## Section A6.8.1 Range-finding study

## Annex Point IIA6.8.1 Rabbit

Official use only

### 86 REFERENCE

**86.1 Reference**

**A6.8.1/01: Doc.No. 00620B-IIA-681a**

[redacted] (2003): Five copper substances – repeated dose toxicity and tolerability study in non-pregnant rabbits; [redacted]; Report no.: DuPont-11638, November 17, 2003.

**86.2 Data protection** Yes

86.2.1 Data owner: Spiess-Urania Chemicals GmbH, Hamburg, Germany

86.2.2 Companies with letter of access: --

86.2.3 Criteria for data protection: Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.

### 87 GUIDELINES AND QUALITY ASSURANCE

**87.1 Guideline study** No

Range-finding study to determine the maximum tolerated dose with regard to clinical toxicity.

**87.2 GLP** Yes

Self-certified laboratory

**87.3 Deviations** Not applicable

### 88 MATERIALS AND METHODS

**88.1 Test material**

(i) Copper hydroxide

(ii) Copper oxide

(iii) Copper oxychloride

(iv) Tribasic copper sulphate

(v) Bordeaux mixture

88.1.1 Lot/Batch number

(i) 380-71-05

(ii) 280802

(iii) 27003B

(iv) 471/2002

(v) 1/170

88.1.2 Specification: Not specified.

88.1.3 Purity

(i) 60.1 % copper

(ii) 87 % copper

(iii) 57 % copper

(iv) 31.12 % copper

(v) 27.38 % copper

**Section A6.8.1** **Range-finding study**

**Annex Point IIA6.8.1** **Rabbit**

| | | | |
|---|---|---|---|
| 88.1.4 | Description | (i) light blue powder<br>(ii) red-brown powder<br>(iii) light green, fine homogeneous powder<br>(iv) greenish-blue solid<br>(v) green powder | |
| 88.1.5 | Stability | No evidence of instability was observed. | |
| **88.2** | **Test animals** | | |
| 88.2.1 | Species | Rabbit | |
| 88.2.2 | Strain | New Zealand White | |
| 88.2.3 | Source | [redacted] | |
| 88.2.4 | Sex | Female (non-pregnant) | |
| 88.2.5 | Age/weight at study initiation | Age: 6 to 6.5 month<br>Body weight: 3382 – 4116 g (day after arrival) | |
| 88.2.6 | Number of animals per group | Part 1: 2 to 4<br>Part 2: 2 | |
| 88.2.7 | Control animals | No | |
| 88.2.8 | Mating period | Not applicable | |
| **88.3** | **Administration/ Exposure** | Oral | |
| 88.3.1 | Duration of exposure | Part 1: 14 days<br>Part 2: 7 days | |
| 88.3.2 | Post-exposure period | None | |
| 88.3.3 | Type | By gavage | |
| 88.3.4 | Concentration | Part 1: 30 mg/kg bw/day for each test substance<br>Part 2: 50/40 mg/kg bw/day for each test substance (lowered to 40 mg/kg bw/day on test day 2 on humane grounds) | X |
| 88.3.5 | Vehicle | 0.5 % aqueous methylcellulose | |
| 88.3.6 | Concentration in vehicle | Not stated in the report. | X |
| 88.3.7 | Total volume applied | 1 mL/ kg bw | |
| 88.3.8 | Controls | None | |
| **88.4** | **Examinations** | | |
| 88.4.1 | Body weight | Yes (daily) | |
| 88.4.2 | Food consumption | Yes (daily) | |
| 88.4.3 | Clinical signs | Yes (daily) | |

**Section A6.8.1** **Range-finding study**

**Annex Point IIA6.8.1** **Rabbit**

| | | |
|---|---|---|
| 88.4.4 | Gross pathology and histopathology | Yes<br>Gross external and visceral examination was performed for all animals. |
| **88.5** | **Further remarks** | None |
| | | **89 RESULTS** |
| **89.1** | **Clinical signs and necropsy** | No mortality or clinical signs were observed in the two animals administered with 30 mg Cu/kg bw/day as **copper hydroxide**. Upon necropsy ulceration and discolouration of the stomach lining was noted in one female. During part 2 of the study, one female died after administration of 50 mg Cu/kg bw/day. The surviving animal showed stomach ulceration upon necropsy.<br><br>One female dosed with 30 mg Cu/kg bw/day as **copper oxide** showed transient diarrhoea. Necropsy observations of the two females of this dose group included discolouration and haemorrhages in the stomach linings. One animal died after administration at the 50 mg/kg level and necropsy revealed discoloration and thickening of the stomach lining. The survivor dosed at 40 mg/kg bw/day for the remaining 6 days showed no adverse clinical signs. Ulceration of the stomach lining was noted upon necropsy.<br><br>Two of three females administered with **copper oxychloride** were found dead during part 1 of the study. The other female showed no adverse clinical signs. One of two females died after administration of the 50 mg/kg bw dose. The other female survived during part 2 of the study. Necropsy observations for both surviving animals included thickening and discoloration of and/or ulceration of the stomach lining.<br><br>One of four females dosed with **tribasic copper sulphate** showed transient diarrhoea. It should be noted that two of these four females were inadvertently under-dosed by about 40 % for 8 days and one of the four was under-dosed for one day. Necropsy observations for three of four females included discolouration and/or ulcerations and/or haemorrhagic areas of the stomach lining. Similar observations were reported upon necropsy for the two females dosed in the 2$^{nd}$ part of the study.<br><br>The two females dosed with **Bordeaux mixture** during part 1 of the study showed no adverse clinical signs or necropsy findings. One female died after administration with 50 mg Cu/kg bw and necropsy revealed discoloration and thickening of the stomach lining. Necropsy findings of the surviving high dose female included also discoloration and thickening in the stomach lining. |

| Section A6.8.1 | Range-finding study |
|---|---|
| Annex Point IIA6.8.1 | Rabbit |

