 (12 per group, randomly selected) for possible analysis of copper, zinc, manganese and iron concentrations; analysis was not performed since it was not considered necessary to meet the objectives of the study.

Clinical observations, body weight, and food consumption were determined weekly throughout the study. Litter examinations (number of live and dead, individual pup weights, clinical observations) were determined at birth, on day 4, and weekly during the lactation period. Estrous cycle parameters (percent days in diestrus, proestrus, and estrus) and estrous cycle length were evaluated for 3 weeks prior to cohabitation in P1 and F1 rats. The age at either vaginal opening or preputial separation was recorded for the F1 generation. Sperm motility, morphology, and concentration in the cauda epididymis, and spermatid concentration in the testis were determined for P1 and F1 rats.

At weaning, a gross postmortem examination was performed on one weanling/sex/litter from F1 and F2 litters and gross lesions were retained; the liver, brain, spleen, and thymus were weighed and gross lesions and target organs (brain, liver) retained. After litter production, all P1 and F1 rats were given a gross pathological examination and the testes, epididymides, right cauda epididymis, seminal vesicles (with

coagulating glands and their fluids), prostate, ovaries, and uterus (with oviducts and cervix), thyroid gland, brain, liver, spleen, adrenal glands, pituitary, and kidneys were weighed. The right testis and epididymis, prostate, seminal vesicles (with coagulating glands), pituitary, ovaries, uterus (with oviducts and cervix), vagina, brain, liver and gross lesions were placed in fixative for all P1 and F1 adults. These tissues as well as target organs and gross lesions from F1 weanlings in the control and 1500 ppm groups were examined microscopically; tissues in the low and intermediate groups were subsequently evaluated as needed to determine a no-adverse-effect level. Reproductive organs from the low and intermediate group P1 and F1 rats with suspected reduced fertility were examined microscopically. Ovarian follicle numbers were evaluated in control and 1500 ppm F1 rats (10/sex/group).

Blood and selected tissues (brain, liver, kidney, pancreas, femur, intestine and heart) were collected from P1 and F1 adults and F1 and F2 weanlings (10/sex/group); plasma and tissues were stored frozen for possible analysis of copper, zinc, manganese and iron concentrations. In addition, selected tissues (kidney, pancreas, femur, intestine and heart) from the same animals were placed in fixative for possible microscopic examination; evaluation was not performed since it was not considered necessary to meet the objectives of the study. The concentration of copper, zinc, manganese and iron was determined in all plasma, brain and liver samples; analysis of other tissues (kidney, pancreas, femur, intestine and heart) was not performed since it was not considered necessary to meet the objectives of the study.

This study was conducted in compliance with OECD test guideline 416 (adopted 22nd January 2001) with the exception of minor deviations listed under point 2.3. These deviations are not considered to have affected the scientific integrity, or outcome of this study.

240.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

The mean achieved dose levels of copper for P1 and F1 generation parental animals of both sexes were within the ranges 1.53-2.65, 7.7-13.3, 15.2-26.7 and 23.6-43.8 mg/kg body weight/day, for the 100, 500, 1000 and 1500 ppm groups, respectively. During the first 2 weeks of lactation, achieved dose levels exceeded these ranges in lactating dams.

The concentration of copper was increased in the liver of F1 males and F1 and F2 male and female weanlings at 1000 and 1500 ppm and in P1 and F1 females at 1500 ppm. Brain copper concentration was increased in F1 females and F1 and F2 male weanlings at 1500 ppm. The concentration of liver iron was decreased in P1 females at 1500 ppm. The concentration of plasma iron was decreased in F2 male and female weanlings at 1500 ppm.

There were no effects considered to be related to copper sulfate treatment on the following parameters at any concentration:

- Mortality and clinical signs of toxicity in P1 and F1 males and females.
- Body weights, weight gain, food consumption, food efficiency in P1 and F1 males and females.
- Sperm and estrous cycle parameters in P1 and F1 males and females.

- Mating, precoital interval, fertility, gestation length, number of implantation sites, and implantation efficiency in the P1 and F1 generations.
- Number of pups born, born alive, alive on day 4, 7, 14, or 21, sex ratio, and survival indices during the lactation period in F1 and F2 litters.
- Body weights and clinical observations in F1 and F2 litters during lactation
- Age at preputial separation in F1 males and vaginal opening in F1 females.
- Ovarian follicle counts in F1 females.
- Weight of testes, epididymides, right cauda epididymis, seminal vesicles, prostate, ovaries, uterus, thyroid gland, brain, liver, adrenal glands, kidneys and pituitary in P1 and F1 males and females.
- Weight of the spleen in P1 males and F1 males and females.
- Weight of liver, brain and thymus in F1 and F2 weanlings.
- Gross observations in P1 and F1 adults and F1 and F2 weanlings.
- Microscopic observations in the liver, brain and reproductive organs in P1 and F1 adults.
- Microscopic observations in the liver and brain in F1 and F2 weanlings.

Potentially adverse effects considered to be related to copper sulfate treatment were limited to the 1500 ppm groups and were comprised of:

- Decreased spleen weight in P1 adult females, and F1 and F2 male and female weanlings (see discussion below).

Discussion on spleen weight changes

The small (9% - 15%) decrease in mean spleen weight parameters in weanlings may have been spurious but was considered most likely test substance related since it was observed in both the F1 and F2 male and female weanlings. This is a conservative interpretation of the data, considering the highly variable nature of weanling spleen weights as illustrated by the clearly spurious, statistically significant, increase (16%) in the mean absolute splenic weight of the low-dose F1 female weanlings.

Since weanling spleens were not examined microscopically, the decrease in spleen weights were considered potentially adverse. Nonetheless, the following would suggest that the decrease in weanling spleen weights may have been a transient physiological alteration such as a marginal decrease in sinusoidal dilatation:

- The ranges of high-dose weanling splenic weights were similar to the respective control ranges. Of the 111 high-dose weanlings, only 4 had spleen weights below the range of their respective control group, and these were within the general range of weanling spleen weights.
- There was no test substance-related effect on thymus weight, suggesting that the lymphoid system was not affected by the test substance.
- All control and high-dose weanling livers had the normal amount of extramedullary hematopoiesis in the livers

(microscopic examination), suggesting that the hematopoietic system was not affected by the test substance.

- There were no test substance-related effects observed in F1 adults.

Similarly, the small (9%) decrease in mean spleen weight parameters in the P1 female adults was also interpreted to be test substance related and potentially adverse. Since spleens were not collected at necropsy, microscopic evaluation of these spleens was also not conducted.

Under the conditions of this study, the no-observed-effect level (NOEL) [a] for reproductive toxicity was 1500 ppm, the highest concentration tested. The NOEL [a] for P1 and F1 rats and F1 and F2 offspring during lactation was 1000 ppm, based on reduced spleen weight in P1 adult females, and F1 and F2 male and female weanlings at 1500 ppm however the transient reduced spleen weights are not considered a reproductive endpoint as it did not affect growth or fertility.

In compliance with the "Definition of reproductive toxicity", OECD document ENV/JM/MONO(2001)6 the spleen effect cannot be considered a reproductive effect as this must include:

- **Adverse effects on sexual function and fertility in adult males and females**
- **Developmental toxicity in the offspring**

For a compound to be considered to be a reproductive toxin "data for animal studies ideally should provide clear evidence of specific reproductive toxicity in the absence of other, systemic, toxic effects"

The dietary concentration of 1000 ppm was equivalent to mean daily intakes of copper of 15.2-23.5 mg/kg body weight/day for male rats during premating and 17.0-26.7 mg/kg body weight/day for female rats during premating and gestation.

a *the study report indicates that the NOEL for this study is defined as the highest dose at which toxicologically important effects attributable to the test substance were not detected. Thus, for this study, the NOEL is equivalent to the NOEL as defined by the United States Environmental Protection Agency and to the no-observed-adverse-effect level (NOAEL) as defined by the European Union.*

240.3 Conclusion *Non-entry field*

240.3.1 LO(A)EL *Non-entry field*

240.3.1.1	Parent males	*give critical effect and concentration* No effects up to 1500 ppm. No reproductive toxicity was seen at any concentration.
240.3.1.2	Parent females	*give critical effect and concentration* 1500 ppm (decreased spleen weight in P1 adult females). No reproductive toxicity was seen at any concentration.
240.3.1.3	F1 males	*give critical effect and concentration* 1500 ppm (decreased spleen weight in F1 male weanlings) No reproductive toxicity was seen at any concentration.
240.3.1.4	F1 females	*give critical effect and concentration* 1500 ppm (decreased spleen weight in F1 female weanlings) No reproductive toxicity was seen at any concentration.
240.3.1.5	F2 males	*give critical effect and concentration* 1500 ppm (decreased spleen weight in F2 male weanlings)
240.3.1.6	F2 females	*give critical effect and concentration* 1500 ppm (decreased spleen weight in F2 female weanlings)
240.3.2 NO(A)EL		*Non-entry field*

		give concentration
240.3.2.1	Parent males	1500 ppm Equivalent to 23.6 mg/kg bw/day for P1 males during premating. *give concentration*
240.3.2.2	Parent females	1000 ppm No reproductive toxicity was seen at any concentration Equivalent to 19.1, 17.0 and 33.8 mg/kg bw/day for P1 females during premating, gestation and the first 2 weeks of lactation, respectively. *give concentration*
240.3.2.3	F1 males	1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F1 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm. *give concentration*
240.3.2.4	F1 females	1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F1 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm. *give concentration*
240.3.2.5	F2 males	1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F2 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm. *give concentration*
240.3.2.6	F2 females	

	1000 ppm
	No reproductive toxicity was seen at any concentration
	Effects were seen in F2 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm.
240.3.3 Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4*
	1
240.3.4 Deficiencies	No
	(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)

Section A6.8.2(06)	**Multigeneration Reproduction Toxicity Study**
Annex Point IIA6.8.2	*Two Generation Reproduction Study in Rats by Oral Administration*

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	[redacted]
Guidelines and Quality Assurance	• [redacted]
Materials and Methods	[redacted]

Results and discussion

[redacted]

Section A6.8.2(06) **Multigeneration Reproduction Toxicity Study**

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral Administration*

[redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks [redacted]

Table A6.8.2(06)-2: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female P1 Adult Rats

	M					F				
Concentration (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	595.4	600.1	603.9	599.5	586.8	328.8	332.2	335.8	333.3	331.9
Spleen (AW)	0.866	0.887	0.892	0.881	0.841	0.643	0.629	0.639	0.605	0.586
Spleen (RW)	**0.146**	**0.148**	**0.148**	**0.147**	**0.143**	**0.195**	**0.190**	**0.190**	**0.182**	**0.177***

- underlined values were interpreted to be test substance-related organ weight effects.
*** statistically significant difference from control at p<0.05 by Dunnett/Tamhane-Dunnett Test**

Table A6.8.2(06)-3: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female F1 Weanlings

	M					F				
Dose (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	58.3	60.1	60.9	56.6	58.7	54.5	56.8	56.2	53.5	55.3
Spleen (AW)	0.256	0.290	0.280	0.238	0.232	0.245	0.283*	0.265	0.236	0.223
Spleen (RW)	**0.439**	**0.479**	**0.460**	**0.417**	**0.394**	**0.449**	**0.498***	**0.470**	**0.429**	**0.403**

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant increase (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Table A6.8.2(06)-4: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female F2 Weanlings

	M					F				
Dose (ppm):	0	100	500	1000	1500	0	100	500	1000	1500
Final Body	56.9	59.3	59.2	59.8	57.3	54.6	56.8	56.8	55.3	54.7
Spleen (AW)	0.253	0.269	0.254	0.252	0.227	0.254	0.265	0.252	0.243	0.217*
Spleen (RW)	**0.444**	**0.453**	**0.430**	**0.421**	**0.397***	**0.465**	**0.466**	**0.444**	**0.440**	**0.396***

-	underlined values were interpreted to be test substance-related organ weight effects.
*	statistically significant decrease (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Section A6.8.2(06) Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral Administration*

	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 A6.8.2(06)-1: Table for animal assignment to dosage groups

		Number of animals				
		Controls	**100 ppm**	**500 ppm**	**1000 ppm**	**15000 ppm**
P1	**m**	30	30	30	30	30
	f	30	30	30	30	30
F1	**m**	30	30	30	30	30
	f	30	30	30	30	30

Table A6.8.2(06)-2: Mean Absolute Spleen Weights (grams) in Male and Female P1 Adult Rats

	Males					**Females**				
Concentration (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	595.4	600.1	603.9	599.5	586.8	328.8	332.2	335.8	333.3	331.9
Spleen	0.866	0.887	0.892	0.881	0.841	0.643	0.629	0.639	0.605	0.586

- underlined values were interpreted to be test substance-related organ weight effects.

Table A6.8.2(06)-3: Mean Absolute Spleen Weights in Male and Female F1 Weanlings

	Males					Females				
Dose (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Weight (grams)										
Final Body	58.3	60.1	60.9	56.6	58.7	54.5	56.8	56.2	53.5	55.3
Spleen	0.256	0.290	0.280	0.238	0.232	0.245	0.283 *	0.265	0.236	0.223

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant increase (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Table A6.8.2(06)-4: Mean Absolute Spleen Weights in Male and Female F2 Weanlings

	Males					Females				
Dose (ppm):	0	100	500	1000	1500	0	100	500	1000	1500
Weight (grams)										
Final Body	56.9	59.3	59.2	59.8	57.3	54.6	56.8	56.8	55.3	54.7
Spleen	0.253	0.269	0.254	0.252	0.227	0.254	0.265	0.252	0.243	0.217 *

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant decrease (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

241 REFERENCE

241.1 Reference

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

[redacted] X (2005). Copper Sulfate Pentahydrate: Multigeneration Reproduction Study in Rats. DuPont Haskell Laboratory for Health and Environmental Sciences. Laboratory Project ID: DuPont-14226. (unpublished)

241.2 Data protection

Yes

(indicate if data protection is claimed)

241.2.1 Data owner

Give name of company

European Copper Institute (ECI).

241.2.2 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)

Wood Preservatives Copper Taskforce.

241.2.3 Criteria for data protection

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

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

242 GUIDELINES AND QUALITY ASSURANCE

242.1 Guideline study

Yes. The study report claims compliance with the following test guidelines:

- United States (U.S.) Environmental Protection Agency (EPA), Office of Prevention, Pesticides, and Toxic Substances (OPPTS) Health Effects Test Guidelines, OPPTS 870.3800 Reproduction and Fertility Effects. (August 1998).
- Organisation for Economic Cooperation and Development (OECD/OCDE). Guidelines for Testing of Chemicals, 416. (22nd January 2001).

Compliance with OECD test guideline 416 (adopted 22nd January 2001) is confirmed with the exception of deviations listed under point 2.3.

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

242.2 GLP

Yes.

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

242.3 Deviations

The following minor deviations occurred from the requirements of OECD guideline No. 416 (adopted 22nd January 2001):

- Animal rooms were maintained at a temperature of 18-26°C instead of the test guideline recommended 19-25%. X
- The guideline indicates that "Twice daily, during the weekend once daily when appropriate, all animals should be observed for morbundity and mortality. However it is not clear from the report if these intervals were respected. The report indicates that "Cage-site examinations were conducted at least once daily throughout the study".
- Testicular histopathological examinations are not fully described.

These deviations are not considered to have affected the scientific integrity, or outcome of this study.

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

243 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.