| | | |
|---|---|---|
| **89.2** | **Body weight and food consumption** | For all copper substances, the body weight change data for animals that survived until test termination indicated dose-related body weight losses during the first week of administration, concurrent to reduced food consumption. Animals generally appeared to recover from the second week of dosing onwards. |
| | | **90 APPLICANT'S SUMMARY AND CONCLUSION** |
| **90.1** | **Materials and methods** | Female non-pregnant rabbits received 30 or 50/40 mg/kg bw/day (reduced to 40 mg/kg bw/day on day 2) of five copper test substances by gavage for 14 or 7 consecutive days, respectively. The study was designed as a range-finding study to determine the maximum tolerated dose relevant for dose selection in subsequent studies. |
| **90.2** | **Results and discussion** | The results of the study indicated that generally similar patterns of toxicity were evident for all copper substances tested. Animals surviving up to study termination showed a compound-related reduction in food consumption and subsequent reduced body weight losses during the first week of dosing. Animals generally appeared to recover from the second week of dosing onwards. Necropsy observations from all groups revealed apparent compound-related stomach lesions including haemorrhages, ulcerations, discoloration and/or thickening of the lining. |
| **90.3** | **Conclusion** | The general pattern and degree of inappetance and weight loss followed by recovery, and the observation of stomach ulceration at necropsy was considered sufficient to show that there were no major differences in the sensitivity of the rabbit to the five copper substances. Doses greater than 30 mg Cu/kg bw/day were considered unsustainable for repeat dosing studies. As there were no major differences between the five substances, further preliminary investigations would be performed on only one substance, copper hydroxide. |
| 90.3.1 | Reliability | 2 |
| 90.3.2 | Deficiencies | No |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE (*)** |
| **Date** | 24/12/2004 |
| **Materials and Methods** | 3.3.4 – doses are expressed as mg **Cu**/kg bw<br>3.3.6 – concentrations were adjusted for copper content: 30 mg Cu/mL for exp 1 and 50/40 mg Cu/mL for exp 2 |
| **Results and discussion** | Agree with applicant's version |

| | |
|---|---|
| **Conclusion** | Agree with applicant's version |
| **Reliability** | 2 |
| **Acceptability** | Acceptable |
| **Remarks** | This study is not really needed in this section as it is not a teratogenicity study. Only a summary in the main teratogenicity study would have been sufficient. |
| | **COMMENTS FROM ...** |
| **Date** | |
| **Materials and Methods** | |
| **Results and discussion** | |
| **Conclusion** | |
| **Reliability** | |
| **Acceptability** | |
| **Remarks** | |

## Section A6.8.1 Range-finding study

## Annex Point IIA6.8.1 Rabbit

| | | | Official use only |
|---|---|---|---|
| | | **91 REFERENCE** | |
| **91.1** | **Reference** | **A6.8.1/02: Doc. No. 00620B-IIA-681b**<br>[redacted] (2003): Copper – A 23-day tolerability study in non-pregnant rabbits; [redacted]; Report no.: DuPont-11762, November 17, 2003. | |
| **91.2** | **Data protection** | Yes | |
| 91.2.1 | Data owner | Spiess-Urania Chemicals GmbH, Hamburg, Germany | |
| 91.2.2 | Companies with letter of access | -- | |
| 91.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I. | |
| | | **92 GUIDELINES AND QUALITY ASSURANCE** | |
| **92.1** | **Guideline study** | No | |
| | | Range-finding study to assess effects of treatment equal in duration to a teratology study in the non-pregnant rabbit. | |
| **92.2** | **GLP** | Yes | |
| | | Self-certified laboratory | |
| **92.3** | **Deviations** | Not applicable | |
| | | **93 MATERIALS AND METHODS** | |
| **93.1** | **Test material** | As given in section 2. | X |
| 93.1.1 | Lot/Batch number | 380-71-05 | |
| 93.1.2 | Specification | As given in section 2. | X |
| 93.1.3 | Purity | 60.1 % | X |
| 93.1.4 | Description | Light blue powder | |
| 93.1.5 | Stability | No evidence of instability was observed. | |
| **93.2** | **Test animals** | | |
| 93.2.1 | Species | Rabbit | |
| 93.2.2 | Strain | New Zealand White | |
| 93.2.3 | Source | [redacted] | |
| 93.2.4 | Sex | Female (non-pregnant) | |
| 93.2.5 | Age/weight at study initiation | Age: 6 to 6.5 month<br>Body weight: not stated | |
| 93.2.6 | Number of animals per group | 5 | |
| 93.2.7 | Control animals | Yes | |
| 93.2.8 | Mating period | Not applicable | |

**Section A6.8.1** **Range-finding study**

**Annex Point IIA6.8.1** **Rabbit**

| | | |
|---|---|---|
| **93.3** | **Administration/ Exposure** | Oral |
| 93.3.1 | Duration of exposure | 23 days |
| 93.3.2 | Post-exposure period | None |
| 93.3.3 | Type | By gavage |
| 93.3.4 | Concentration | 0, 7.5, 15, 30 mg Cu/kg bw/day |
| 93.3.5 | Vehicle | 0.5 % aqueous methylcellulose |
| 93.3.6 | Concentration in vehicle | 0, 7.5, 15, 30 mg/mL |
| 93.3.7 | Total volume applied | 1 mL/ kg bw |
| 93.3.8 | Controls | Vehicle only |
| **93.4** | **Examinations** | |
| 93.4.1 | Body weight | Yes (daily) |
| 93.4.2 | Food consumption | Yes (daily) |
| 93.4.3 | Clinical signs | Yes (daily) |
| 93.4.4 | Gross pathology and histopathology | Yes<br>Gross external and visceral examination was performed for all animals. |
| **93.5** | **Further remarks** | None |

**94 RESULTS**

| | | |
|---|---|---|
| **94.1** | **Clinical signs and necropsy** | Two females receiving the highest tested dose level were found dead after 2 or 3 days of administration. For the animal found dead on day 3, clinical observations prior to death included lethargy, weakness, and abnormal gait or mobility. Necropsy findings for these animals and for one survivor included haemorrhages and/or discoloration of the stomach lining. No compound-related mortality was observed at doses of ≤ 15 mg Cu/kg bw/day. |
| **94.2** | **Body weight and food consumption** | Administration of 7.5 mg Cu/kg bw/day for 23 consecutive days produced no effects on body weight or food consumption. Compound-related reductions in food consumption and consequently body weight were observed at the mid and high dose level. Body weight losses and reductions in food consumption were observed during the first week of dosing. Thereafter animals generally resumed eating and gained weight. |

**95 APPLICANT'S SUMMARY AND CONCLUSION**

| | | |
|---|---|---|
| **95.1** | **Materials and methods** | Female non-pregnant rabbits received 0, 7.5, 15 or 30 mg Cu/kg bw/day as copper hydroxide by gavage for 23 consecutive days. The study was designed as a range-finding study to assess effects of treatment equal in duration to a teratology study in the non-pregnant rabbit. |

| Section A6.8.1 | Range-finding study |
|---|---|
| Annex Point IIA6.8.1 | Rabbit |

| | | |
|---|---|---|
| **95.2** | **Results and discussion** | Compound-related effects on body weight and food consumption were observed after administration of 15 or 30 mg Cu/kg bw/day for 23 consecutive days. Two females of the high dose group died during the first 3 days of dosing. Necropsy findings for these animals and for one survivor included haemorrhages and/or discoloration of the stomach lining. No compound-related effects were observed at 7.5 mg Cu/kg bw/day. |
| **95.3** | **Conclusion** | |
| 95.3.1 | Reliability | 2 |
| 95.3.2 | Deficiencies | No |

| **Evaluation by Competent Authorities** | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | **EVALUATION BY RAPPORTEUR MEMBER STATE (*)** |
| **Date** | 02/12/2004 |
| **Materials and Methods** | 3.1 and 3.1.2 Not for Copper Carbonate<br>3.1.3 purity: 92.3 % (Metallic Copper Equivalent 60.1 %) |
| **Results and discussion** | Agree with applicant's version |
| **Conclusion** | Agree with applicant's version |
| **Reliability** | 2 |
| **Acceptability** | Acceptable |
| **Remarks** | This study is not really needed in this section as it is not a teratogenicity study. Only a summary in the main teratogenicity study would have been sufficient. |
| | **COMMENTS FROM ...** |
| **Date** | |
| **Materials and Methods** | |
| **Results and discussion** | |
| **Conclusion** | |
| **Reliability** | |
| **Acceptability** | |
| **Remarks** | |

## Section A6.8.1 Pilot developmental toxicity study

## Annex Point IIA6.8.1 Rabbit

| | | | Official use only |
|---|---|---|---|
| | | **96 REFERENCE** | |
| **96.1** | **Reference** | **A6.8.1/03: Doc.No. 00620B-IIA-681c**<br>[redacted] (2003): Copper hydroxide – pilot developmental toxicity study in rabbits; [redacted]<br>Report no.: DuPont-11861, November 17, 2003. | |
| **96.2** | **Data protection** | Yes | |
| 96.2.1 | Data owner | Spiess-Urania Chemicals GmbH, Hamburg, Germany | |
| 96.2.2 | Companies with letter of access | -- | |
| 96.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I. | |
| | | **97 GUIDELINES AND QUALITY ASSURANCE** | |
| **97.1** | **Guideline study** | No<br>Pilot study to provide preliminary information on the potential maternal and developmental toxicity of copper hydroxide. | |
| **97.2** | **GLP** | Yes<br>Self-certified laboratory | |
| **97.3** | **Deviations** | Not applicable | |
| | | **98 MATERIALS AND METHODS** | |
| **98.1** | **Test material** | As given in section 2. | X |
| 98.1.1 | Lot/Batch number | 021121/1 | |
| 98.1.2 | Specification | As given in section 2. | X |
| 98.1.3 | Purity | 61.14 % copper | X |
| 98.1.4 | Description | Free flowing blue product, needles | |
| 98.1.5 | Stability | No evidence of instability was observed. | |
| **98.2** | **Test animals** | | |
| 98.2.1 | Species | Rabbit | |
| 98.2.2 | Strain | New Zealand White | |
| 98.2.3 | Source | [redacted] | |
| 98.2.4 | Sex | Female (pregnant) | |
| 98.2.5 | Age/weight at study initiation | Age: 5 to 6.5 month old<br>Body weight: 2885 to 4330 g (day of gestation) | |
| 98.2.6 | Number of animals per group | 5 females (control group),<br>8 females (7.5 and 30 mg/kg bw),<br>9 females (15 mg/kg bw) | |
| 98.2.7 | Control animals | Yes | |

**Section A6.8.1** **Pilot developmental toxicity study**

**Annex Point IIA6.8.1** **Rabbit**

| | | |
|---|---|---|
| 98.2.8 | Mating period | Not stated in the report. |
| **98.3** | **Administration/ Exposure** | Oral |
| 98.3.1 | Duration of exposure | day 7 to 28 of gestation |
| 98.3.2 | Postexposure period | Animals were sacrificed on day 29 of gestation. |
| 98.3.3 | Type | By gavage |
| 98.3.4 | Concentration | 0, 7.5, 15 or 30 mg Cu/kg bw/day |
| 98.3.5 | Vehicle | 0.5 % aqueous methylcellulose |
| 98.3.6 | Concentration in vehicle | 0, 7.5, 15, 30 mg/mL |
| 98.3.7 | Total volume applied | 1 mL/ kg bw |
| 98.3.8 | Controls | Vehicle only |
| **98.4** | **Examinations** | |
| 98.4.1 | Body weight | Yes (daily) |
| 98.4.2 | Food consumption | Yes (daily) |
| 98.4.3 | Clinical signs | Yes (daily) |
| 98.4.4 | Gross pathology and histopathology | Yes<br>Gross external and visceral examination was performed for all animals. |
| 98.4.5 | Examination of uterine content | gravid uterine weight, number of corpora lutea, number of implantations |
| 98.4.6 | Examination of foetuses | General: No. of live and dead foetuses, No. of resorptions foetal weight, sex ratio, external alterations<br>Skelet: No<br>Soft tissue: No |
| **98.5** | **Further remarks** | None |