243.1 Test material Copper sulphate pentahydrate

Or give name used in study report

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

Aldrich Lot 17919TA

243.1.2 Specification Deviating from specification given in section 2 as follows.

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

243.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Colour: blue Physical form: crystal
243.1.2.2	Purity	*Give purity in % active substance* [redacted]
243.1.2.3	Stability	*Describe stability of test material* ***The stability of the test substance over the course of the study was confirmed by purity analyses conducted near the beginning and the end of the study. Analyses were conducted at:*** Exygen Research 3058 Research Drive State College, Pennsylvania 16801 U.S.A.
243.2 Test Animals		Non-entry field
243.2.1 Species		Rat. *if other, state reason for non standard species*
243.2.2 Strain		Crl:CD®(SD)IGS BR
243.2.3 Source		Charles River Laboratories, Inc., Raleigh, North Carolina, US.
243.2.4 Sex		Male and female.
243.2.5 Age/weight at study initiation		The P1 generation animals were approximately 8 weeks old at the start of treatment, and in the body weight ranges of approximately 262-332 g (males) and approximately 166-231g (females).
243.2.6 Number of animals per group		*Give number should be enough to yield 20 pregnant females per group* 30 rats/sex/concentration. See Table A.6.8.2-1.
243.2.7 Mating		*Start of Cohabitation* Animals were cohoused after approximately 10 weeks of exposure to the test substance. The day animals were first cohoused was designated as day 1 of cohabitation. *Duration of Cohabitation Period* Animals were cohoused until evidence of copulation was observed or until 2 weeks had elapsed. The cohabitation period ended in the morning of day 15 of cohabitation. *Evidence of Copulation* Once daily, each female was examined for the presence of an

	intravaginal copulation plug or sperm in the vaginal lavage sample, either of which was considered evidence of copulation. The presence of an intravaginal plug and/or sperm was recorded. The day evidence of copulation was observed was designated as day 0 of gestation. *Cohousing* Each female was continually housed on a 1:1 basis with a randomly selected, nonsibling male of the same dietary concentration level, in the male's cage. On the day copulation was confirmed, the female was transferred back to individual cage housing.
243.2.8 Duration of mating	*2 weeks or other* 2 weeks (see 3.2.7)
243.2.9 Deviations from standard protocol	*I.e. second mating of parent or F1 generations, standardisation of litter size* None.
243.2.10 Control animals	Yes. See Table A.6.8.2-1.
243.3 Administration/ Exposure	Oral *Fill in respective route in the following, delete other routes*
243.3.1 Animal assignment to dosage groups	*See table below* See to Table A6.8.2-1.
243.3.2 Duration of exposure before mating	*10 weeks or other (mice at least 56 days, rats 70 days)* At least 70 days for both P1 and F1 animals.

From beginning of the study until sacrifice of parent, F1, F2-generation or other

Treatment Schedule

243.3.3 Duration of exposure in general P, F1, F2 males, females

Gen.	Approximate Age at Start of Dosing (days)	Approximate No. of Study Days Before Mating	Duration of Dosing
P1	56	70 (at least)	Until sacrifice
F1	21	70 (at least)	Until sacrifice

Sacrifice Schedule

243.3.3 Duration of exposure in general P, F1, F2 males, females

Animals	Gen.	Schedule
Adult Males	P1 F1	Test days 109-113 Test day 119
Pregnant Females	P1, F1	On day of weaning litters (Day 21 Postpartum)
Nonpregnant Females	P1, F1	Approximately Day 28 after the end of cohabitation
Culled Pups	F1, F2	Day 4 Postpartum
Weanlings	F1, F2	On day of weaning (except F1 rats selected as parental rats)

~~Oral~~

243.3.4 Type Via the diet.

243.3.5 Concentration 0, 100, 500, 1000, 1500 ppm in the diet.

Diet Preparation Analysis

Diet preparation analysis demonstrated that the test substance was stable in the diet under study conditions. The homogeneity data support that the mixing procedure was adequate for all dietary levels. The concentration verification data indicate that the test substance was present at the targeted levels during the study.

243.3.6 Vehicle	*Moistened with water, aqueous solution, corn oil or other* No vehicle was used to mix the substance in the diet.
243.3.7 Concentration in vehicle	Not applicable.
243.3.8 Total volume applied	Not applicable.
243.3.9 Controls	*Vehicle, plain diet or other* Plain diet.
243.4 Examinations	*Non-entry field*
243.4.1 Clinical signs	Cage-site examinations were conducted at least once daily throughout the study. Moribund rats were sacrificed. At least once weekly throughout the premating feeding, gestation, and lactation periods, each of the P1 and F1 parental rats was individually handled and carefully examined for abnormal behavior and/or appearance.
243.4.2 Body weight	*Premating Period* All P1 and F1 rats were weighed once a week. All rats evaluated for developmental landmarks (vaginal patency, preputial separation) were weighed on the day of achievement. *Gestation and Lactation Periods* P1 and F1 dams were weighed on days 0, 7, 14, and 21 of each period. Females without evidence of copulation, those that copulated and did not deliver a litter, and males were weighed on a weekly schedule.
243.4.3 Food/water consumption	<u>Food consumption</u> *Premating and Cohabitation Periods* Individual food consumption was determined weekly for all P1 and F1 rats throughout the period, ending on test day 70. Food consumption was not measured during cohabitation for males and females or after cohabitation for males. *Gestation and Lactation Periods* Individual food consumption of pregnant P1 and F1 females was recorded on gestation days 0, 7, 14, and 21 and on lactation days 0, 7, and 14. Food consumption was not measured for males or females without evidence of copulation. From these determinations and body weight data, individual daily food consumption, food efficiency, and mean daily intake of the test substance were calculated. <u>Water consumption</u> Tap water was provided *ad libitum*. Water consumption was not measured.

243.4.4 Oestrus cycle

Vaginal lavage samples were collected daily from all P1 and F1 female rats in order to determine the stages of the estrous cycle. Vaginal lavage samples were collected beginning 3 weeks prior to start of cohabitation and continuing until copulation was confirmed or the cohabitation period ended. The vaginal lavage sample collected on the day copulation was confirmed was not used for estrous cycle evaluation. Vaginal lavage samples were also collected from all P1 and F1 parental female rats at the time of sacrifice. Vaginal lavage samples were examined microscopically for determination of the stage of the estrous cycle (diestrus, estrus, proestrus).

243.4.5 Sperm parameters

Sperm parameters for all P1 and F1 parental males were evaluated. At sacrifice, the right epididymis was weighed, the cauda was removed, weighed, and placed in 32°C phosphate buffered saline with 10 mg/mL bovine serum albumin, pricked with a needle to facilitate the release of sperm, and placed in an incubator at 35°C for 5 minutes. After incubation, an aliquot was placed in a sample chamber which was loaded into a Hamilton Thorne Integrated Visual Optical System (IVOS). The percentage of motile cells among at least 200 cells examined per animal was determined.

The right cauda epididymis was further incubated at 35°C for approximately 15 minutes. An aliquot was stained with eosin, and smears were prepared on microscope slides. Sperm smears were examined to determine the frequency of morphologically abnormal sperm, expressed as percentage of normal cells among at least 200 cells examined per animal. The specific types of abnormal morphologies, if any, were not categorized. The excised right cauda and the right caput and corpus epididymis were then placed in Bouin's solution.

The left epididymis and left testis were flash frozen in liquid nitrogen and stored at –65° to –85°C until analyzed. After thawing, the cauda epididymis was excised, weighed, and homogenized. After thawing, the testis was decapsulated and the parenchyma was weighed and homogenized. Sperm count per cauda epididymis and per gram cauda epididymis, and spermatid count per testis and per gram testis were determined using the IVOS.

243.4.6 Offspring

Lactation Procedures

The day when delivery was complete was designated day 0 postpartum. At each examination period (days 0, 4, 7, 14, and 21 postpartum), offspring were individually handled and examined for abnormal behavior and appearance; any dead or abnormal pups were recorded. Dams that had no live pups remaining during lactation were sacrificed.

Day 0 Postpartum

Live and dead pups in each litter were counted by sex as soon as possible after delivery was completed. Live pups in each litter were individually weighed.

Day 4 Postpartum

Pups in each litter were counted by sex and individually weighed. Then, litters were culled randomly to 8 (4/sex when possible) and the number of pups of each sex recorded. Extra offspring were euthanized (by decapitation) and discarded without pathological examination. Litters of 8 offspring or fewer were not reduced.

Days 7 and 14 Postpartum

Pups in each litter were counted by sex and individually weighed.

Day 21 Postpartum (Weaning - Postnatal day 21)

Pups in each litter were counted by sex and individually weighed. Offspring in the F1 litters of each treatment level were randomly selected (one rat/sex/litter when possible) to serve as parents for the F2 generation and were placed in individual cages. For groups without sufficient litters, additional pups were chosen from randomly selected litters within the group to achieve the required group size. Selection of rats within litters was random.

P1 and F1 Adult Rats

Weights of the following organs were recorded (paired organs weighed together) for all P1 and F1 adult animals sacrificed by design. Group means and organ weight ratios (organ wt./final body wt. and organ wt./brain wt.) were calculated.

Male	Female	Both Sexes
Testes Epididymides Right Cauda Epididymis Seminal Vesicles (with coagulating glands) Prostate	Ovaries Uterus (with oviducts and cervix)	Liver Brain Kidneys Spleen Adrenal Glands Pituitary Gland[a] Thyroid Gland[a]

a Pituitary and thyroid glands were weighed after fixation. Nursing Offspring

Organ weights from pups were not recorded.

F1 and F2 Weanlings

The liver, brain, spleen, and thymus weights were recorded from one weanling/sex/litter. Group means and organ weight ratios (organ wt./final body wt. and organ wt./brain wt.) were calculated.

Final body weight data, for animals that were sacrificed by design, were used for calculation of organ/body weight ratios.

243.4.8 Histopathology P and F1

See 3.5 for Euthanasia and Postmortem examination procedures.

Tissues designated for microscopic evaluation were embedded in paraffin, cut at a nominal thickness of 5 micrometers, stained with hematoxylin and eosin (H&E), and examined microscopically by a veterinary pathologist.

P1 and F1 Adult Rats

Reproductive organs, gross observations, and potential target organs (liver and brain) were processed and evaluated microscopically in all control and high-dose P1 and F1 adult rats. Tissue from rats in the low- and intermediate P1 and F1 adult dose groups did not require examination to determine a no-observed-adverse-effect level.

In addition, the reproductive organs from all mated animals that failed to produce a litter (i.e. reproductive failures) were evaluated microscopically. These included 18 P1 pairs and 9 F1 pairs.

Most gross lesions in P1 and F1 adults were saved and evaluated microscopically. Selected gross observations for which a microscopic diagnosis would not be additive (e.g. osteoarthritis, pododermatitis, tail chronic dermatitis, calculus, and deformities of the teeth, toe, tail, or ear pinnae) were saved, but not processed for microscopic evaluation.

Nursing Offspring

Microscopic examination of tissues from pups (nursing offspring) that died (found dead, sacrificed in extremis, or accidentally killed) during the lactation period was not performed.

F1 and F2 Weanlings

Tissues collected at necropsy (liver, brain, and gross observations) were processed and evaluated microscopically from selected (one/sex/litter) F1 and F2 high-dose and control weanlings. Examination of tissues from other groups was not required to establish a no-observed-adverseeffect level.

243.4.9 Histopathology See 3.4.8.
F1 not selected for mating, F2

243.5 Further remarks Euthanasia and Postmortem Examinations

P1 and F1 Adult Rats

All P1 and F1 generation rats that were not found dead, were sacrificed by carbon dioxide euthanasia and exsanguination. All P1 and F1 rats received a gross pathological examination. The uteri of all cohabited females were examined for the presence and number of implantation sites.

Blood (approximately 2 mL) was collected from the caudal vena cava from the first 10 rats (the first 10 consecutive animal numbers in each group) surviving to scheduled sacrifice in each of the P1 and F1 male and female groups. Scheduled sacrifice for these rats was in the morning (to optimize copper content in the blood samples). Blood samples were placed into EDTA tubes, processed to plasma, and frozen at approximately -80°C.

The following tissues were collected from all P1 and F1 adult animals and preserved in appropriate fixative for possible future histopathological examination:

Male	Female	Both Sexes
Testis[a]	Ovaries	Brain[be]
Epididymis[a]	Uterus (with oviducts)	Liver[be]
Prostate Seminal Vesicles	Vagina	Gross Observations[c]
Coagulating Glands	Cervix	Kidney[de]
		Pancreas[de]
		Femur[de] Intestine (first 10 cm after stomach)[de] Heart[de]

f) For rats sacrificed by design, the right testis and epididymis were placed in Bouin's solution. The left testis and epididymis were frozen for sperm assessment. All other tissues (reproductive and non-reproductive) collected from male and female rats were placed in formalin.
g) Potential target organs.
h) Gross observations observed at necropsy for which histopathology was not appropriate or would not be additive were generally not collected.
i) Collected from same 10 rats from which blood was collected.
j) An additional sample from the same animal was frozen in liquid nitrogen and stored at approximately -80°C for possible analysis.

Nursing Offspring

Pups were euthanized by decapitation (for pups up to

lactation day 4) or by carbon dioxide euthanasia and exsanguination. Pups that died (found dead, sacrificed in extremis, or accidentally killed) during the lactation period underwent a gross pathological evaluation and the carcass was preserved in an appropriate fixative.

At culling on lactation day 4, twelve randomly selected pups (6 male and 6 female) per dose group had samples of liver and brain collected, frozen in liquid nitrogen and stored at approximately -80°C.

F1 and F2 Weanlings

All F1 and F2 weanlings, except for F1 weanlings selected for continuation to the F1 generation adults, were sacrificed by carbon dioxide anesthesia and exsanguination. A gross pathological evaluation was performed on all weanlings with external abnormalities or clinical signs and from one randomly selected pup/sex/litter. All gross lesions and potential target organs (brain and liver) were preserved in formalin. Microscopic examination of preserved tissues was conducted on F1 and F2 high-dose and control groups. Examination of tissues in other groups was not required to establish a no-observed-adverse-effect level.

Blood (approximately 0.5 mL) was collected from the caudal vena cava from the first 10 rats (the first 10 consecutive animal numbers in each group that were selected for organ weights) surviving to scheduled sacrifice in each of the F1 and F2 male and female groups. Scheduled sacrifice for these rats was in the morning (to optimize copper content in the blood samples). Blood samples were placed into EDTA tubes, processed to plasma, and frozen at approximately -80°C.

In addition, the following tissues were collected from the same 10 weanlings/sex/dose from which blood was collected: brain, liver, kidney, pancreas, femur, intestine (first 10 cm of intestine after stomach), and heart. Tissues samples were frozen in liquid nitrogen (approximately -80°C) for possible chemical analysis, and additional samples were stored in formalin for possible microscopic evaluation.

<u>Ovarian Follicular Counts</u>

A quantitative evaluation of primordial and growing follicles was conducted on the first 10 lactating F1 females (surviving to scheduled sacrifice) from control and highdose (1500 ppm) groups. No treatment-related change was observed; therefore, counting of follicles in the low and intermediate groups to determine a no-effect level was not

required. Six ovarian cross sections (5 µm thick) were taken from the central area of the ovary using a step section technique. Primordial and growing follicles (up to but not including antral follicles) were enumerated for up to 12 ovarian sections per animal.

F1 Generation - Developmental Landmarks *Vaginal Patency*

F1 female rats designated for mating were examined for vaginal patency once daily beginning on postnatal day 21 until achievement or postnatal day 43, whichever came first. Body weight was recorded on the day of achievement.

Preputial Separation

F1 male rats designated for mating were examined for preputial separation beginning on postnatal day 35 until achievement or postnatal day 55, whichever came first. Body weight was recorded on the day of achievement.

Copper Analysis in Water and Feed *Water*

Samples of drinking water were taken near the beginning and end of the study and sent to Atlantic Coast Laboratories, Inc., to determine copper concentration. Inductively Coupled Argon Plasma Emission Spectroscopy (ICP) was used to measure the concentrations of copper in drinking water samples. The concentration of copper in samples collected on March 26, 2004 and November 5, 2004 were 0.014 and 0.024 ppm, respectively. The range of targeted concentrations added to the diet in this study was approximately 25-382 ppm copper (equivalent to 100-1500 ppm copper sulfate).

Feed

Samples of control feed were analyzed for copper content at Haskell Laboratory. The copper concentration in the control diet agreed with the specified amount of copper that was reported by the supplier. All reported results for the study diet samples were corrected for the analyzed value of the copper in the control diet. The concentration of copper in samples collected on March 3, 2004, June 23, 2004, and October 27, 2004 were 17.9, 11.4, and 11.8 ppm, respectively. The range of targeted concentrations added to the diet in this study was approximately 25-382 ppm copper (equivalent to 100-1500 ppm copper sulfate).

Tissue Metal Concentrations

Plasma, brain and liver samples were sent to DuPont

Corporate Center for Analytical Science, DuPont Experimental Station for analysis of copper, zinc, manganese, and iron concentration.