**99 RESULTS**

## Section A6.8.1 Pilot developmental toxicity study

## Annex Point IIA6.8.1 Rabbit

| | | |
|---|---|---|
| **99.1** | **Maternal toxic effects** | Low incidences of diarrhoea were observed at all dose levels and were considered to be treatment-related. Treatment-related mortality was observed at a dose of 30 mg Cu/kg bw/day. One rabbit was sacrificed *in extremis* on day 9 of gestation and another rabbit was found dead on day 26 of gestation. Gross necropsy revealed stomach haemorrhages or a small liver that was moderately autolysed. Histopathology of selected tissues of the rabbit sacrificed non-scheduled indicated that the cause of death was related to a haemolytic event that resulted in haemoglobin nephropathy and probably renal failure.<br><br>Clearly compound-related reductions of maternal body weight, weight change and food consumption were observed at 15 and 30 mg Cu/kg bw/day. Generally these findings occurred during the first week of dosing and animals recovered thereafter. No treatment-related effects on maternal body weight or food consumption were observed at 7.5 mg Cu/kg bw/day. |
| **99.2** | **Developmental toxic effects** | There was evidence of compound-related developmental toxicity at 30 mg Cu/kg bw/day. Mean foetal weights were reduced by 12 % relative to the control group. Foetal resorptions appeared slightly increased at this level and 4 foetuses (2 each from 2 litters) were observed with omphalocele (protrusion of intestines at the umbilicus). No evidence of developmental toxicity was observed at the other dose levels. One foetus of the 7.5 mg Cu/kg bw/day group had anasarca, domed head and a short tail. This finding was considered to be incidental since only one foetus showed these changes and no dose-response was observed. All other reproductive outcome and foetal data were comparable to the control group for all dose levels tested. |
| | | **100 APPLICANT'S SUMMARY AND CONCLUSION** |
| **100.1** | **Materials and methods** | Pregnant rabbits received 0, 7.5, 15 or 30 mg Cu/kg bw/day as copper hydroxide by gavage during day 7 to 28 of gestation. The animals were sacrificed on day 29 of gestation and the uterine contents were examined and described. The study was designed as a pilot study to assess the potential maternal and developmental toxicity of copper hydroxide. |
| **100.2** | **Results and discussion** | Under the conditions of this study, no treatment-related maternal toxicity was observed at 7.5 mg Cu/kg bw/day and no treatment-related developmental toxicity occurred at 7.5 and 15 mg Cu/kg bw/day. Observed effects at the 30 mg/kg/day dose level included maternal mortality, and gastric ulcer, haemolytic anaemia and renal damage upon necropsy. Reduced mean foetal weights, slightly increased resorptions and increased malformations were observed at this dose level. In addition, reductions of maternal food consumption and body weights were noted at 15 and 30 mg Cu/kg bw/day. |
| **100.3** | **Conclusion** | |
| 100.3.1 | Reliability | 2 |
| 100.3.2 | Deficiencies | No |

| Evaluation by Competent Authorities | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | EVALUATION BY RAPPORTEUR MEMBER STATE (*) |
| **Date** | 02/12/2004 |
| **Materials and Methods** | 3.1 and 3.1.2 Not for copper Carbonate<br>3.1.3 purity: (Metallic Copper Equivalent 61.1 %) |
| **Results and discussion** | Agree with applicant's version |
| **Conclusion** | |
| **Reliability** | 2 |
| **Acceptability** | Acceptable |
| **Remarks** | This study is not really needed in this section as it is a range-finding teratogenicity study. Only a summary in the main teratogenicity study would have been sufficient. |
| | COMMENTS FROM ... |
| **Date** | |
| **Materials and Methods** | |
| **Results and discussion** | |
| **Conclusion** | |
| **Reliability** | |
| **Acceptability** | |
| **Remarks** | |

## Section A6.8.1 Teratogenicity test

## Annex Point IIA6.8.1 Rabbit

| | | | Official use only |
|---|---|---|---|
| | | **101 REFERENCE** | |
| **101.1** | **Reference** | **A6.8.1/04: Doc.No. 00620B-IIA-681d**<br>[redacted] 2003): Copper hydroxide –developmental toxicity study in rabbits; [redacted]; Report no.: DuPont-11862, November 17, 2003. | |
| **101.2** | **Data protection** | Yes | |
| 101.2.1 | Data owner | Spiess-Urania Chemicals GmbH, Hamburg, Germany | |
| 101.2.2 | Companies with letter of access | -- | |
| 101.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I. | |
| | | **102 GUIDELINES AND QUALITY ASSURANCE** | |
| **102.1** | **Guideline study** | Yes<br>OECD 414 (1981); method B.31 (87/302/EEC), USEPA OPPTS 870.3700 (1998), MAFF guideline 59 NohSan Np. 4200 (1985) | |
| **102.2** | **GLP** | Yes<br>Self-certified laboratory | |
| **102.3** | **Deviations** | No | |
| | | **103 MATERIALS AND METHODS** | |
| **103.1** | **Test material** | As given in section 2. | X |
| 103.1.1 | Lot/Batch number | 021121/1 | |
| 103.1.2 | Specification | As given in section 2. | X |
| 103.1.3 | Purity | 61.14 % copper | X |
| 103.1.4 | Description | Free flowing blue product, needles | |
| 103.1.5 | Stability | No evidence of instability was observed. | |
| **103.2** | **Test animals** | | |
| 103.2.1 | Species | Rabbit | |
| 103.2.2 | Strain | New Zealand White | |
| 103.2.3 | Source | [redacted] | |
| 103.2.4 | Sex | Female | |
| 103.2.5 | Age/weight at study initiation | Age: approx. 5 month<br>Body weight: 2988 to 4412 g (on day 0 of gestation) | |
| 103.2.6 | Number of animals per group | 22 females<br>(1 rabbit of the high dose group was removed on day 7 of gestation, prior to dosing, due to a self-inflicted injury) | |
| 103.2.7 | Control animals | Yes | |

## Section A6.8.1 Teratogenicity test

**Annex Point IIA6.8.1** **Rabbit**

| | | |
|---|---|---|
| 103.2.8 | Mating period | Not specified |
| **103.3** | **Administration/ Exposure** | Oral |
| 103.3.1 | Duration of exposure | day 7 – 28 of gestation |
| 103.3.2 | Post-exposure period | Sacrifice on day 29 of gestation. |
| 103.3.3 | Type | By gavage |
| 103.3.4 | Concentration | 6, 9, or 18 mg Cu/kg bw/day |
| 103.3.5 | Vehicle | 0.5 % aqueous methylcellulose |
| 103.3.6 | Concentration in vehicle | 0, 6, 9, or 18 mg Cu/mL |
| 103.3.7 | Total volume applied | 1 mL/ kg bw |
| 103.3.8 | Controls | Vehicle only |
| **103.4** | **Examinations** | |
| 103.4.1 | Body weight | Yes (daily) |
| 103.4.2 | Food consumption | Yes (daily) |
| 103.4.3 | Clinical signs | Yes (daily) |
| 103.4.4 | Gross pathology and histopathology | Yes<br>Gross external and visceral examination was performed for all animals. |
| 103.4.5 | Examination of uterine content | gravid uterine weight, number of corpora lutea, number of implantations, number of resorptions |
| 103.4.6 | Examination of foetuses | General: Litter Size, number of live and dead foetuses, foetal weight, sex ratio, external alterations, retarded renal development (according to Woo and Hoar)<br>Skeleton: Yes<br>Soft tissue: Yes |
| **103.5** | **Statistics** | Linear contrast of means (Maternal weight, weight change, food consumption); Jonckheere's test (Live and dead foetuses, resorptions, implantations, incidence of foetal alterations), Cochran-Armitage test (Incidence of pregnancy, clinical observations, maternal mortality, females with total resorptions, abortions/early deliveries); Linear contrast of least square means (foetal weight, sex ratio) |
| **103.6** | **Further remarks** | Analysis of dose formulations confirmed stability, homogeneity and verified the accuracy of formulation. |

**104 RESULTS**

**Section A6.8.1** **Teratogenicity test**

**Annex Point IIA6.8.1** **Rabbit**

| | | |
|---|---|---|
| 104.1 | **Maternal toxic effects** | Three females of the high dose group were found dead during the study. One of them showed diarrhoea, red cageboard-staining, weakness, and irregular respiration prior to death. Gross necropsy of the three animals included stomach haemorrhage and/or ulceration, dark discolouration or mottling of lung tissue, pale liver, gelatinous tan rectal discharge, and brown liquid in chest cavity. In addition, two females of this dose group aborted. Diarrhoea was observed for one of them and the other animal showed red discoloured stomach lining upon necropsy. No mortality occurred at the low and mid dose levels. All animals of the test substance groups showed diarrhoea. Statistically significant adverse effects on maternal body weights, body weight changes and food consumption were observed at 9 and 18 mg Cu/kg bw/day. Marked reduction in food consumption and body weight losses were observed during the 1st week of dosing. The results are presented in Table A6.8.1-1. |
| 104.2 | **Teratogenic/ embryotoxic effects** | The number of foetuses, and numbers of early and late embryonic deaths were not adversely affected by maternal treatment. Mean foetal weight was slightly lower at 18 mg/kg bw/day (9 % lower than controls). The difference from control was considered treatment-related, but it was not statistically significant. The results are presented in Table A6.8.1- 2.<br><br>There was a total of four foetuses with malformations: one control foetus showed fused ribs, one foetus at 6 mg/kg bw/day showed ectopic kidney, and two foetuses (from separate litters) at 18 mg/kg bw/day showed hemivertebra. These malformations were considered spontaneous and unrelated to treatment. There was a slight increase in incidence of foetuses at 18 mg/kg bw/day with retarded ossification of skull and pelvic bones. However, there was no correlation with foetal weight, and the biological significance of such a slight increase is uncertain, as there was no increase in the incidence of retarded sternebral ossification. Retarded sternebral ossification is a more common indicator of foetal immaturity. Rib alterations occurred at a very high incidence across all groups in this study; almost all litters were affected. The biological significance of an increase in incidence of a very common finding is uncertain. The results are presented in Table A6.8.1- 3. |
| | | **105 APPLICANT'S SUMMARY AND CONCLUSION** |
| 105.1 | **Materials and methods** | Copper hydroxide was administered at concentrations of 0, 6, 9, or 18 mg Cu/kg bw to pregnant rabbits by gavage from day 7 to day 28 of gestation. Potential effects on maternal and developmental parameters were assessed. The study was conducted according to OECD 414 (1981); method B.31 (87/302/EEC), and USEPA OPPTS 870.3700 (1998). |

| Section A6.8.1 | Teratogenicity test |
|---|---|
| Annex Point IIA6.8.1 | Rabbit |

| | | |
|---|---|---|
| **105.2** | **Results and discussion** | Administration of copper to pregnant rabbits at 18 mg/kg bw/day was associated with marked initial bodyweight loss, inappetance, abortion and death. Pups in litters from surviving dams showed slightly lower mean foetal weight, and slightly increased incidence of a common skeletal variant. Maternal treatment at 9 mg/kg bw/day was associated with initial bodyweight loss and inappetance; pups also showed slightly increased incidence of a common skeletal variant, but mean foetal weights were not adversely affected. Maternal administration of copper hydroxide was not associated with increased incidence of foetal malformations, pre-implantation losses, or foetal (embryonic) deaths. The maternal and foetal no observed effect level was 6 mg/kg bw/day, based on maternal weight loss, inappetance, and an increased incidence of a common skeletal variant in foetuses at 9 mg/kg bw/day. |
| **105.3** | **Conclusion** | Under the conditions of the current study, there was no evidence of test substance-related teratogenicity. |
| 105.3.1 | LO(A)EL maternal toxic effects | 9 mg Cu/kg bw/day |
| 105.3.2 | NO(A)EL maternal toxic effects | 6 mg Cu/kg bw/day |
| 105.3.3 | LO(A)EL developmental effects | 9 mg Cu/kg bw/day |
| 105.3.4 | NO(A)EL developmental effects | 6 mg Cu/kg bw/day |
| 105.3.5 | Reliability | 1 |
| 105.3.6 | Deficiencies | No |

| Evaluation by Competent Authorities | |
|---|---|
| | Use separate "evaluation boxes" to provide transparency as to the comments and views submitted |
| | EVALUATION BY RAPPORTEUR MEMBER STATE (*) |
| **Date** | 03/12/2004 |
| **Materials and Methods** | 3.1 – 61.4 % is the copper content, not the purity of the copper hydroxide. |
| **Results and discussion** | Agree with applicant's version |
| **Conclusion** | Agree with applicant's version |
| **Reliability** | 1 |
| **Acceptability** | Acceptable |
| **Remarks** | Omphalocoeles which were detected at relatively high incidence in the range-finding study at 30 mgCu/kg bw were not seen in this study. |

| | COMMENTS FROM ... |
|---|---|
| **Date** | |
| **Materials and Methods** | |
| **Results and discussion** | |
| **Conclusion** | |
| **Reliability** | |
| **Acceptability** | |
| **Remarks** | |

Table A6.8.1- 1: Maternal effects

| Parameter | Control | low dose | mid dose | high dose | dose response |
|---|---|---|---|---|---|
| Number of dams examined | 22 | 22 | 22 | 21 | |
| Clinical findings during application of test substance | | | | | |
| Alopecia | 2 | 5 | 2 | 1 | - |
| Diarrhea | 0 | 5* | 5* | 9* | + |
| Irregular respiration | 0 | 0 | 0 | 1 | - |
| Sore | 1 | 0 | 0 | 0 | - |
| Stain cageboard | 0 | 0 | 0 | 3* | - |
| Stain tail | 0 | 3 | 2 | 5* | + |
| Weak | 0 | 0 | 0 | 1 | - |
| Mortality of dams (%) | 0 | 0 | 0 | 14 %* | - |
| Abortions | 0 | 1 | 0 | 2 | - |
| Body weight gain (day 7-29 of gestation) [g] | 261.5 ± 130.42 | 208.5 ± 158.50 | 179.5 ± 160.97 | 72.6 ± 211.64* | + |
| Food consumption (day 7-29 of gestation) [g/day] | 144.1 ± 10.61 | 134.8 ± 21.36 | 120.3 ± 32.62* | 100.6 ± 32.02* | + |
| Pregnancies (No. of dams pregnant) | 21 | 21 | 21 | 21 | - |