Brain and liver tissue samples were prepared for analysis by weighing a subsample into a digestion vessel and adding concentrated nitric acid. The mixture was then microwave digested at 190°C for 30 min so that a clear solution resulted. Plasma samples were diluted with 2% nitric acid before analysis. Analysis was done by inductively-coupled plasma (ICP) atomic emission spectroscopy. Copper, manganese, iron, and zinc emission signals were monitored at 324.8, 257.6, 259.9, and 213.9 nm, respectively. The intensity of the emitted signal is directly proportional to the concentration of the metal ion in solution. Calibration was done using standards prepared from certified metal reference materials.

Due to an instrument malfunction during analysis, no data is available for plasma samples from P1 males and F1 male and female weanlings. The analysis could not be repeated because all of the samples had been consumed; "quantity not sufficient" (QNS) is reported for all of these samples.

244 RESULTS AND DISCUSSION

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

244.1 Effects

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

244.1.1 Parent males

Intake of Test Substance - P1 males (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

1.53, 7.7, 15.2 and 23.6 mg/kg/day for P1 males during premating.

Clinical Observations and Mortality - P1 males

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed at any dose level.

Mean Body Weights and Body Weight Gains - P1 males

No test substance-related effects on body weight or body weight gain were observed at any dose level. Occasional findings of statistically significant increases in body weight gain were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Food Consumption and Food Efficiency - P1 males

There were no test substance-related effects on food consumption or food efficiency in P1 males during premating at any dose level. Occasional findings of statistically significant increases in food consumption were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship. The statistically significant decreases in food efficiency in P1 males on days

0-7 and for the entire premating period (days 0-70) at 1500 ppm were considered spurious and due to a slightly higher food consumption on days 0-7 in this group.

Sperm Parameters - P1 males

There were no test substance-related effects on sperm motility, morphology, epididymal sperm or testicular spermatid numbers at any dose level.

Reproductive Indices and Precoital Interval

The following parameters (where relevant) were not affected by test substance administration: precoital interval length, mating, fertility, gestation length, number of implantation sites, or implantation efficiency at any dose level.

Cause of Death P1 adult rats

There were no test substance-related deaths in the study. Of the 120 P1 adult males, one animal was sacrificed in extremis on day 14 because of a fractured nose.

Organ Weight Data - P1 adult rats

There were no treatment related organ weight changes. Gross Findings - P1 Adult Rats

At the terminal sacrifice, there were no test substance-related gross observations. All gross observations were consistent with normal background lesions in rats of this age and stock.

Microscopic Findings - P1 Adult Rats

In P1 adult rats, there were no test substance-related microscopic findings in the liver, brain or reproductive organs. All microscopic findings were considered to be incidental lesions commonly found in rats of this age and stock.

P1 Adult Reproductive Failures

The failure of 18 P1 adult pairs to produce litters was not related to test substance exposure. Gross and microscopic evaluation revealed a morphological explanation of their infertility in 3 P1 individuals. (absence of recent corpora lutea). The cause of the reproductive failure in the remaining pairs was not determined.

244.1.2 Parent females

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

Intake of Test Substance - P1 females (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

1.92, 9.6, 19.1 and 29.5 mg/kg/day for P1 females during premating;

1.67, 8.6, 17.0 and 26.2 mg/kg/day for P1 females during gestation;

3.39, 17.7, 33.8 and 55.7 mg/kg/day for P1 females during the first 2 weeks of lactation.

Clinical Observations and Mortality - P1 females

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed during premating,

gestation, or lactation at any dose level.

Mean Body Weights and Body Weight Gains - P1 females

There were no test substance-related effects on body weight or body weight gain during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant decreases in body weight gain were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Food Consumption and Food Efficiency - P1 females

There were no test substance-related effects on food consumption or food efficiency in P1 females during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant increases in food consumption or decreases in food efficiency were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Estrous Cycle Parameters - P1 females

There were no test substance-related effects on the mean percent days in estrus, diestrus, or proestrus, or mean cycle length at any dose level.

In P1 females, the mean percent days in estrus at 1000 and 1500 ppm were slightly higher than the control value (47 and 40%, respectively, vs. 30% for the control group). Since the increase was greater at 1000 than 1500 ppm, was not associated with any change in mean estrous cycle length or adverse reproductive outcome, and was not observed in F1 females, it was not considered test substance-related.

The distribution of estrous cycle stages at sacrifice was similar across groups.

Reproductive Indices and Precoital Interval

See 4.1.1.

Cause of Death - P1 Adult Rats

There were no deaths amongst P1 adult females.

Organ Weight Data P1 adult rats (see Table A6.8.2-2)

In P1 female adult rats, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group. Mean absolute and relative (organ wt./body wt.) values were both decreased 9%, as compared to control values. Only the decrease in mean relative spleen weight was statistically significant. No differences were observed in P1 male spleen weight values. X

All other individual and mean organ weight differences in P1 rats were considered to be spurious and unrelated to test substance administration. X

Gross Findings - P1 Adult Rats X

See 4.1.2.

Microscopic Findings - P1 Adult Rats

See 4.1.2.

P1 Adult Reproductive Failures

	See 4.1.2.
244.1.3 F1 males	*No effects / describe significant effects referring to data in results table*

Section A6.8.2(06) **Multigeneration Reproduction Toxicity Study**

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral*
Administration

Intake of Test Substance - F1 males (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

2.25, 11.5, 23.5 and 36.1 mg/kg/day for F1 males during premating. Clinical Observations and Mortality - F1 males

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed at any dose level.

Mean Body Weights and Body Weight Gains - F1 males

No test substance-related effects on body weight or body weight gain were observed at any dose level.

Food Consumption and Food Efficiency - F1 males

There were no test substance-related effects on food consumption or food efficiency in F1 males during premating. Occasional findings of statistically significant increases in food consumption and decreases in food efficiency (due to slightly higher food consumption) were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Sperm Parameters - F1 males

There were no test substance-related effects on sperm motility, morphology, epididymal sperm or testicular spermatid numbers at any dose level.

Reproductive Indices and Precoital Interval

The following parameters (where relevant) were not affected by test substance administration: precoital interval length, mating, fertility, gestation length, number of implantation sites, or implantation efficiency at any dose level.

F1 Offspring Data

Litter Size, Sex Ratio and Pup Survival

There were no test substance-related effects on the number of pups born, born alive, alive on day 4, 7, 14, or 21, nor were there any effects on sex ratio, or survival indices during the lactation period at any concentration level.

Clinical Observations in pups

There were no test substance-related clinical observations in F1 litters at any concentration level. Clinical observations observed in pups in this study occurred at low incidences and/or were not dose-related.

Pup Weights

No test substance-related effects were observed on F1 pup weights at any dose level. The increase in pup weight in F1 litters at 100 ppm on lactation day 7 was

considered spurious since it was not dose-related.

Developmental Landmarks

There were no test substance-related effects on the age at preputial separation in F1 males at any dose level.

Cause of Death - F1 Adult Rats

There were no deaths amongst F1 adult males.

Organ Weight

Data *F1 Adult*

Rats

In F1 male (and female) adult rats, there were no test substance-related organ weight effects. All individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

F1 Weanlings (see Table A6.8.2-3)

In F1 male (and female) weanlings, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group, that was not statistically significant. Mean absolute spleen weights were decreased 9% in both sexes, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were decreased 10% and 11% in male and female F1 weanlings, respectively, as compared to controls.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross

Findings

F1 Adult

Rats

At the terminal sacrifice, there were no test substancerelated gross observations. All gross observations were consistent with normal background lesions in rats of this age and stock.

F1 Weanlings

There were no test substance-related gross observations in F1 weanlings. Gross observations occurred at low incidences, were randomly distributed across control and treatment groups, and/or were lesions common to rats of this stock and age.

F1 Pups

There were no test substance-related gross observations in F1 pups. Observations in pups of lungs not expanded and no milk spot in the stomach are nonspecific lesions that are commonly seen in all pups that are born dead, and thus are not considered to be test substance related.

Microscopic

Findings *F1*

Adult Rats

In F1 adult rats, there were no test substance-related microscopic findings in the liver, brain or reproductive organs. All microscopic findings were considered to be incidental lesions commonly found in rats of this age and stock.

F1 Weanlings

There were no test substance-related microscopic findings in the liver and brain of the F1 weanlings. The few lesions present (amongst F1 and F2 weanlings) are common spontaneous lesions in this age and stock of rat.

F1 Adult Reproductive Failures

The failure of 9 F1 adult pairs to produce litters was not related to test substance exposure. The cause of reproductive failure in one F1 female was dystocia. The cause of the reproductive failure in the remaining pairs was not determined.

244.1.4 F1 females

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

Intake of Test Substance - F1 females (see 4.2)

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

2.65, 13.3, 26.7 and 43.8 mg/kg/day for F1 females during premating; 1.69, 8.5, 17.1 and 26.5 mg/kg/day for F1 females during gestation;

3.27, 17.6, 35.2 and 55.4 mg/kg/day for F1 females during the first 2 weeks of lactation.

Clinical Observations and Mortality - F1 females

There was no test substance-related mortality during the study. No test substance-related clinical observations were observed during premating,
gestation, or lactation at any dose level.

Mean Body Weights and Body Weight Gains - F1 females

There were no test substance-related effects on body weight gain during premating, gestation, or lactation at any dose level. Occasional findings of statistically significant increases in body weight or body weight gain were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Food Consumption and Food Efficiency - F1 females

There were no test substance-related effects on food consumption during premating, gestation or lactation at any dose level. Occasional findings of statistically significant increases in food consumption and decreases in food efficiency (due to slightly higher food consumption) were considered spurious due to their small magnitude, sporadic nature and/or lack of dose-response relationship.

Estrous Cycle Parameters - F1 females

There were no test substance-related effects on the mean percent days in estrus, diestrus, or proestrus, or mean cycle length any dose level. The distribution of estrous cycle stages at sacrifice was similar across groups.

Reproductive Indices

and Precoital Interval See 4.1.3.

F1 Offspring Data

See 4.1.3.

Developmental Landmarks

There were no test substance-related effects on the age at vaginal opening in F1 females at any dose level. In F1 females, the mean age at vaginal opening at 1500 ppm was significantly increased (33.6 vs. 32.1 days for the control group) but the delay was small (1.5 days) and was within the laboratory's historical control range (see below). Therefore, the apparent delay in vaginal opening was not considered test substancerelated.

Historical Control Data for Haskell Laboratory 1999-2003:

Study Start Date	Mean Day of Vaginal Opening
24-Sep-03	32.3
26-Sep-02	31.3
24-Apr-02	33.9
15-Oct-02	31.3
15-Mar-02	31.7
20-Feb-02	33.0
15-Mar-01	31.4
12-Dec-00	32.1
18-Sep-00	32.3
29-Jul-99	32.8
17-Jun-99	32.1
8-Apr-99	33.1
15-Mar-99	32.5
Mean	32.3
Standard	0.8
Minimum	31.3
Maximum	33.9

Ovarian Follicle Counts - F1 females

There was no significant difference in the total number of primordial and pre-antral follicles between the control and 1500 ppm F1 adult females.

Cause of Death - F1 females

There were no test substance-related deaths in the study. Of the 120 F1 females the following premature deaths occurred:

- one animal from the 500 ppm group was sacrificed in extremis on day 109 due to dystocia,
- one animal from the 500 ppm group was found dead on day 17 due to pyelonephritis,
- one animal from the 1000 ppm group was sacrificed in extremis on day 119 for morbidity of undetermined cause.

Organ Weight Data

F1 Adult Rats

In F1 female (and male) adult rats, there were no test substance-related organ weight effects. All individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

F1 Weanlings (see Table A6.8.2-3)

In F1 female (and male) weanlings, there was a small decrease in the spleen mean organ weight parameters in the 1500 ppm dietary exposure group, that was not statistically significant. Mean absolute spleen weights were decreased 9% in both sexes, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were decreased 10% and 11% in male and female F1 weanlings, respectively, as compared to controls.
All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross

Findings

F1 Adult

Rats

See 4.1.4.

F1 Weanlings

See 4.1.4.

F1
Pups
See
4.1.4.

Microscopic

Findings *F1*

Adult Rats See

4.1.4.

F1

Weanling

s See 4.1.4.

F1 Adult Reproductive

Failures See 4.1.4.

244.1.5 F2 males

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

F2 Offspring Data

Litter Size, Sex Ratio and Pup Survival

There were no test substance-related effects on the number of pups born, born alive, alive on day 4, 7,

14, or 21, nor were there any effects on sex ratio, or survival indices during the lactation period at any concentration level.

Clinical Observations in pups

There were no test substance-related clinical observations in F1 litters at any concentration level. Clinical observations observed in pups in this study occurred at low incidences and/or were not dose-related.

Pup Weights

No test substance-related effects were observed on F2 pup weights at any dose level.

Organ Weight Data - F2 Weanlings (see Table A6.8.2-4)

The F2 weanlings had a decrease in mean spleen weight parameters, at the high dose (1500 ppm), that was similar to that observed in the F_1 weanlings. Mean absolute spleen weights were decreased 10% and 15% in males and females, respectively, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were also decreased 10% and 15% in males and females, respectively, as compared to controls. Except for the male mean absolute spleen weight decrease (10%), these differences were statistically significant.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross

Findings

F2

Weanlings

There were no test substance-related gross observations in F2 weanlings. Gross observations occurred at low incidences, were randomly distributed across control and treatment groups, and/or were lesions common to rats of this stock and age.

F2 Pups

There were no test substance-related gross observations in F2 pups. Observations in pups of lungs not expanded and no milk spot in the stomach are nonspecific lesions that are commonly seen in all pups that are born dead, and thus are not considered to be test substance related. X

Microscopic Findings - F2 Weanlings

There were no test substance-related microscopic findings in the liver and brain of the 21 weanlings. The few lesions present (amongst F1 and F2 weanlings) are common spontaneous lesions in this age and stock of rat.

244.1.6 F2 females

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

F1

Offspring

Data See

4.1.5.

Organ Weight Data – F2 Weanlings (see Table A6.8.2-4)

The F2 weanlings had a decrease in mean spleen weight

parameters, at the high dose (1500 ppm), that was similar to that observed in the F1 weanlings. Mean absolute spleen weights were decreased 10% and 15% in males and females, respectively, as compared to controls. Mean relative (organ wt./body wt.) spleen weights were also decreased 10% and 15% in males and females, respectively, as compared to controls. Except for the male mean absolute spleen weight decrease (10%), these differences were statistically significant.

All other individual and mean organ weight differences were considered to be spurious and unrelated to test substance administration.

Gross Findings

F2 Weanlings

See 4.1.5.

F2 Pups

See 4.1.5.

Microscopic Findings – F2 Weanlings

See 4.1.5.

244.2 Other

Intake of Test Substance

The overall mean daily intake of copper in the 100, 500, 1000 and 1500 ppm groups, respectively, was:

- 1.53, 7.7, 15.2 and 23.6 mg/kg/day for P1 males during premating;
- 1.92, 9.6, 19.1 and 29.5 mg/kg/day for P1 females during premating;
- 1.67, 8.6, 17.0 and 26.2 mg/kg/day for P1 females during gestation;
- 3.39, 17.7, 33.8 and 55.7 mg/kg/day for P1 females during the first 2 weeks of lactation;
- 2.25, 11.5, 23.5 and 36.1 mg/kg/day for F1 males during premating;
- 2.65, 13.3, 26.7 and 43.8 mg/kg/day for F1 females during premating;
- 1.69, 8.5, 17.1 and 26.5 mg/kg/day for F1 females during gestation; and
- 3.27, 17.6, 35.2 and 55.4 mg/kg/day for F1 females during the first 2 weeks of lactation

Tissue Metal Concentrations

P1 Male Rats

No test substance-related changes in the concentration of copper, iron, manganese or zinc were observed in the liver or brain at any dose level. Plasma concentration data was not available for P1 males; however, this had no impact on the study since plasma data was available for P1 females, F1 males and females, and F2 male and female weanlings. The statistically significant decrease in liver iron concentration at 1500 ppm was considered spurious since there was high interindividual animal variability across groups for this parameter and because the change did not correlate, as in P1 females, with increased liver copper concentration.