* statistically significant trend ( $p < 0.05$)

Table A6.8.1- 2: Litter response (Caesarean section data)

| Parameter | Control | low dose | medium dose | high dose | dose response + / - |
|---|---|---|---|---|---|
| No. mated | 22 | 22 | 22 | 21 | |
| No. pregnant | 21 | 21 | 21 | 21 | - |
| No. aborted/delivered early | 0 | 1 | 0 | 2 | - |
| No. found dead | 0 | 0 | 0 | 3* | - |
| No. accidentally killed | 0 | 0 | 0 | 1 | - |
| No. with total resorptions | 0 | 1 | 0 | 0 | - |
| Total number of litters | 21 | 19 | 21 | 15 | - |
| Means per litter: | | | | | |
| Mean corpora lutea | 10.0 ± 2.06 | 10.2 ± 1.51 | 9.1 ± 1.96 | 10.1 ± 2.26 | - |
| Implantation/Nidations | 8.8 ± 2.04 | 9.0 ± 1.33 | 7.8 ± 2.04 | 9.0 ± 2.36 | - |
| Resorptions | | | | | |
| Total | 1.0 ± 1.47 | 1.0 ± 1.45 | 0.2 ± 0.54 | 0.6 ± 0.91 | - |
| Early | 0.8 ± 1.47 | 0.7 ± 1.41 | 0.2 ± 0.51 | 0.4 ± 0.63 | - |
| Late | 0.1 ± 0.48 | 0.3 ± 0.56 | 0.0 ± 0.22 | 0.2 ± 0.56 | - |
| No. of dead fetuses | 0 | 0 | 0 | 0 | - |
| No. of Live fetuses | | | | | |
| Total | 7.9 ± 2.63 | 8.0 ± 1.73 | 7.6 ± 2.04 | 8.4 ± 2.03 | - |
| Males | 3.9 ± 1.77 | 4.7 ± 2.10 | 3.9 ± 1.59 | 4.3 ± 2.12 | - |
| Females | 4.0 ± 1.73 | 3.3 ± 1.45 | 3.7 ± 1.74 | 4.1 ± 1.88 | - |
| Mean fetal weight | | | | | |
| Total | 42.95 ± 2.98 | 41.71 ± 4.51 | 43.93 ± 5.88 | 38.91 ± 4.82 | - |
| Males | 43.25 ± 3.14 | 42.63 ± 4.46 | 43.32 ± 5.47 | 38.84 ± 4.95 | - |
| Females | 42.67 ± 3.77 | 40.85 ± 4.86 | 43.69 ± 6.06 | 39.16 ± 5.54 | - |
| Fetal sex ratio | 0.48 ± 0.16 | 0.58 ± 0.18 | 0.50 ± 0.22 | 0.48 ± 0.23 | - |

* significant trend ($p \leq 0.05$)

Table A6.8.1- 3: Examination of the fetuses

| Parameter | Control | low dose | medium dose | high dose | dose response |
|---|---|---|---|---|---|
| No. examined (no. of litters) | 165 (21) | 152 (19) | 159 (21) | 126 (15) | |
| No. of fetuses (litters) with: | | | | | |
| External malformations | 0 | 0 | 0 | 0 | - |
| External developmental variations | 0 | 0 | 0 | 0 | - |
| External variations due to retarded development | 0 | 0 | 0 | 0 | - |
| Skeletal malformations | | | | | |
| Rib- fused | 1 (1) | 0 | 0 | 0 | - |
| Vertebra - hemi | 0 | 0 | 0 | 2* (2) | - |
| Skeletal developmental variations | | | | | |
| Rib- supernumerary | 105 (21) | 102 (19) | 127 (20) | 110 (15)* | - |
| Sternebra - fused | 1 (1) | 2 (2) | 0 | 0 | - |
| Skeletal variations due to retarded development | | | | | |
| Mandible- retarded ossification | 0 | 0 | 0 | 1 (1) | - |
| Pelvis- retarded ossification | 0 | 1 (1) | 1 (1) | 2 (1) | - |
| Skull- bent | 0 | 2 (2) | 0 | 0 | - |
| Skull- retarded ossification | 0 | 0 | 1 (1) | 5 (2)* | + |
| Sternebra- retarded ossification | 65 (16) | 60 (12) | 76 (18) | 51 (12) | - |
| Vertebra- retarded ossification | 1 (1) | 0 | 0 | 0 | - |
| Visceral malformations | | | | | |
| Kidney- ectopic | 0 | 1 (1) | 0 | 0 | - |
| Visceral developmental variations | 0 | 0 | 0 | 0 | - |
| Visceral variations due to retarded development | | | | | |
| Kidney: Small papilla- size 2 | 0 | 1 (1) | 5 (3) | 1 (1) | - |
| Kidney: Papilla- size 3 | 2 (2) | 5 (5) | 1 (1) | 1 (1) | - |
| Total (%) with retardation | 68 (41%) | 67 (44%) | 80 (50%) | 57 (45%) | - |
| Total (%) with variations | 125 (76%) | 124 (82%) | 143 (90%) | 118 (94%) | - |

* statistically significant trend ($p < 0.05$)

| | |
|---|---|
| **Section A6.8.1** | **Teratogenicity test** |
| **Annex Point IIA6.8.1** | **Rat** |

| JUSTIFICATION FOR NON-SUBMISSION OF DATA | | | Official use only |
|---|---|---|---|
| **Other existing data [ X ]** | **Technically not feasible [ ]** | **Scientifically unjustified [ ]** | |
| **Limited exposure [ ]** | **Other justification [ X ]** | | |

| | | |
|---|---|---|
| **Section A6.8.1**<br>**Annex Point IIA6.8.1** | **Teratogenicity test**<br>**Rat** | |
| **Detailed justification:** | A developmental study in rats that conforms in all aspects with the required test guidelines (EU method B.31 and/or OECD 414) is not available. However, it is nevertheless proposed to waive the conduct of such a study for the following reasons:<br><br>1) Copper has been shown to be free of developmental toxicity in the rabbit;<br><br>2) Copper has been reported in review by the "Joint FAO/WHO Expert Committee on food additives" to be free of developmental toxicity in the rat (de la Iglesia, F.W. et al. ,1972a), as summarised below in key study format. However, access to an original copy of this report was not possible;<br><br>3) Copper is generally considered a trace element essential for human health rather than a reprotoxic agent, and for which the human required daily intake corresponds to 3 mg/day;<br><br>4) for animal welfare reasons, further testing of developmental toxicity is not considered to be required, since the studies listed under (2) above and under point (5) below may be interpreted together to demonstrate a lack of developmental toxicity;<br><br>5) the following animal test results related to developmental toxicity are summarised briefly further below (full summaries were largely not considered to be required since most studies on their own do not represent a true "key study"):<br><br>- studies in rats: Haddad *et al.*, 1991 (A6.8.1/06); Marois and Buvet, 1972 (A6.8.1/07);<br><br>- studies in mice: Lecyk, 1980 (A6.8.1/08); O'Shea and Kaufman, 1979 (A6.8.1/09);<br><br>- studies in hamsters: Ferm and Hanlon, 1974 (A6.8.1/10); Di Carlo, 1980 (A6.8.1/11). | X |

| | |
|---|---|
| **Section A6.8.1** | **Teratogenicity test** |
| **Annex Point IIA6.8.1** | **Rat** |

| | |
|---|---|
| **Undertaking of intended data submission [ ]** | |

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

| | |
|---|---|
| | EVALUATION BY RAPPORTEUR MEMBER STATE (*) |
| **Date** | 03/12/2004 |
| **Evaluation of applicant's justification** | It seems that Cu can be the cause of some developmental abnormalities in particular experimental/exposure conditions. Cu could be teratogenic in mice and hamster. It was also observed some teratogenic potential in vitro developmental tests in rats and mice. Moreover, humans with Wilson's disease usually have complicated pregnancies. |
| **Conclusion** | According to the data available in the documents IVA, some developmental abnormalities were produced after Cu IV or IP exposures. These effects are not expected after oral exposure due to the binding to proteins and the first pass through liver. For the risk assessment of Copper Carbonate, teratogenic effects cannot be dismissed but for the risk characterisation, if it can be proven that exposures other than by oral route can be considered as negligible, then no risks are expected for this end-point. |
| **Remarks** | |

| | |
|---|---|
| | COMMENTS FROM ... |
| **Date** | |
| **Evaluation of applicant's justification** | |
| **Conclusion** | |
| **Remarks** | |

## Section A6.8.1 Teratogenicity test

**Annex Point IIA6.8.1** **Rat**

| | | | Official use only |
|---|---|---|---|
| | | **106 REFERENCE** | |
| **106.1** | **Reference** | **A6.8.1/05: Doc.No. URA-97-08740-057 (see ref. A6.4.1/03)**<br>Anonymous (1982): Joint FAO/WHO Expert Committee on food additives: Copper toxicological evaluation of certain food additives; WHO Food additives Series 17. | |
| **106.2** | **Data protection** | No | |
| 106.2.1 | Data owner | Published data | |
| 106.2.2 | Companies with letter of access | -- | |
| 106.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA. | |
| | | **107 GUIDELINES AND QUALITY ASSURANCE** | |
| **107.1** | **Guideline study** | No | |
| **107.2** | **GLP** | No<br>The study was conducted prior to implementation of GLP. | |
| **107.3** | **Deviations** | Not applicable | |
| | | **108 MATERIALS AND METHODS** | |
| **108.1** | **Test material** | Copper gluconate | |
| 108.1.1 | Lot/Batch number | Not stated | |
| 108.1.2 | Specification | Not stated | |
| 108.1.3 | Purity | Not stated | |
| 108.1.4 | Description | Not stated | |
| 108.1.5 | Stability | Not stated | |
| **108.2** | **Test animals** | | |
| 108.2.1 | Species | Rat | |
| 108.2.2 | Strain | Wistar | |
| 108.2.3 | Source | Not stated | |
| 108.2.4 | Sex | Female | |
| 108.2.5 | Age/weight at study initiation | Age: Not stated<br>Body weight: Not stated | |
| 108.2.6 | Number of animals per group | Not stated | |
| 108.2.7 | Control animals | Yes | |
| 108.2.8 | Mating period | Not stated | |

## Section A6.8.1 Teratogenicity test

**Annex Point IIA6.8.1** **Rat**

| | | |
|---|---|---|
| **108.3** | **Administration/ Exposure** | Oral |
| 108.3.1 | Duration of exposure | day 5 to 15 of gestation |
| 108.3.2 | Post-exposure period | Not stated |
| 108.3.3 | Type | By gavage |
| 108.3.4 | Concentration | 0.1, 3, 30 mg copper gluconate/kg bw/day |
| 108.3.5 | Vehicle | Not stated |
| 108.3.6 | Concentration in vehicle | Not stated |
| 108.3.7 | Total volume applied | Not stated |
| 108.3.8 | Controls | Not specified |
| **108.4** | **Examinations** | |
| 108.4.1 | Body weight | Yes (weekly) |
| 108.4.2 | Food consumption | Yes (weekly) |
| 108.4.3 | Clinical signs | Not stated |
| 108.4.4 | Examination of uterine content | Number of *corpora lutea*, implantation sites, implantation loss |
| 108.4.5 | Examination of foetuses | General: Litter Size, foetal viability, resorption sites, foetal weight and length<br>Skeleton: Yes<br>Soft tissue: Yes |
| **108.5** | **Further remarks** | The results cited in the reference are based on the following report, which is not bibliographically available to the applicant: de la Iglesia, F.W. et al. (1972a): Teratology and embryotoxicity study of W10219A (Copper gluconate) in rats, Warner-Lambert, Sheridan, Canada, 250-0653).<br><br>The extrapolation from copper gluconate to copper hydroxide is considered not be restricted in any way, since the moiety of interest is the copper ion itself, which may be expected to be released from both compounds during passage of the GI tract after oral uptake. Despite the somewhat limited bioavailability for poorly soluble copper compounds, the extrapolation from the readily bioavailable copper gluconate will only lead to a more conservative but nevertheless valid assessment. |
| | | **109 RESULTS** |
| **109.1** | **Maternal toxic effects** | Weekly body weights and food intake were similar among all groups. Implantation data (corpora lutea, implantation sites, implantation loss) were not affected by copper treatment. |