P1 Female Rats

A test substance-related increase in the concentration of copper and a decrease in the concentration of iron were observed in the liver at 1500 ppm. No change in the concentration of copper or iron was observed in the plasma or brain at any dose level.

No test substance-related changes in the concentration of manganese or zinc were observed in the liver, plasma or brain.

F1 Male Rats

The concentration of copper was increased in the liver at 1000 and 1500 ppm. Although of small magnitude compared to the increase observed in P1 and F1 females, the increase was considered possibly test substance-related since a few animals in these groups had concentrations that were 2-3 fold higher than the control group mean. However, this is an expected physiological response of homeostatic

control and the absence of liver pathology (histological data) confirms that this is not an adverse effect. No test substance-related change in the concentration of copper was observed in the plasma or brain at any dose level. The statistically significant increase in the concentration of copper in the plasma at 1500 ppm was considered spurious due to the small magnitude of the change and considering the variability of the plasma values across groups.

No test substance-related changes in the concentration of iron, manganese or zinc were observed in the liver, plasma or brain. The statistically significant increases in liver zinc concentration at 500 ppm and in copper concentration in the brain at 1000 ppm were considered spurious since they were not dose-related.

F1 Female Rats

A test substance-related increase in the concentration of copper was observed in the liver and brain at 1500 ppm. No change in the concentration of copper was observed in the plasma at any dose level.

No test substance-related changes in the concentration of iron, manganese or zinc were observed in the liver, plasma or brain. The statistically significant increase in manganese concentration in the brain at 1500 ppm was considered spurious due to the small magnitude of the change and considering the inter-animal variability of across groups. The statistically significant decrease in plasma zinc concentration at 1000 ppm was considered spurious since it was not dose-related.

F1 and F2 Weanlings

A test substance-related increase in the concentration of copper was observed in the liver of F1 and F2 male and female weanlings at 1000 and 1500 ppm. The concentration of copper was slightly increased in the brain of F1 and F2 male (but not female) weanlings at 1500 ppm, and was considered possibly test substance-related. No change in the concentration of copper was observed in the plasma of F2 weanling at any dose level. Plasma concentration data was not available for the F1 weanlings; however, this had no impact on the study since plasma data was available for P1 females, F1 males and females, and F2 male and female weanlings.

The concentration of iron in the plasma was decreased in F2 male and female weanlings at 1500 ppm and was considered possibly test substance-related.

The statistically significant increase in manganese concentration in the brain of F2 male and female weanlings at 1500 ppm was considered spurious due to the small magnitude of the change and considering the inter-animal variability across groups. The statistically significant increase in zinc concentration in the liver in F1 male and female weanlings at 1000 and 1500 ppm was considered spurious since it was small and the magnitude of the change was independent of dose. The statistically significant increase in zinc concentration in the brain of F2 male weanlings at 1500 ppm was considered spurious since it was small and was not observed in F2 females or F1 males or females at this dose level. The statistically significant decrease in zinc concentration in the liver of F2 female weanlings at 100 ppm was considered spurious since it was not dose-related.

245 APPLICANT'S SUMMARY AND CONCLUSION

245.1 Materials and methods

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

A two-generation reproduction study, which involved the production of one set of litters in each generation, was conducted with copper sulphate pentahydrate. Throughout the study, Crl:CD®(SD)IGS BR rats (30/sex/concentration) were fed diets containing 0, 100, 500, 1000 or 1500 ppm copper sulfate. Following at least 70 days of diet administration (premating), the P1 and F1 generation males and females were co-housed within their respective treatment groups to produce F1 and F2 litters, respectively. Dams were allowed to deliver and rear their offspring until weaning (postpartum day 21). At weaning, 30 F1 rats/sex/group were randomly selected to comprise the F1 generation and were given the same dietary concentration level as their respective P1 generation sires and dams. F1 and F2 litters were culled to 4 pups/sex/litter (litter size permitting) on postnatal day 4; all remaining pups were discarded without further evaluation. Brain and liver samples were collected from culled pups on postnatal day 4 (12 per group, randomly selected) for possible analysis of copper, zinc, manganese and iron concentrations; analysis was not performed since it was not considered necessary to meet the objectives of the study.

Clinical observations, body weight, and food consumption were determined weekly throughout the study. Litter examinations (number of live and dead, individual pup weights, clinical observations) were determined at birth, on day 4, and weekly during the lactation period. Estrous cycle parameters (percent days in diestrus, proestrus, and estrus) and estrous cycle length were evaluated for 3 weeks prior to cohabitation in P1 and F1 rats. The age at either vaginal opening or preputial separation was recorded for the F1 generation. Sperm motility, morphology, and concentration in the cauda epididymis, and spermatid concentration in the testis were determined for P1 and F1 rats.

At weaning, a gross postmortem examination was performed on one weanling/sex/litter from F1 and F2 litters and gross lesions were retained; the liver, brain, spleen, and thymus were weighed and gross lesions and target organs (brain, liver) retained. After litter production, all P1 and F1 rats were given a gross pathological examination and the testes, epididymides, right cauda epididymis, seminal vesicles (with coagulating glands and their fluids), prostate, ovaries, and uterus (with oviducts and cervix), thyroid gland, brain, liver, spleen, adrenal glands, pituitary, and kidneys were weighed. The right testis and epididymis, prostate, seminal vesicles (with coagulating glands), pituitary, ovaries, uterus (with oviducts and cervix), vagina, brain, liver and gross lesions were placed in fixative for all P1 and F1 adults. These tissues as well as target organs and gross lesions from F1 weanlings in the control and 1500 ppm groups were examined microscopically; tissues in the low and intermediate groups were subsequently evaluated as needed to determine a no-adverse-effect level. Reproductive organs from the low and intermediate group P1 and F1 rats with suspected reduced fertility were examined microscopically. Ovarian follicle numbers were evaluated in control and 1500 ppm F1 rats (10/sex/group).

Blood and selected tissues (brain, liver, kidney, pancreas, femur, intestine and heart) were collected from P1 and F1 adults and F1 and F2 weanlings (10/sex/group); plasma and tissues were stored frozen for possible analysis of copper, zinc, manganese and iron concentrations. In addition, selected tissues (kidney, pancreas, femur, intestine and heart) from the same animals were placed in fixative for possible microscopic

examination; evaluation was not performed since it was not considered necessary to meet the objectives of the study. The concentration of copper, zinc, manganese and iron was determined in all plasma, brain and liver samples; analysis of other tissues (kidney, pancreas, femur, intestine and heart) was not performed since it was not considered necessary to meet the objectives of the study.

This study was conducted in compliance with OECD test guideline 416 (adopted 22nd January 2001) with the exception of minor deviations listed under point 2.3. These deviations are not considered to have affected the scientific integrity, or outcome of this study.

245.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

The mean achieved dose levels of copper for P1 and F1 generation parental animals of both sexes were within the ranges 1.53-2.65, 7.7- 13.3, 15.2-26.7 and 23.6-43.8 mg/kg body weight/day, for the 100, 500, 1000 and 1500 ppm groups, respectively. During the first 2 weeks of lactation, achieved dose levels exceeded these ranges in lactating dams.

The concentration of copper was increased in the liver of F1 males and F1 and F2 male and female weanlings at 1000 and 1500 ppm and in P1 and F1 females at 1500 ppm. Brain copper concentration was increased in F1 females and F1 and F2 male weanlings at 1500 ppm. The concentration of liver iron was decreased in P1 females at 1500 ppm. The concentration of plasma iron was decreased in F2 male and female weanlings at 1500 ppm.

There were no effects considered to be related to copper sulfate treatment on the following parameters at any concentration:

- Mortality and clinical signs of toxicity in P1 and F1 males and females.
- Body weights, weight gain, food consumption, food efficiency in P1 and F1 males and females.
- Sperm and estrous cycle parameters in P1 and F1 males and females.
- Mating, precoital interval, fertility, gestation length, number of implantation sites, and implantation efficiency in the P1 and F1 generations.
- Number of pups born, born alive, alive on day 4, 7, 14, or 21, sex ratio, and survival indices during the lactation period in F1 and F2 litters.
- Body weights and clinical observations in F1 and F2 litters during lactation
- Age at preputial separation in F1 males and vaginal opening in F1 females.
- Ovarian follicle counts in F1 females.
- Weight of testes, epididymides, right cauda epididymis, seminal vesicles, prostate, ovaries, uterus, thyroid gland, brain, liver, adrenal glands, kidneys and pituitary in P1 and F1 males and females.
- Weight of the spleen in P1 males and F1 males and females.
- Weight of liver, brain and thymus in F1 and F2 weanlings.
- Gross observations in P1 and F1 adults and F1 and F2

weanlings.

- Microscopic observations in the liver, brain and reproductive organs in P1 and F1 adults.
- Microscopic observations in the liver and brain in F1 and F2 weanlings.

Potentially adverse effects considered to be related to copper sulfate treatment were limited to the 1500 ppm groups and were comprised of:

- weight in P1 adult females, and F1 and F2 male and female weanlings (see discussion below).

Discussion on spleen weight changes

The small (9% - 15%) decrease in mean spleen weight in weanlings may have been spurious but was considered most likely test substance related since it was observed in both the F1 and F2 male and female weanlings. This is a conservative interpretation of the data, considering the highly variable nature of weanling spleen weights as illustrated by the clearly spurious, statistically significant, increase (16%) in the mean absolute splenic weight of the low-dose F1 female weanlings.

Since weanling spleens were not examined microscopically, the decrease in spleen weights were considered potentially adverse. Nonetheless, the following would suggest that the decrease in weanling spleen weights may have been a transient physiological alteration such as a marginal decrease in sinusoidal dilatation:

- The ranges of high-dose weanling splenic weights were similar to the respective control ranges. Of the 111 high-dose weanlings, only 4 had spleen weights below the range of their respective control group, and these were within the general range of weanling spleen weights.
- There was no test substance-related effect on thymus weight, suggesting that the lymphoid system was not affected by the test substance.
- All control and high-dose weanling livers had the normal amount of extramedullary he livers (microscopic examination), suggesting that the hematopoietic system was not affected by the test substance.
- There were no test substance-related effects observed in F1 adults.

Similarly, the small (9%) decrease in mean spleen weight parameters in the P1 female adults was also interpreted to be test substance related and potentially adverse. Since spleens were not collected at necropsy, microscopic evaluation of these spleens was also not conducted.

Under the conditions of this study, the no-observed-effect level (NOEL) [a] for reproductive toxicity was 1500 ppm, the highest concentration tested. The NOEL [a] for P1 and F1 rats and F1 and F2 offspring during lactation was 1000 ppm, based on reduced spleen weight in P1 adult females, and F1 and F2 male and female weanlings at 1500 ppm however the transient reduced spleen weights are not considered a reproductive endpoint as it did not affect growth or fertility.

In compliance with the "Definition of reproductive toxicity", OECD document ENV/JM/MONO(2001)6 the spleen effect cannot be

considered a reproductive effect as this must include:

- **Adverse effects on sexual function and fertility in adult males and females**
- **Developmental toxicity in the offspring**

For a compound to be considered to be a reproductive toxin "data for animal studies ideally should provide clear evidence of specific reproductive toxicity in the absence of other, systemic, toxic effects"

The dietary concentration of 1000 ppm was equivalent to mean daily intakes of copper of 15.2-23.5 mg/kg body weight/day for male rats during premating and 17.0-26.7 mg/kg body weight/day for female rats during premating and gestation.

a *the study report indicates that the NOEL for this study is defined as the highest dose at which toxicologically important effects attributable to the test substance were not detected. Thus, for this study, the NOEL is equivalent to the NOEL as defined by the United States Environmental Protection Agency and to the noobserved-adverse-effect level (NOAEL) as defined by the European Union.*

245.3 Conclusion *Non-entry field*

245.3.1 LO(A)EL *Non-entry field*

245.3.1.1 Parent males *give critical effect and concentration*
No effects up to 1500 ppm.

No reproductive toxicity was seen at any concentration.

245.3.1.2 Parent females *give critical effect and concentration*
1500 ppm (decreased spleen weight in P1 adult females).

No reproductive toxicity was seen at any concentration.

245.3.1.3 F1 males *give critical effect and concentration*
1500 ppm (decreased spleen weight in F1 male weanlings)

No reproductive toxicity was seen at any concentration.

245.3.1.4 F1 females *give critical effect and concentration*
1500 ppm (decreased spleen weight in F1 female weanlings)

No reproductive toxicity was seen at any concentration.

245.3.1.5 F2 males *give critical effect and concentration*
1500 ppm (decreased spleen weight in F2 male weanlings)

245.3.1.6 F2 females *give critical effect and concentration*
1500 ppm (decreased spleen weight in F2 female weanlings)

245.3.2 NO(A)EL *Non-entry field*

245.3.2.1	Parent males	*give concentration* 1500 ppm Equivalent to 23.6 mg/kg bw/day for P1 males during premating.
245.3.2.2	Parent females	*give concentration* 1000 ppm No reproductive toxicity was seen at any concentration Equivalent to 19.1, 17.0 and 33.8 mg/kg bw/day for P1 females during premating, gestation and the first 2 weeks of lactation, respectively.
245.3.2.3	F1 males	*give concentration* 1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F1 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm.
245.3.2.4	F1 females	*give concentration* 1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F1 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm.
245.3.2.5	F2 males	*give concentration* 1000 ppm No reproductive toxicity was seen at any concentration Effects were seen in F2 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm.
245.3.2.6	F2 females	*give concentration*

1000 ppm

No reproductive toxicity was seen at any concentration

Effects were seen in F2 weanlings. See 4.2 for the mg/kg bw/day equivalent for adults at 1000 ppm.

245.3.3 Reliability

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

1

245.3.4 Deficiencies

No

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

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

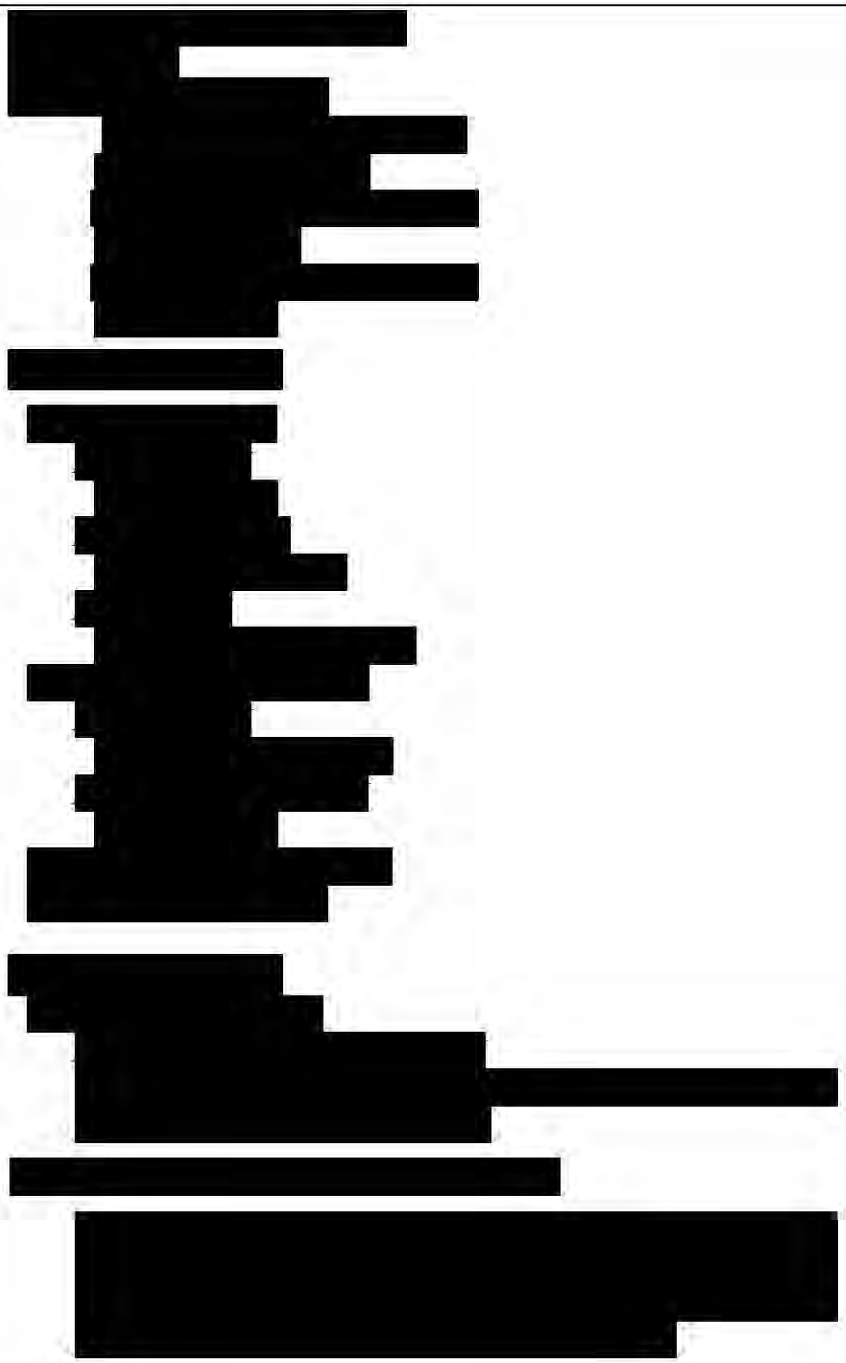
EVALUATION BY RAPPORTEUR MEMBER STATE

Section A6.8.2(06) **Multigeneration Reproduction Toxicity Study**

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral Administration*

Date	[redacted]
Guidelines and Quality Assurance	• [redacted]
Materials and Methods	[redacted]

Results and discussion



Section A6.8.2(06) **Multigeneration Reproduction Toxicity Study**

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral Administration*

[redacted]

Reliability [redacted]

Acceptability [redacted]

Remarks [redacted]

Table A6.8.2(06)-2: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female P1 Adult Rats

	M					Fe				
Concentration (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	595.4	600.1	603.9	599.5	586.8	328.8	332.2	335.8	333.3	331.9
Spleen (AW)	0.866	0.887	0.892	0.881	0.841	0.643	0.629	0.639	0.605	0.586
Spleen (RW)	**0.146**	**0.148**	**0.148**	**0.147**	**0.143**	**0.195**	**0.190**	**0.190**	**0.182**	**0.177***

- underlined values were interpreted to be test substance-related organ weight effects.
*** statistically significant difference from control at p<0.05 by Dunnett/Tamhane-Dunnett Test**

Table A6.8.2(06)-3: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female F1 Weanlings

	M					Fe				
Dose (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	58.3	60.1	60.9	56.6	58.7	54.5	56.8	56.2	53.5	55.3
Spleen (AW)	0.250	0.290	0.280	0.238	0.232	0.245	0.283*	0.265	0.236	0.223
Spleen (RW)	**0.439**	**0.479**	**0.460**	**0.417**	**0.394**	**0.449**	**0.498***	**0.470**	**0.429**	**0.401**

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant increase (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Table A6.8.2(06)-4: Mean Absolute (AW) and relative (RW) Spleen Weights (grams) in Male and Female F2 Weanlings

	M					Fe				
Dose (ppm):	0	100	500	1000	1500	0	100	500	1000	1500
Final Body	56.9	59.3	59.2	59.8	57.3	54.6	56.8	56.8	55.3	54.7
Spleen (AW)	0.253	0.269	0.254	0.252	0.227	0.254	0.265	0.252	0.243	0.217*
Spleen (RW)	**0.444**	**0.453**	**0.429**	**0.421**	**0.397**	**0.465**	**0.466**	**0.443**	**0.439**	**0.396***

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant decrease (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Section A6.8.2(06) **Multigeneration Reproduction Toxicity Study**

Annex Point IIA6.8.2 *Two Generation Reproduction Study in Rats by Oral Administration*

	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 A6.8.2(06)-1: Table for animal assignment to dosage groups

		Number of animals				
		Controls	**100 ppm**	**500 ppm**	**1000 ppm**	**15000 ppm**
P1	**m**	30	30	30	30	30
	f	30	30	30	30	30
F1	**m**	30	30	30	30	30
	f	30	30	30	30	30

Table A6.8.2(06)-2: Mean Absolute Spleen Weights (grams) in Male and Female P1 Adult Rats

	Males					**Females**				
Concentration (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Final Body	595.4	600.1	603.9	599.5	586.8	328.8	332.2	335.8	333.3	331.9
Spleen	0.866	0.887	0.892	0.881	0.841	0.643	0.629	0.639	0.605	0.586

- underlined values were interpreted to be test substance-related organ weight effects.

Table A6.8.2(06)-3: Mean Absolute Spleen Weights in Male and Female F1 Weanlings

	Males					**Females**				
Dose (ppm):	**0**	**100**	**500**	**1000**	**1500**	**0**	**100**	**500**	**1000**	**1500**
Weight (grams)										
Final Body	58.3	60.1	60.9	56.6	58.7	54.5	56.8	56.2	53.5	55.3
Spleen	0.256	0.290	0.280	0.238	0.232	0.245	0.283 *	0.265	0.236	0.223

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant increase (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Table A6.8.2(06)-4: Mean Absolute Spleen Weights in Male and Female F2 Weanlings

	Males					**Females**				
Dose (ppm):	0	100	500	1000	1500	0	100	500	1000	1500
Weight (grams)										
Final Body	56.9	59.3	59.2	59.8	57.3	54.6	56.8	56.8	55.3	54.7
Spleen	0.253	0.269	0.254	0.252	0.227	0.254	0.265	0.252	0.243	0.217 *

- underlined values were interpreted to be test substance-related organ weight effects.
* statistically significant decrease (parametric comparison to control: Dunnett/Tamhane-Dunnett Test)

Route of exposure	Test type Method Guideline	Species Strain Sex no/group	Exposure Period	Doses	Critical effect	Reference
Oral (diet)	Not a guideline study No GLP	Mouse (C57BL and DBA), male & female, 7-22 females/group (no data on the number of males)	1 month plus gestation up to day 19	0, 0.5, 1, 1.5, 2, 3 or 4 g/kg (0, 27, 53, 80, 106, 159 or 213 mg Cu/kg bw/day	No effect on number of successful matings and number of litters	Lecyk, 1980. Toxicity of CuSO4 in mice embryonic development. Zoologica Poloniae. Vol 28: 101-105.
Oral (diet)	Not a guideline study No GLP	Mink (male & female), groups of 4 males and 12 females	8 months plus gestation, lactation	0, 25, 50, 100 & 200 ppm CuSO4 feed (app. 0, 3, 6, 12 & 24 mgCu/kg bw/day	Neither reproductive nor general toxic effects on parental animals were observed. Kit weight at 4 weeks was significantly reduced in the 12 mgCu/kg bw/day but not the 24 mgCu/kg bw/day group. Kit mortality in the 12 & 24 mgCu/kg bw/day appeared to be increased and in all treated groups litter mass (at weaning) was reduced.	Aulerich *et al*., 1982. Effects of supplemental dietary copper on growth, reproductive performance and kit survival of standard dark mink and the acute toxicity of copper to mink. J. Animal Sci. Vol 55(2): 337-343.
Oral (diet)	Not a multi-gen study GLP	Rat (F344/N), mouse (B6C3F1) 10 animals per sex and group	13 weeks 92 days	Rats: 0, 500, 1000, 2000, 4000, 8000 ppm, equivalent to, 0, 8, 18, 17, 34, 67 & 138 mgCu/kg/bw/ day (estimated intake). Mice: 0, 1000, 2000, 4000, 8000, 16000 ppm equivalent to, 0, 44, 97, 187, 398, 815 mgCu/kg/bw/day in males & 0, 52, 126, 267, 536, 1058 mgCu/kg/bw/ day females.	No effects were seen on testis, epididymis or cauda epididymis weight, spermatid counts or sperm motility in males of either species, at any tested dose. The length of the oestrous cycle in females was unaffected.	NTP, 1993. NTP technical report on toxicity studies on cupric sulphate administered in drinking water and feed to F344/N rates and B6C3F1 mice. US Department of Commerce, National Technical Information Service. Publication No. 93-3352.
IUD	Not a guideline study No GLP	Rat (Holtzman), parent generation females, 12/ group; Hamster, parent generation females, 11/ group; Rabbit (New Zealand White), parent generation females, 9/group.	Rat, from day 6 of gestation until sacrifice of parent. Hamster, from day 6 of gestation until sacrifice of parent. Rabbit, from day 7 of gestation until sacrifice of parent.	Rate of dissolution of the Cu wire was approx 2.75 µg Cu/day in rats and hamsters and 5.5 µg Cu/day in rabbits.	The fertility of rats, hamsters and rabbits of the parent, F_1 and F_2 generations was unaffected by exposure of parent animals to copper from wire placed into the uterus after implantation of the embryos. Similarly, no adverse effects (teratogenicity or growth and development) attributable to the exposure of parent females to copper were seen in F_1 or F_2 animals.	Chang and Tatum, 1973. Absence of teratogenicity of intrauterine copper wire in rats, hamsters and rabbits. Contraception, Vol 7(5): 413-434.

COMMENTS FROM OTHER MEMBER STATE *(specify)*

Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Official use only

246 REFERENCE

246.1 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).

Murthy, R.C., Lal, S., Saxena, D.K., Shukla, G.S., Mohd Ali, M and Chandra, S.V. Effect of Manganese and Copper Interaction on Behaviour and Biogenic Amines in Rats Fed a 10% Casein Diet. Chem. Biol. Interactions, **37:** 299 – 308 (published).

246.2 Data protection

No.
(indicate if data protection is claimed)

246.2.1 Data owner

Give name of company

Public domain.

246.2.2 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.

246.2.3 Criteria for data protection

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

No data protection claimed.

247 GUIDELINES AND QUALITY ASSURANCE

247.1 Guideline study

No. This was a non-regulatory study designed to investigate the effects of potentially neurotoxic magnesium and copper on behaviour and on biogenic amines in rats maintained on a 10% casein diet (i.e. a low-protein diet). For the purposes of this summary, only information relevant to the potential neurotoxicity of copper is presented herein.

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

247.2 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)

247.3 Deviations

Yes. Refer to section 5.3.4 for a general discussion of deviations and deficiencies.

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

248 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.

248.1 Test material

Cu^{2+} as copper sulphate ($CuSO_4.5H_2O$).

or give name used in study report

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
~~**IUCLID: 5.9/05**~~	~~**A6.9(01), Neurotoxicity of copper**~~

248.1.1 Lot/Batch number		*List lot/batch number if available* Not stated.
248.1.2 Specification		Deviating from specification given in section 2 as follows *(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):*
248.1.2.1	Description	*If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)* Copper sulphate ($CuSO_4.5H_2O$).
248.1.2.2	Purity	*Give purity in % of active substance* [redacted]
248.1.2.3	Stability	*Describe stability of test material* Not stated.
248.2 Reference Substance (positive control)		None.
248.3 Test Animals		Non-entry field
248.3.1 Species		Rat
248.3.2 Strain		Not stated.
248.3.3 Source		Industrial Toxicology, Research Centre, India.
248.3.4 Sex		Male.
248.3.5 Rearing conditions		*As the test relies partly on effects on the mobility of the test animals, rearing conditions should permit free mobility.* Rats were kept in stainless steel cages in an air-conditioned room with a 12 hour light and 12 hour dark cycle was maintained.
248.3.6 Age/weight at study initiation		*Young adults (age 8 – 12 months) recommended* Bodyweight at study initiation was approximately 60 g. Age not stated.
248.3.7 Number of animals per group		*Give number per treatment and vehicle group* *Minimum 12 animals each per treatment and vehicle control group:* *3 animals each at 2 time points for biochemical determinations 6 animals for necropsy after 21 days* *Minimum for positive control group 9 animals:* *3 animals for biochemical determinations 6 animals for necropsy after 21 days* 21 control animals received a diet containing 21% casein. 21animals were given feed containing 21% casein supplemented with 250 ppm Cu (as $CuSO_4.5H_2O$) for 30 days, providing 5 mg Cu/rat/day. 21 control animals received a diet containing 10% casein. 21 animals 250 ppm Cu (as $CuSO_4.5H_2O$) was given in a low-protein diet (10% casein) for 30 days, providing 5 mg Cu/rat/day.
248.3.8 Control animals		Yes.

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
~~**IUCLID: 5.9/05**~~	~~**A6.9(01), Neurotoxicity of copper**~~
248.4 Administration	Oral in the diet.
248.4.1 Exposure	Administration in the diet for 30 days.
248.4.2 Dose Levels	One vehicle group plus one treatment group for each diet (see section 3.3.7).
248.4.3 Vehicle	Diet. The study was designed to investigate the potential for the neurotoxicity of Cu (and Mn) to vary with the protein content of the diet. *give justification, if not water*
248.4.4 Concentration in vehicle	The diet containing 21% casein was supplemented with 250 ppm Cu (as $CuSO_4.5H_2O$), providing 5 mg Cu/rat/day (equivalent to about 20 mg Cu/kg bw/day). The diet containing 10% casein was supplemented with 250 ppm Cu (as $CuSO_4.5H_2O$), providing 5 mg Cu/rat/day (equivalent to about 40 mg Cu/kg bw/day).
248.4.5 Total volume applied	Not applicable.
248.4.6 Postexposure period	None.
248.4.7 Anticholinergic substances used	Not applicable. *Specify substance used for protecting test animals against acute cholinergic effects, if applicable, state concentration [mg/kg b.w.]*
248.4.8 Controls	Relevant diets.
248.5 Examinations	Non-entry field.
248.5.1 Body Weight	Weighing before application and after application in weekly intervals.
248.5.2 Signs of Toxicity	*behaviour abnormalities with special respect to* *Ataxia (measured on a scale with at least four levels) Paralysis* *effects observed in a period of forced motor activity (such as ladder climbing) in the hens selected for pathology. These hens should be observed in that way at least twice a week.* After 30 days of treatment, batches of 6 animals selected randomly from each group were subjected to the following behavioural test procedures under standard laboratory conditions: Spontaneous motor activity (SMA) was assessed in groups of 6 animals using an actophotometer in which the frequency with which the animals interrupt the light beams was measured; Learning ability was assessed in groups of 6 animals in a pole-climbing chamber employing a 5 second sound signal and 5 second electric foot-shock. The percentages of conditioned avoidance (CAR) and unconditioned escape (UER) responses and escape failure (EF) were calculated; Relearning capacity and memory were analysed in each animal in a Y-Maze employing shock-motivated brightness discrimination response.

248.5.3 Observation schedule: Animals were assessed at the end of the 30 day treatment period.

248.5.4 Clinical Chemistry: Yes

Number of animals: Six animals from each group

Time points: At the end of the 30 day treatment period.

Parameters: After the treatment period, the brains were removed from six animals from each group and the following parameters were examined:
Biogenic amines
Protein content
Metal content

248.5.5 Pathology: No

248.5.6 Histopathology: No

248.6 Further remarks: Statistical significance between control and experimental values were calculated by Student's t-test; *P*-values < 0.05 were considered significant.

249 RESULTS AND DISCUSSION

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

249.1 Body Weight

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

Exposure of test animals to Cu^{2+} did not adversely affect the pattern of growth rate in either dietary group, relative to controls. Approximate bodyweight values over the first 4 weeks (extrapolated from graphically presented data are as follows (each point represents the mean weight of 6 animals):

Group	Bodyweight (grams)				
	Week 0	Week 1	Week 2	Week 3	Week 4
Low Protein Diet					
Control	105.00	111.25	115.00	132.50	141.25
Test	105.00	113.75	127.50	133.75	136.25
High Protein Diet					
Control	190.00	202.50	215.00	255.00	275.00
Test	190.00	210.00	225.00	250.00	287.50

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.9/05	**A6.9(01), Neurotoxicity of copper**

249.2 Clinical signs of toxicity

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

state time of onset, type, severity, duration and reversibility of behaviour abnormalities with special respect to:

Ataxia (measured on a scale with at least four levels)

Paralysis

Give toxic effects observed in a period of forced motor activity (such as ladder climbing) in the hens selected for pathology.

Deaths or killings of moribund animals

Exposure of test animals to Cu^{2+} had no significant effect on locomotor activity. Approximate activity counts (extrapolated from data presented in a bar chart) are as follows (each point represents the mean of 2 – 3 experiments):

Group	Activity Counts/15 minutes
Low Protein Diet	
Control	160
Test	167
High Protein Diet	
Control	195
Test	210

Exposure of test animals to Cu^{2+} had no significant effect on learning ability, as assessed by the deficit in the acquisition of CAR and the incidence of EF responses. Approximate % CAR and % EF values (extrapolated from data presented in a bar chart) are as follows (each point represents the mean of 2 – 3 experiments):

Group	Percentage of CAR	Percentage of EF
Low Protein Diet		
Control	22.50	11.25
Test	25.00	12.50
High Protein Diet		
Control	40.00	21.25
Test	33.75	18.75

Exposure of test animals to Cu^{2+} had no significant effect on relearning and memory processes, as indicated by RI and AC. Approximate % RI and AC values (extrapolated from data presented in a bar chart) are as

follows (each point represents the mean of 2 – 3 experiments):

Group	Percentage of RI	AC
Low Protein Diet		
Control	34.29	3.59
Test	28.57	3.0
High Protein Diet		
Control	57.15	4.72
Test	52.15	4.26

249.3 Clinical Chemistry *No effects / describe significant effects referring to data in results table*

Cu-treatment was associated with an increase in the brain content of dopamine (DA) and norepinephrine (NE) in rats receiving the 21% casein diet, but decreased the level of 5-hydroxytryptamine (5-HT) in protein-deprived rats (**Table A6.9(01)-1**).

Brain protein content of metal-treated rats at the termination of the experiment did not differ significantly from controls (79.06 ± 9.64 mg/g fresh weight).

The administration of Cu was associated with decreased levels of calcium and zinc in the brains of rats fed both diets. The Cu content of the brain was also elevated in Cu-treated animals (**Table A6.9(01)-2**).

249.4 Pathology Not applicable.

249.5 Histopathology Not applicable.

249.6 Other *Describe any other significant effects*

Brain weights of protein-deprived and Cu-treated animals at the termination of the experiment did not differ significantly from controls (1.58 ± 0.03 g).

250 APPLICANT'S SUMMARY AND CONCLUSION

250.1 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 potentially neurotoxic magnesium and copper on behaviour and on biogenic amines in rats maintained on a 10% casein diet (i.e. a low-protein diet). For the purposes of this summary, only information relevant to the potential neurotoxicity of copper is presented in this summary.

88 male rats with an initial bodyweight of 60 g were kept in stainless steel cages in an air-conditioned room where a cycle of 12 hours light and 12 hours dark was maintained. The rats were randomly divided into two dietary groups of equal number ($N = 44$). One group was fed with a synthetic diet containing 21% casein (normal protein diet) and the other received a diet containing 10% casein (low protein diet).

After 30 days, rats from each dietary regimen were further subdivided into 2 groups of 22 animals. Animals in one (control) group from each regimen continued on the relevant diet only. The second (test) group from each regimen was given a diet supplemented with 250 mg Cu/kg diet as $CuSO_4.5H_2O$. This was equivalent to about 20 mg/kg bw/day in rats on the normal protein diet and 40 mg/kg bw/day in those on the low-protein diet. All groups received *ad libitum* drinking water. Food and water intakes were recorded. According to the consumption of food, the dose of Cu administered to test animals was 5.0 ± 0.5 mg Cu/rat/day. After 30 days of treatment, batches of 6 animals selected randomly from each group were subjected to the following behavioural test procedures under standard laboratory conditions:

Spontaneous motor activity (SMA): Rats were placed individually in an actophotometer which measured the frequency with which animals interrupted the light beams. Counts were made for 15 minutes per rat.

Learning ability: This was tested in a pole-climbing chamber, using a 5 second sound signal and electric foot shock. Each animal received 20 trials and the % of conditioned avoidance (CAR) and unconditioned escape (UER) responses and escape failure (EF) were calculated.

Relearning capacity and memory: These were analysed in a Y-maze employing shock motivated brightness discrimination response. Each animal was subjected to 40 trials/day for 2 consecutive days. The relearning index (RI) and the increase in positive change (**A**C) were calculated as follows: RI = ((Ts – RLs)/Ts) x 100, where Ts is the number of shocks received during test (1st day), RLs is number of shocks received during relearning (2nd day), **A**C is RLc – Tc where RLc is number of +ve changes during relearning and Tc is number of +ve changes during test.

Additional investigations were carried out following sacrifice of 6 animals from each group and removal and processing of their brains:

Quantification of biogenic amines: Concentrations of the following biogenic amines were estimated: dopamine (DA), norepinephrine (NE) and 5-hydroxytryptamine (5-HT). Fluorescence was measured in a Carl-Zeiss PMQ 3 C specrofluorometer.

Quantification of protein and metal: Protein was estimated using bovine serum albumin as standard. Metals were estimated by atomic absorption spectrophotometry.

250.2 Results and discussion

Statistical significance between control and experimental values were calculated by Student's *t*-test; *P* values < 0.05 were considered significant.

Summarize relevant results; discuss dose-response relationship.

Bodyweight: Exposure of test animals to Cu^{2+} did not adversely affect the pattern of growth rate in either dietary group, relative to that of the controls.

Brain weights of protein-deprived and Cu-treated animals at the termination of the experiment did not differ significantly from controls (1.58 ± 0.03 g).

Spontaneous motor activity: Approximate activity counts (extrapolated

from data presented in a bar chart) were 160 and 167 counts/minute respectively for control and test animals on the low protein regimen. Approximate counts for animals receiving the high protein diet were 195 and 210 counts/minute for control and test animals, respectively. It was concluded that exposure of test animals to Cu^{2+} had no significant effect on locomotor activity.

Learning ability: Approximate values for deficit in the acquisition of CAR (extrapolated from data presented in a bar chart) were 22.5 and 25.0 % respectively for control and test animals on the low protein regimen. Approximate counts for animals receiving the high protein diet were 40.0 and 33.75 % for control and test animals, respectively. It was concluded that exposure of test animals to Cu^{2+} had no significant effect on learning ability, as assessed by the deficit in the acquisition of CAR

Approximate values for the incidence of EF responses (extrapolated from data presented in a bar chart) were 11.25 and 12.50 % respectively for control and test animals on the low protein regimen. Approximate counts for animals receiving the high protein diet were 21.25 and 18.75 % for control and test animals, respectively. It was concluded that exposure of test animals to Cu^{2+} had no significant effect on learning ability, as assessed by the incidence of EF responses.

Relearning capacity and memory: Approximate values for RI (extrapolated from data presented in a bar chart) were 34.29 and 28.57 % respectively for control and test animals on the low protein regimen. Approximate counts for animals receiving the high protein diet were 57.15 and 52.15 % for control and test animals, respectively. It was concluded that exposure of test animals to Cu^{2+} had no significant effect on relearning and memory processes, as indicated by RI.

Approximate values for **A**C (extrapolated from data presented in a bar chart) were 3.59 and 3.0 % respectively for control and test animals on the low protein regimen. Approximate counts for animals receiving the high protein diet were 4.72 and 4.26 % for control and test animals, respectively. It was concluded that exposure of test animals to Cu^{2+} had no significant effect on relearning and memory processes, as indicated by **A**C.

Quantification of biogenic amines: Cu-treatment was associated with an increase in the brain content of DA (+16%) and NE (+17%) in rats receiving the 21% casein diet, but decreased the level of 5-HT (-20%) in protein-deprived rats.

Quantification of protein and metal: Brain protein content of metaltreated rats at the termination of the experiment did not differ significantly from controls (79.06 ± 9.64 mg/g fresh weight).

The administration of Cu was associated with decreased levels of calcium in the brains of rats fed both the low- and high-protein diets (- 30 and -35%, respectively). Decreased levels of zinc were also seen in both dietary groups (-22 and -16% for low- and high-protein diets, respectively). The Cu content of the brain was elevated in all Cu-treated animals (+174 and +172% for low- and high-protein diets, respectively).

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.9/05	**A6.9(01), Neurotoxicity of copper**
250.3 Conclusion	The administration of 250 ppm Cu^{2+} as $CuSO_4$ in the diet of male rats had no significant effect on locomotor activity, learning ability or relearning ability and memory, irrespective of whether the diet was protein-deficient or –normal. The rate of Cu administration was equivalent to about 20 mg Cu/kg bw/day in rats on the normal protein diet and 40 mg Cu/kg bw/day in those on the low-protein diet. Analysis of biogenic amines in the brain did, however reveal an increase in the dopamine and norepinephrine levels of animals receiving the protein-adequate diet and a decrease in 5-hydroxytryptamine levels of those on a low-protein diet. The neurotoxicological significance of these findings is unclear, given that there were no associated effects on behaviour.
250.3.1 LOAEL	*give critical effect and value* Not established (behavioural effects) 250.3.2 NOAEL *give value* 20 mg Cu/kg bw/day for rats on a normal protein diet and 40 mg Cu/kg bw/day for those on the low-protein diet (behavioural effects).
250.3.3 Reliability *appropriate*	*Based on the assessment of materials and methods include* *reliability indicator 0, 1, 2, 3, or 4* 2

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.9/05	**A6.9(01), Neurotoxicity of copper**

250.3.4 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 neurotoxicty studies (e.g. OECD 424), including the following:

- The test substance was inadequately characterised;
- Only one $CuSO_4$ test concentration were used;
- Only male animals were used;
- The frequency and nature of clinical observations is unclear;
- The range of functional tests used was limited;
- No formal histopathological studies were reported;
- Data on individual animals were not reported.

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 neurotoxicity of copper.

Overall, this is an adequately-reported study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper neurotoxicity. 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].

Section A6.9	**Neurotoxicity**
Annex Point IIA6.9	*Specify section no., heading, route and species as appropriate*
IUCLID: 5.9/05	**A6.9(01), Neurotoxicity of copper**

Conclusion	• [redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

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

Official use only

251 REFERENCE

251.1 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).

Pratt, W.B., Omdahl, J.L. and Sorenson, R.J., (1985). Lack of Effects of Copper Gluconate Supplementation. The American Journal of Clinical Nutrition, **42:** 681 – 682 (Published).

252 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

253 MATERIALS AND METHODS

253.1 Substance

Give any available information on substance/product, such as identity, physical form (e.g. powder, grain size, particle size/distribution), purity

[redacted]

253.2 Persons exposed Non-entry field

253.2.1 Sex: Male and female.

253.2.2 Age/weight: Mean age 42 years.

253.2.3 Known Diseases: *State if any; state, if healthy*

All the subjects suffered from back pain.

253.2.4 Number of persons 7 (3 men and 4 women). 253.2.5 Other information None X

253.3 Exposure Oral

Indicate respective route, delete other routes 253.3.1 Reason of exposure

As part of a study of back pain management.

253.3.2 Frequency of exposure: Multiple doses. Subjects were dosed twice a day.
specify frequency

253.3.3 Overall time period of exposure: *if applicable*
12 weeks.

253.3.4 Duration of single exposure: *if applicable*
Not applicable.

253.3.5 Exposure concentration/dose *give all available information*: Subjects received 5 mg of copper twice a day in capsule form.

253.3.6 Other information: Control subjects received placebo capsules.

253.4 Examinations

give type of examination and time after exposure for each examination

Subjects were seen every 2 weeks to evaluate their progress. Blood, serum, urine and hair samples were collected at the beginning of the study, after 6 weeks of supplementation and at the end of the 12 week study.

253.5 Treatment

give any available information on the medical treatment of intoxicated persons

Not applicable; there were no adverse effects on treated individuals. The study was approved by the Human research Review Committee.

253.6 Remarks

The study was double blind.

254 RESULTS

Describe findings. If appropriate, include table.

254.1 Clinical Signs

Describe any relevant effects observed

There were no clinical signs associated with treatment.

254.2 Results of examinations

Describe the results of e.g. clinical chemistry, blood analysis and urinalysis

There was no significant change in the level of copper, zinc or magnesium of the serum, urine or hair samples of the seven subjects during the 12 weeks of the study. There was also no significant change in the haematocrit, mean corpuscular volume, serum cholesterol, serum triglyceride, SGOT, serum alkaline phosphatase, serum GGT, or serum LDH (**Table A6.12.2(01)-1**). Serum potassium did change from a mean of 4.3 mEq/L to 4.0 mEq/L ($p < 0.05$). The incidence of nausea, diarrhoea, and heartburn was the same in the seven subjects receiving the copper gluconate as it was among the seven other subjects receiving the placebo capsules.

254.3 Effectivity of medical treatment

Not applicable to the present study.

254.4 Outcome

Describe outcome / manifestation of symptoms / disease

It was found that 10 mg/day of copper as copper gluconate had no detectable effect on the seven subjects. It was concluded that treated individuals excrete excess amounts of absorbed copper not needed to meet tissue needs or to maintain liver stores under homeostatic conditions.

254.5 Other

Describe any other significant observations

None.

255 APPLICANT'S SUMMARY AND CONCLUSION

255.1 Materials and methods

Briefly describe circumstances

As part of a double-blind study of back pain management, 7 adult patients received an oral dose of 5 mg copper (as copper gluconate capsules) twice a day for 12 weeks. Seven others received a placebo capsule over the exposure period.

	Subjects were seen every 2 weeks to evaluate their progress. Blood, serum, urine and hair samples were collected at the beginning of the study, after 6 weeks of supplementation and at the end of the 12 week study. Parameters assessed were haematocrit, mean corpuscular volume, serum cholesterol, serum triglyceride, SGOT, serum alkaline phosphatase, serum GGT, serum LDH and serum potassium. Copper, zinc and magnesium levels were also assessed in serum, urine and hair.
255.2 Results and discussion	*Summarize relevant results* There was no toxicologically significant change in the level of copper, zinc or magnesium of the serum, urine or hair samples of the seven subjects during the 12 weeks of the study. Similarly, there was no toxicologically significant change in the haematocrit, mean corpuscular volume, serum cholesterol, serum triglyceride, SGOT, serum alkaline phosphatase, serum GGT, or serum LDH. Serum potassium changed from a mean of 4.3 mEq/L to 4.0 mEq/L. The incidence of nausea, diarrhoea, and heartburn was the same in subjects receiving copper gluconate as it was in the control group. It was concluded that 10 mg/day of copper as copper gluconate had no detectable adverse effect on the seven test subjects.
255.3 Conclusion	*Give general conclusions* 10 mg copper/day, administered orally as copper gluconate for 12 weeks, had no detectable adverse effect on the livers or gastrointestinal tracts of the seven test subjects.

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]

Section A6.12.2(1)	**Human Case Report**
Annex Point IIA6.12	*specify subsection number*
IUCLID : 5.9/01	**A6.12.2(1) Copper Tolerance**

Reliability	[redacted]
Acceptability	[redacted]
Remarks	
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*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*
Remarks	

Table A6.12.2(01)-1. A comparison of serum levels before and after 12 weeks of supplementation with 10 mg of copper/da

	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Copper (µg/100 ml)	Zinc (µg/100 ml)	Magnesium (mg/100 ml)
Before Cu supplement	199.6 ± 26.8 mg/dL	112.4 ± 37 mg/dL	126 ± 22 µg/100 ml	1.47 ± 0.12 µg/100 ml	18.4 ± 1.5 mg/100 ml
After 12 week supplement	212.6 ± 40.7 mg/dL	101.8 ± 40 mg/dL	123 ± 16 µg/100 ml	1.44 ± 0.38 mg/ml	19.4 ± 1.6 mg/100 ml

Section A6.12.2(2) **Human Case Report**

Annex Point IIA6.12.2 *specify subsection number*

A6.12.2(2): Vineyard sprayer's lung

Official use only

256 REFERENCE

256.1 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).

Pimentel JC & Marques F (1969) 'Vineyard sprayer's lung': a new occupational disease. Thorax, 24: 678-688 (published).

257 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

258 MATERIALS AND METHODS

258.1 Substance

Give any available information on substance/product, such as identity, physical form (e.g. powder, grain size, particle size/distribution), purity

'Bordeaux Mixture',
[redacted]

258.2 Persons exposed

258.2.1 Sex: Males

258.2.2 Age/weight:
Case 1: 35 years old
Case 2: 36 years old
No information on body weight available.

258.2.3 Known Diseases
State if any; state, if healthy

Both patients had (12 and 14 months) previously been diagnosed with tuberculosis. As antituberculous therapy clearing was not complete and because of persistently negative sputum for tubercle bacilli, patients were admitted to hospital for further diagnostic investigations. Upon admission one patient was apparently asymptomatic; the other had evident respiratory symptoms.

No further information on health status was given.

258.2.4 Number of persons 2 case reports are described.

258.2.5 Other information None

258.3 Exposure

Inhalation
Although no exposure data is available it assumed that inhalation to the 'Bordeaux mixture' occurred during spraying.
Indicate respective route, delete other routes

258.3.1 Reason of exposure Occupational

258.3.2 Frequency of exposure
specify frequency
Multiple

258.3.3 Overall time period of exposure

if applicable

No precise information is available.

Patient 1 was said to have sprayed vineyards for many years.

Vine spraying was the main occupation of patient 2.

258.3.4 Duration of single exposure

if applicable

No information provided.

258.3.5 Exposure concentration/dose

measured, estimated, not available
give all available information

No information provided.

258.3.6 Other information

The authors report that in Portugal, as a rule, six to eight treatments were made, but in some parts of the country there may be as many as 12 to 14 each season. The 'Bordeaux Mixture' is sprayed on the vines using either manual sprayers or, in mechanized vineyards, atomizers or other low-flow apparatus.

258.4 Examinations

give type of examination and time after exposure for each examination

The following examinations were performed on one or both patients:

- Examination of general condition,
- Test for tubercle bacilli in sputum,
- Chest radiography,
- Bronchoscopy,
- Lung function tests,
- Thoracotomy,
- Surgical lung biopsy and microscopic examination.

Further details are presented with results in point 4.2.

258.5 Treatment

give any available information on the medical treatment of intoxicated persons

Treatment during the period of hospitalisation was not discussed.

258.6 Remarks

Experimental work

This publication also reports experimental work in guineapigs which had been conducted in order to show a relationship between the pulmonary lesions and inhalation of the 'Bordeaux mixture'.

The authors concluded that experimental reproduction in the guinea-pig of lesions similar to those found in man confirmed the relationship between the inhalation of 'Bordeaux mixture' and the disease.

Due to significant reporting deficiencies, this experimental work is not discussed here.

259 RESULTS

259.1 Clinical Signs

Describe findings. If appropriate, include table.
Describe any relevant effects observed

Case 1

On admission, the patient's general condition was good, but he developed dyspnoea on moderate exertion.

Case 2

The patient was admitted to hospital showing weakness, loss of weight, and cough productive of thick, yellowish sputum.

259.2 Results of examinations

Describe the results of e.g. clinical chemistry, blood analysis and urinalysis

Case 1

Bronchoscopy was non-contributory. Blood count and sedimentation rate were within normal limits. Lung function tests showed some restriction and moderately decreased ventilatory parameters. At thoracotomy, the right lung showed extensive blue patches in which nodules and bands could be palpated due to their greater consistency in relation to the surrounding lung. The biopsy specimen showed that these corresponded to blue nodules and bands or to greyish white fibrous-appearing areas.

Microscopically the lesions had a focal distribution and corresponded to three different patterns, with all transitions between them: a varying number of alveoli filled with desquamated macrophages, granulomas in the alveolar septa, and fibro-hyaline nodules which seem to be the scars of the granulomas. The intra-alveolar macrophages frequently formed large sheets, and a yellowish-brown granular material was found in their cytoplasm. This did not give the haemosiderin reactions (Perls' technique) but was positive for copper techniques (rubeanic acid and benzidine). The alveolar septa in these areas were either infiltrated by lymphocytes, plasma cells, or histiocytes, and a number of fibroblasts, or were more or less fibrotic and, in some areas, hyalinized.

The granulomas developed in the septa and form rounded, well-limited nodules made up of histiocytes frequently containing the material already mentioned, and a few lymphocytes and plasma cells. Sometimes a few foreign body giant-cells containing copper or cholesterol inclusions were seen in the granulomas. Finally, all transitions between these granulomas and their fibro-hyaline scars

made up of whorls of collagen fibres concentrically placed and very similar to 'silicotic nodules' were found.
Polarized light showed no silica in these nodules and chemical studies showed no increase of this substance in the lung. Some of the cicatricial nodules contained appreciable quantities of the copper-containing material mentioned. Some of the sections showed large fibro-hyaline plaques due to the conglomeration of the cicatricial nodules with notable collagen formation (van Gieson's technique).

Microscopic study of the less involved areas of the lung showed small dispersed foci, similar to those described, around the bronchioli and vessels and sometimes in the interlobular septa.

Case 2

Sputum was negative for tubercle bacilli, as it always had been. The chest film showed increased pulmonary markings and an area of consolidation in the right upper lobe, considered to be a tuberculoma. Bronchoscopy was negative, as were the bronchial secretions for both tubercle bacilli and tumour cells. The blood count was within normal limits and the sedimentation rate was never above 35 mm/hour. Right upper lobectomy was done and blue areas were seen on the pleural surface of the lung, especially of the upper lobe.

Pathological examination of the lobe showed numerous blue foci, in plaques, one of which was 2.5 cm in its largest diameter and corresponded to the consolidation seen on the radiograph. There was also a fine, well-defined nodulation, standing out on the cut surface of the lung. Large sheets of fibrosis and marked thickening of the reticulum were seen in some areas. Microscopy showed, alongside the more diffuse lesions made up of large cicatricial foci preserving or destroying alveolar structure, various sized foci presenting three main patterns: areas of desquamative pneumonia, granulomas, and fibrohyaline scars. The desquamated cells formed sheets and contained a granulomatous, yellowish-brown material, Perls' negative, but giving the histochemical reactions for copper (rubeanic acid and benzidine). The septa that limit these sheets were partly infiltrated with lymphocytes, plasma cells, and fibroblasts and were partially transformed into fibrous bands, some times partially or totally hyalinized. The granulomas were rounded and made up of histiocytes, fibroblasts and some lymphocytes and plasma cells. The majority of the histiocytes had within their cytoplasm the same material seen in the macrophages. All transitions were seen between these granulomatous foci and the nodular, well-defined scars made up of concentrically disposed bands of hyalinized fibrous tissue. These nodules did not

contain silica and had plentiful copper-containing material. Some of them were partially broken down or contained small calcium deposits.

259.3 Effectivity of medical treatment

Treatment during hospitalisation was not discussed.

259.4 Outcome

Describe outcome / manifestation of symptoms / disease

Case 1: During the four months the patient was in hospital and under observation, dyspnoea disappeared and there was considerable radiological improvement. Lung function tests showed a slight improvement of ventilation.

Case 2: The outcome following these investigations is not given. The authors report that about 14 months before admission the subject had been (wrongly) diagnosis with tuberculosis, for which he was treated in a sanatorium for 10 months with streptomycin, isoniazid, and PAS, and slowly improved. On returning to his working conditions the symptoms soon reappeared.

259.5 Other

Describe any other significant observations

None.

Section A6.12.2(2)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*
	A6.12.2(2): Vineyard sprayer's lung

260 APPLICANT'S SUMMARY AND CONCLUSION

260.1 Materials and methods

Briefly describe circumstances

In the first case, a 35 year old male rural worker, who for many years had sprayed vineyards and cleaned the tartar from wine presses, was admitted to the Thoracic Surgery Centre of the Sanatorio D. Carlos I (IANT) in October 1965 for investigation of diffuse lung lesions. One year previously, tuberculosis had been diagnosed at mass radiography and the patient had been treated for nine months with streptomycin, isoniazid, and PAS. There was considerable improvement but not complete clearing on the radiograph and, as his sputum had always been persistently negative for tubercle bacilli, surgical lung biopsy was proposed.

In the second case, a 36 year old rural worker, whose main occupation was vine spraying, was admitted to the Thoracic Surgery Centre of the Sanatorio D. Carlos I (IANT) for weakness, loss of weight, and cough productive of thick, yellowish sputum. About 14 months before admission he had complained of the same symptoms and a chest radiograph led to the diagnosis of tuberculosis, for which he was treated in a sanatorium for 10 months with streptomycin, isoniazid, and PAS, and slowly improved. On returning to his working conditions the symptoms soon reappeared.

The following examinations were performed on one or both patients:

- Examination of general condition,
- Test for tubercle bacilli in sputum,
- Chest radiography,
- Bronchoscopy,
- Lung function tests,
- Thoractomy,
- Surgical lung biopsy and microscopic examination.

Section A6.12.2(2)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*

A6.12.2(2): Vineyard sprayer's lung

260.2 Results and discussion *Summarize relevant results*

Thoracotomy showed the intensely blue aspect of the visceral pleura which could not be explained by known pathological conditions. The lesions had a focal distribution and their density varied in different areas. The histological picture was well defined and seemed to progress through three principal stages: desquamation, intra alveolar macrophages, histiocytic granulomas in the inter-alveolar septa, and the scars of these lesions, the greater part of which showed as fibro-hyaline nodules somewhat similar to those found in silicosis. The granulomas sometimes contained foreign body giant-cells and rarely were of sarcoid type. The nodular scars may show areas of softening and calcification. The presence of the yellowish or yellowish- green material giving copper histochemical reactions was notable. X

The results of the pulmonary function tests in both cases agreed with the predominantly interstitial localization of the pulmonary damage.

The authors considered that there may be a relationship between the inhalation of ture' and the lung lesions seen. However, they concluded that there was no human or experimental data to show that the copper contained in the inhaled mixture was responsible for the lesions.

There was limited evidence of clearing of part of the lesions when the patient was removed from contact with the offending agent.

Section A6.12.2(2)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*

260.3 Conclusion

A6.12.2(2): Vineyard sprayer's lung

Give general conclusions

Pimentel and Marques (1969) report two cases of Portuguese rural workers who, for many years, had sprayed vineyards with 'Bordeaux mixture'. Both patients had previously been diagnosed with tuberculosis. As antituberculous therapy clearing was not complete and because of persistently negative sputum for tubercle bacilli, patients were admitted to hospital for further diagnostic investigations.

Surgical lung biopsy revealed copper containing lung lesions showing a well-defined histological picture characterised by three stages: intra-alveolar desquamation of macrophages, formation of predominantly histiocytic granulomas in the septa, and the healing of these lesions generally under the form of fibro-hyaline nodules.

The authors considered that there may be a relationship between the inhalation of 'Bordeaux mix' and the lesions seen. However, they concluded that there was no data to show that the copper contained in the inhaled mixture was responsible for the lesions.

No information on confounding variables, notably smoking status, was given. Only limited information was given on the 'Bordeaux mixture' and no exposure data was provided.

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]

Section A6.12.2(2) **Human Case Report**

Annex Point IIA6.12.2 *specify subsection number*

A6.12.2(2): Vineyard sprayer's lung

Results and discussion	[redacted]
Conclusion	[redacted]
Reliability	[redacted]
Acceptability	[redacted]
Remarks	

	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*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*
Remarks	

Section A6.12.2(3) **Human Case Report**

Annex Point IIA6.12.2 *specify subsection number*

IIA 6.12.2(3): Vineyard sprayer's lung

Official use only

261 REFERENCE

261.1 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).

Pimentel JC, Menezes AP. (1975) Liver granulomas containing copper in vineyard sprayer's lung - A new Etiology of Hepatic Granulomatosis. Am. Rev. Respir. Dis. 111:189-195. (published).

262 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

263 MATERIALS AND METHODS

263.1 Substance

Give any available information on substance/product, such as identity, physical form (e.g. powder, grain size, particle size/distribution), purity

'Bordeaux Mixture': [redacted]

263.2 Persons exposed

263.2.1 Sex: Males

263.2.2 Age/weight: Case 1: 57 years old
Case 2: 59 years old Case 3: 52 years old
No information on body weight available.

263.2.3 Known Diseases: *State if any; state, if healthy*
Unknown

263.2.4 Number of persons: 3 case reports are described.

263.2.5 Other information: Case 2: described as an alcoholic.
Case 3: described as having moderate drinking habits.

263.3 Exposure

Inhalation
Although no exposure data is available it assumed that inhalation to the 'Bordeaux mixture' occurred during spraying.
Indicate respective route, delete other routes

263.3.1 Reason of exposure: Occupational

263.3.2 Frequency of exposure: *specify frequency*
Multiple

263.3.3 Overall time period of exposure: *if applicable*
Case 1: 3 years (from the age of 50 to 53).
Case 2: 12 years.
Case 3: Unclear, assumed to be 15 years.

263.3.4 Duration of single exposure: *if applicable*
No information on the duration of single exposure provided.

Section A6.12.2(3) **Human Case Report**

Annex Point IIA6.12.2 *specify subsection number*

IIA 6.12.2(3): Vineyard sprayer's lung

263.3.5 Exposure concentration/dose

measured, estimated, not available
give all available information

No information on the exposure concentration provided.

263.3.6 Other information None

263.4 Examinations

give type of examination and time after exposure for each examination

Examinations are described for each patient with results in point 4.2.

263.5 Treatment

give any available information on the medical treatment of intoxicated persons

Treatment was described for patient 3 only, who received wide spectrum antimicrobial drugs.

263.6 Remarks

Bordeaux mixture used to spray vines typically contains 0.05-2% copper neutralized with soda lime [Pimentel JC & Marques F. (1969) "Vineyard sprayer's lung": a new occupational disease. Thorax, 24: 678-688; see section A.6.12.2(1)].

The authors note that hepatic granulomas are lesions made up of macrophages and epithelioid or similar cell types; their structure can vary from nodular aggregates to well-developed follicles, sarcoid or tubercie type. They can be caused by infections of various origin, reactions to drugs and other compounds, or diseases of unknown nature such as sarcoidosis, Wegener's granulomatosis, or Hodgkin's disease. The structure of the hepatic granulomas seen in these various situations is quite similar, and it is possible to determine their origin only on morphologic criteria, when acid-fast bacilli, fungi, or ova of parasites are identified within the lesions. For this reason, the granulomas observed in liver biopsies can be difficult to interpret, even after exhaustive clinical and laboratory investigations; in approximately one fourth of the cases it is impossible to determine the cause of the lesions.

264 RESULTS

Describe findings. If appropriate, include table.
Describe any relevant effects observed

264.1 Clinical Signs

Case 1: Frequent episodes characterised by fever, muscular pains, weakness, cough, and mucoid sputum during a period of three years prior to admission to hospital. The patient was intensely dyspnoeic and cyanotic on admission. Subcrepitant rales and disseminated wheezes could be heard over the upper third of both lungs fields.

Case 2: The patient was hospitalised for a febrile syndrome of unknown origin that had started 15 days before. During hospitalisation, the patient's condition deteriorated progressively with mental confusion, high fever, profuse sweating, abundant mucoid sputum, coma, and death.

Case 3: Chills and fever, joint and muscular pains, weakness and anorexia were evident during a 3 week period prior to hospitalisation.

Section A6.12.2(3)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*

IIA 6.12.2(3): Vineyard sprayer's lung

264.2 Results of examinations

Describe the results of e.g. clinical chemistry, blood analysis and urinalysis

Case 1: A chest roentgenogram showed diffuse bilateral reticular and micronodular shadows. Pulmonary function studies showed a restrictive ventilatory defect with normoxemia and slight respiratory alkalosis. Serum protein electrophoresis showed discrete hypergamma globulinemia (2.0 g per 100 ml). Liver function tests revealed a thymol turbidity of 13 units, cadmium reaction, 3 plus; serum alkaline phosphatase, 5 Bodansky units; serum glutamic oxaloacetic transaminase (SGOT), 50 units per ml; serum glutamic pyruvic transaminase (SGPT), 16 units per ml. The patient died of bilateral spontaneous pnueumothorax. Autopsy showed bilateral diffuse pulmonary fibrosis with numerous dark blue micronodules throughout all the lobes and marked bullous emphysema in the lower lobes. Histology of the lung showed numerous histiocytic granulomas and fibro-hyaline nodular scars, frequently conglomerated, in the intralveolar septa. These lesions contained abundant inclusions of copper.

Case 2: Examination of the abdomen showed a liver 4 finger-breaths below the right costal margin, with an irregular surface and hard consistency. A chest roentgenogram showed reticular and micronodular shadows, predominantly in the lower half of the right lung. Repeated blood cultures and *Brucella* and *Salmonella* agglutinin titers were negative. Serum protein electrophoresis showed hypoalbuminemia (2.6 g per 100 ml) and an increase in the gammaglobulin fraction (2.8 g per 100 ml). Liver function tests revealed total cholesterol, 120 mg per 100 ml (66 % esterificated), thymol turbidicity, thymol turbidity, 31 units; cephaline-cholesterol flocculation and Takata reaction, 3 plus; serum alkaline phosphatase, 5.5 Bodansky units; SGOT, 364 units per ml; SGPT, 110 units per ml; prothrombin time, 84 %. The patient's condition deteriorated progressively with mental confusion, high fever, profuse sweating, abundant mucoid sputum, coma, and death. Autopsy showed numerous blue nodules and extensive fibrosis in both lungs. On histocytic examination there were numerous histiocytic granulomas localized in the septa and extensive nodular scars, often conglomerated, that in some areas had softened and broken down. Histochemical methods showed that these lesions contained considerable amounts of copper. The liver was enlarged and finely nodular on the surface and on the section, with the usual aspect found in micronodular cirrhosis. There was also a slightly enlarged spleen, oesophageal varices, and a small amount of fluid in the peritoneal cavity.

Case 3: A chest roentgenogram showed increased lung markings and a homogeneous density in the lower third of the right lung field, interpreted as pneumonia with pleural reactions. No acid-fast bacilli or other bacteria could be found in smears and cultures of the patient's sputum. The liver was enlarged 2 fingerbreadths below the right costal margin, with smooth rounded edge. Serum protein electrophoresis showed hypergamaglobulinemia (2.4 g per 100 ml); thymol turbidity, 14 units; cephalin-cholesterol flocculation, 3 plus; Takata reaction, weakly positive; serum alkaline phosphatase, 6 Bodansy units; SGOT, 36 units per ml; SGPT, 34 units per ml. A percutaneous liver biopsy was performed. The patient was treated with wide spectrum antimicrobial drugs with improvement of both clinical and radiologic status. To attempt __ to determine the cause of the increased lung markings, a lung biopsy was performed 3 weeks later. Histology showed histiocytic granulomas that in some areas were in an advance condition of sclerosis and hyalinization. These lesions contained abundant inclusions of copper.

Section A6.12.2(3)	Human Case Report
Annex Point IIA6.12.2	*specify subsection number*
	IIA 6.12.2(3): Vineyard sprayer's lung
264.3 Effectivity of medical treatment	Medical treatment was not described for case 1 or 2. Case 3: The patient was treated with wide spectrum antimicrobial drugs with improvement of both clinical and radiologic status.
264.4 Outcome	*Describe outcome / manifestation of symptoms / disease* Case 1 and 2: died, see point 4.2 Case 2: outcome is not described.
264.5 Other	*Describe any other significant observations* None

265 APPLICANT'S SUMMARY AND CONCLUSION

Briefly describe circumstances

Pimentel and Menezes (1975) described pulmonary and liver lesions which occurred in three rural workers who had been exposed to 'Bordeaux mixture' fungicide in their work as vineyard sprayers for 3 to 15 years. Patients were examined for pulmonary and hepatic changes (described under point 4.2).

265.1 Materials and methods

Summarize relevant results

Two of the cases resulted in death. Reported signs and symptoms in the three cases included fever, joint and muscular pains, weakness, cough, mucoid sputum, dyspnoea and weight loss. The pulmonary lesions were characterised by the presence of granulomas and fibro-hyaline nodular scars containing inclusions of copper. Hepatic changes in all three cases were characterised by proliferation and swelling of Kupffer cells and the development of histiocytic or sarcoid granulomas. The presence of copper in these hepatic lesions was confirmed using histochemical techniques. Micronodular cirrhosis also occurred in one case and fatty liver in another case; both of these hepatic lesions were attributed to alcohol consumption. With the exception of the case in which cirrhosis occurred, hepatic lesions were reported as not being accompanied by significant clinical or biochemical changes.

265.2 Results and discussion

Author's discussion: The cases presented suggest that in all patients in whom pulmonary and hepatic granulomas of unknown cause co-exists or in whom the diagnosis of sarcoidosis is made by exclusion, the possibility of the existence of a disease caused by inhalation of pathogenic dusts must be considered; an attempt must be made to determine the nature of the disease by looking for the possible aggressive substance within the lesions, using the appropriate histochemical techniques.

Section A6.12.2(3)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*
	IIA 6.12.2(3): Vineyard sprayer's lung
265.3 Conclusion	*Give general conclusions*
	Pimentel and Menezes (1975) described pulmonary and liver lesions seen in three subjects that had been exposed to 'Bordeaux mixture' fungicide in their work as vineyard sprayers for 3 to 15 years. Two of the cases resulted in death. Reported signs and symptoms in the three cases included fever, muscular pains, weakness, cough, mucoid sputum, dyspnoea and weight loss. The pulmonary lesions were characterised by the presence of granulomas and fibro-hyaline nodular scars containing inclusions of copper. Hepatic changes in all three cases were characterised by proliferation and swelling of Kupffer cells and the development of histiocytic or sarcoid granulomas. The presence of copper in these hepatic lesions was confirmed using histochemical techniques. No information was provided regarding the smoking status of the three individuals. One case was reported to be "alcoholic" and another case was described as having "moderate drinking habits". Only limited information was given on the 'Bordeaux mixture' and no exposure data was provided.

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

	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss if deviating from view of rapporteur member state*

Section A6.12.2(3)	**Human Case Report**
Annex Point IIA6.12.2	*specify subsection number*
	IIA 6.12.2(3): Vineyard sprayer's lung
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A6.12.2(4) **Human Case Report**

Annex Point IIA6.12.2 *Specify subsection number*

A.6.12.2(4): Vineyard sprayer's lung

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266 REFERENCE

266.1 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).
Pimentel JC, Menezes AP. (1977) Liver disease in vineyard sprayers. Gastroenterology 72:275-283. (published).

267 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

268 MATERIALS AND METHODS

268.1 Substance

Give any available information on substance/product, such as identity, physical form (e.g. powder, grain size, particle size/distribution), purity
'Bordeaux Mixture': [redacted]

268.2 Persons exposed

268.2.1 Sex: Not specified, subjects described as 'rural workers'.

268.2.2 Age/weight: No information provided.

268.2.3 Known Diseases *State if any; state, if healthy*
All workers had "Vineyards sprayer's lung".
[Vineyard Sprayers lung is described by Pimentel JC & Marques F (1969) 'Vineyard sprayer's lung': a new occupational disease. Thorax, 24: 678-688, see section A.6.12.2(1)].
In most cases examination of livers were conducted at autopsy. No further information on the health status is provided. However, the authors indicate that all cases with other possible causes of liver damage, such as hepatitis, alcoholism, and exposure to hepatotoxic substances, including pesticides containing inorganic arsenic, were excluded.

268.2.4 Number of persons 30 vineyard workers. 268.2.5 Other information None.

268.3 Exposure

Indicate respective route, delete other routes
Inhalation
Although no exposure data is available it is assumed that inhalation to the 'Bordeaux mixture' occurred during spraying.

268.3.1 Reason of exposure Occupational

268.3.1 Reason of exposure Occupational

Single/multiple
Multiple

268.3.5 Exposure concentration/dose: Spraying was carried out from 15 to 100 days per year.

measured, estimated, not available give all available information

Bordeaux mixture contained 1-2% copper sulphate.

268.3.6 Other information: 600 litres of mixture were sprayed each day by each worker.

268.4 Examinations

give type of examination and time after exposure for each examination

The livers of 30 rural workers who sprayed vineyards with Bordeaux mixture were studied. The spleens of 4 of them were also examined.

The morphological changes in the liver were classified according to predominant aspects, and sometimes, the clinical and laboratory findings.

The specimens were fixed in a 10% neutral formaldehyde solution and embedded in paraffin. The following staining techniques were used: haematoxylin-eosin, van Gieson, Wilder, periodic acid-Schiff, Perls, Unna-Pappanheim, Gomori for fungi, and Ziehi-Neelsen. The sulphide-silver (slightly modified), rubeanic acid, and benzidine methods were used for the histochemical localization of copper. Conventional, polarized light, phase contrast, and interference microscopy were used in these examinations. In the case of angiosarcoma, hepatic copper levels were estimated by atomic absorption spectrophotometry in tumour and in tumour free portions of the liver.

Normal livers were used as controls.

give any available information on the medical treatment of intoxicated persons

268.5 Treatment Treatment is not discussed in this study; most samples were taken at autopsy.

268.6 Remarks

Experimental work

Experimental work was carried out in young male guinea pigs (three groups of 6), fed on a diet appropriate for their development and kept at 18 to 22°C. These animals were exposed daily for 5 min to aerosols of 0.4% aqueous solutions of two fungicide formulations: group 1 received copper oxychloride (containing 50% copper) and group 2 received oxychloride (37.5% Cu) plus zineb (16%). The flow of the nebulizer was 0.2 to 0.3 ml per min. A third group represented control animals. The animals were killed after 60, 120, 200, 270, and 420 periods of exposure. The histological techniques used were the same as those applied to the human material.

After 70 days of exposure, animals showed copper inclusions within swollen Kupffer cells and histiocytes in the portal tracts and subcapsular areas. In 3 animals killed after 270 days of exposure, a close association was noted between the lesion reported and perisinusoidal and portal fibrosis.

269 RESULTS

Describe findings. If appropriate, include table.

269.1 Clinical Signs

Describe any relevant effects observed

Clinical signs were not reported.

269.2 Results of examinations

Examination of liver tissue samples from subjects, taken either at autopsy or surgical biopsy, revealed the following morphological changes:

- focal or diffuse swelling and proliferations of Kupffer cells in all 30 cases,
- histiocytic or sarcoid like granulomata (7 seven cases),
- fibrosis of variable degree in the perisinusoidal, portal and subcapsular areas, accompanied by atypical proliferation of the sinusoidal lining cells (8 cases),
- micronodular cirrhosis (3 cases),
- idiopathic portal hypertension (2 cases),
- angiosarcoma of the liver (1 case).

Abundant deposits of copper were revealed by histochemical techniques within hepatic and pulmonary lesions in these patients.

269.3 Effectivity of medical treatment

Treatment was not discussed in this report.

Section A6.12.2(4) **Human Case Report**

Annex Point IIA6.12.2 *Specify subsection number*

A.6.12.2(4): Vineyard sprayer's lung

269.4 Outcome

Describe outcome / manifestation of symptoms / disease

Outcome was not investigated in this report. In most cases samples were examined at necropsy.

269.5 Other

Describe any other significant observations

None

270 APPLICANT'S SUMMARY AND CONCLUSION

270.1 Materials and methods

Briefly describe circumstances

The livers of 30 rural workers who sprayed vineyards with Bordeaux mixture for periods that varied from 3 to 45 years (mean 18 years) were studied. The spleens of 4 of them were also examined.

The morphological changes in the liver were classified according to predominant aspects, and sometimes, the clinical and laboratory findings. All cases with other possible causes of liver damage, such as hepatitis, alcoholism, and exposure to hepatotoxic substances, including pesticides containing inorganic arsenic, were excluded.

One case of proliferation of Kupffer cells, 2 of granuloma, and 3 of liver fibrosis were studied by percutaneous biopsy. In 2 cases of idiopathic portal hypertension surgical biopsies were done and, in all other cases, the liver was examined at autopsy. The spleen was studied after splenectomy in 2 cases of idiopathic portal hypertension and at autopsy in the other cases.

Section A6.12.2(4)	**Human Case Report**
Annex Point IIA6.12.2	*Specify subsection number* **A.6.12.2(4): Vineyard sprayer's lung**
270.2 Results and discussion	*Summarize relevant results* Liver disease with inclusions of copper was identified in all 30 cases. The following changes were seen: diffuse or focal swelling of Kupffer cells with granular inclusions of copper (30 cases), histiocytic or sarcoid-type granulomas containing variable amounts of copper (7 cases), liver fibrosis (8 cases), cirrhosis (3 cases), idiopathic portal hypertension (2 cases) and liver angiosarcoma (1 case). Author's discussion: Copper is a normal component of hepatic parenchymal cells. It may occur there in higher than normal levels in various conditions such as Wilson's disease, cryptogenic cirrhosis, and primary biliary cirrhosis. Copper may also occur within the reticuloendothelial elements of the liver or in granulomatous lesions. Our observations in vineyard sprayers demonstrate that copper enters the body via a nonphysiological route (inhalation), and in a non physiological form ('Bordeaux mixture'), is deposited inside the reticuloendothelial cells of the liver, and produces different kinds of lesions. Copper particles inhaled during the spraying of copper sulphate may reach the liver by the bloodstream. The digestive route does not seem to be involved, as exposure occurs almost exclusively by inhalation. The identification of copper in other areas, such as the lymphatic system, spleen, or kidney may be explained also by a haematogenous dissemination of the inhaled material. The observations on the human and experimental material suggest an etiological relationship between exposure to copper sulphate and the lesions described. A morphological resemblance was noted between the liver disease of vineyard sprayers and the hepatic lesions reported in workers exposed to inorganic arsenic and to vinyl chloride.

Section A6.12.2(4)	Human Case Report
Annex Point IIA6.12.2	*Specify subsection number* **A.6.12.2(4): Vineyard sprayer's lung**

270.3 Conclusion

Give general conclusions

Pimentel and Menezes, (1977) investigated liver disease in a group of former vineyard workers with 'vineyard sprayer's lung'. Thirty subjects were investigated who had sprayed vineyards with 'Bordeaux mixture' for a mean duration of 18 years (range 3-45 years). Liver tissue samples from subjects, taken either at autopsy or surgical biopsy showed: diffuse or focal swelling of Kupffer cells with granular inclusions of copper occurred in all 30 cases, histiocytic or sarcoid-type granulomas containing variable amounts of copper (7 cases), liver fibrosis (8 cases), cirrhosis (3 cases), idiopathic portal hypertension (2 cases) and liver angiosarcoma (1 case). The authors conclude that their observations "suggest an etiological relationship between exposure to copper sulphate and the lesions described". The study report provided little information on the cases studied, for example the basis of selection. Only limited information was given on the 'Bordeaux mixture' and no exposure data was provided.

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]
[redacted]	

COMMENTS FROM ... *(specify)*

Date	*Give date of comments submitted*
Materials and Methods	*Discuss if deviating from view of rapporteur member state*

Section A6.12.2(4)	**Human Case Report**
Annex Point IIA6.12.2	*Specify subsection number*

A.6.12.2(4): Vineyard sprayer's lung

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

Remarks

Official use only

271 REFERENCE

271.1 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).
Tanner MS, Portmann B, Mowat AP, Williams R, Pandit AN, Mills CF, Bremner I (1979) Increased hepatic copper concentration in Indian Childhood Cirrhosis. Lancet 1:1203-5 (published).

272 GUIDELINES AND QUALITY ASSURANCE (NOT APPLICABLE)

273 MATERIALS AND METHODS

273.1 Substance
Give any available information on substance/product, such as identity, physical form (e.g. powder, grain size, particle size/distribution), purity
[redacted]

273.2 Persons exposed

273.2.1 Sex: The proportion of male and female children was not reported.

273.2.2 Age/weight: 0.9 - 16 years (10 children between age 1 and 3 years). See Table A.6.12.2(4)-1.

273.2.3 Known Diseases
State if any; state, if healthy
All children had been diagnosed with liver disease.

273.2.4 Number of persons: 19

273.2.5 Other information: All children were from India: 8 children were from Pune, 8 from Madras, and one from Benares, Bombay, and Vellore.

273.3 Exposure
Oral/Inhalation/Dermal
Indicate respective route, delete other routes
Exposure is not investigated in this study.

273.3.1 Reason of exposure
occupational, accidental, abuse, attempted suicide
ee point 3.3.
S

273.3.2 Frequency of exposure
Single/multiple
specify frequency
See point 3.3.

273.3.3 Overall time period of exposure
if applicable
See point 3.3.

273.3.4 Duration of single exposure
if applicable
See point 3.3.

273.3.5 Exposure concentration/dose
measured, estimated, not available
give all available information
See point 3.3.

273.3.6 Other information: None