**Section A6.8.1** **Teratogenicity test**

**Annex Point IIA6.8.1** **Rat**

| | | |
|---|---|---|
| **109.2** | **Teratogenic/ embryotoxic effects** | The mean number of foetuses/litter, foetal viability and resorption sites in the treated groups did not differ from the control group. Measurements of foetal weight and length as well as the incidence of skeletal abnormalities and soft tissue abnormalities were not affected by copper treatment. Based on these results, it was concluded that copper gluconate at the dose levels tested was neither embryotoxic nor teratogenic in the rat. |
| **109.3** | **Other effects** | Not stated |
| | | **110 APPLICANT'S SUMMARY AND CONCLUSION** |
| **110.1** | **Materials and methods** | Copper gluconate was administered via stomach tube to gravid Wistar rats from days 5 to 15 of the gestation period at dosages of 0, 0.1, 3 and 30 mg/kg bw/day. |
| **110.2** | **Results and discussion** | Weekly body weights and food intake were similar among all groups. Implantation data (corpora lutea, implantation sites, implantation loss) were not affected by copper treatment. The mean number of foetuses/litter, foetal viability and resorption sites in the treated groups did not differ from the control group. Measurements of foetal weight and length as well as the incidence of skeletal abnormalities and soft tissue abnormalities were not affected by copper treatment. |
| **110.3** | **Conclusion** | Based on these results, it was concluded that copper gluconate at the dose levels tested was neither embryotoxic nor teratogenic in the rat. |
| 110.3.1 | LO(A)EL maternal toxic effects | Not stated |
| 110.3.2 | NO(A)EL maternal toxic effects | 30 mg/kg bw/d |
| 110.3.3 | LO(A)EL embryotoxic / teratogenic effects | Not stated |
| 110.3.4 | NO(A)EL embryotoxic / teratogenic effects | 30 mg/kg bw/d |
| 110.3.5 | Reliability | 0<br>Not assignable, since only a short summary in secondary literature is available. |
| 110.3.6 | Deficiencies | Yes<br>Insufficient reporting |

## Section A6.8.1 Developmental toxicity

**Annex Point IIA6.8.1** **Supportive data**

The most reliable data concerning developmental toxicity of copper compounds are provided by reference A6.8.1/04, teratogenicity test in rabbits. Concerning teratogenicity in rats, only a short summary of an original source to which the applicant has no direct access is available from secondary literature, yet a reliable source, i.e. the Joint FAO/WHO Expert Committee on food additives. However, several further animal studies have been published which have investigated various aspects of developmental toxicity of copper compounds.

The applicant also wishes to note that the following data were already submitted in the context of an application for inclusion of the active substance Copper hydroxide into Annex I of Directive 91/414/EEC. Since these data are not considered to represent key studies, they are cited below in abbreviated format as supportive data only:

### Effects on pregnancy in the rat:

**A6.8.1/06**

**Report:** Haddad, D.S., Al-Alousi, L.A. and Kantarjian, A.H. (1991). The effect of copper loading on pregnant rats and their offspring. *Functional and Developmental Morphology*, **1**, 17-22.

**Guidelines:** Not stated.
Deviations from recommended: not applicable.

**GLP:** No.

**Materials and methods:**
Water loaded with copper acetate was administered to Wistar albino rats at increasing stepwise concentration of the copper acetate to 0.185% over a period of seven weeks. A group of control animals received demineralised water. At the end of seven weeks 7 rats from each group were sacrificed to serve as non-pregnant controls. The remaining rats were mated singly. The pregnant females were divided into three groups. The first group with 7 controls and 14 experimental rats were sacrificed at 11.5 days of gestation; the second group with 7 controls and 14 experimental rats were sacrificed at 21.5 days of gestation and the third group with 7 controls and 14 experimental rats were allowed to litter. Blood samples were collected for the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase levels.

Histopathology was performed on liver and kidneys, including staining for copper and iron. Samples of liver were subjected to atomic absorption spectrophotometry for copper levels. Embryos from the dams killed after 11.5 days were examined for growth and development and 21.5 day foetuses and newborn pups were counted, weighed and examined for external malformations. Two foetuses and two newborn pups (from each litter) were processed and examined for visceral malformations. Histopathological examination was performed on sections of liver and kidney from one foetus and one newborn pup. The remaining foetuses and newborn pups were processed for skeletal assessment. Statistical analyses were performed.

**Findings:**
General observations: There were no treatment related clinical signs throughout dosing and maternal weight gains for the treated animals were similar to those in the controls. Pregnancy rate was not adversely affected by maternal treatment.

Duration of gestation: There was no difference in the duration of gestation between the controls and the copper loaded group.

Clinical chemistry: There were no differences in the serum AST, ALT and alkaline phosphatase activities between the control and the copper loaded groups.

Table A6.8.1- 4 Clinical chemistry parameters

| Parameter | Controls | Copper loaded |
|---|---|---|
| AST (IU/L) | 27.3 | 25.9 |
| ALT (IU/L) | 14.2 | 17.3 |
| Alkaline phosphatase (IU/L) | 11.8 | 9.6 |

Histopathology: Liver and kidney sections from the control animals showed normal histology with no copper deposits. Liver sections from the copper loaded rats showed copper deposition in the hepatocytes and to a lesser extent in the Kupffer cells; copper was present as clusters or granules in the cytoplasm. Analysis of copper content showed that copper levels of treated rats was higher than controls (207.7 µg/g dry weight in treated compared to 23.4 µg/g dry weight in controls). Lesions included hypertrophy of the hepatocytes with cloudy eosinophilic cytoplasm, areas of focal necrosis surrounded by inflammatory foci of polymorph and lymphocyte infiltration, the presence of sinusoidal dilatation and the appearance of cytoplasmic vacuolation. In the kidneys, copper deposition was present in the proximal convoluted tubules. Lesions were confined to the proximal convoluted tubules, characterised as cloudy swelling due to hydropic degeneration and obliteration of the lumen with occasional desquamation of the epithelial cells. Liver and kidney sections stained for iron showed no deposits. The histological changes indicated that the levels of copper loading were in excess of the maximum tolerated dose. Foetal and newborn liver and kidney sections showed a normal histological pattern with no copper deposits.

Foetal and newborn examinations: At 11.5 days gestation, overt embryonic development was similar in most parameters analysed. However, there were minor changes in mean somite number, mean crown-rump length and mean yolk sac diameter were slightly decreased when compared with the controls. These changes indicated a slight delay in development for time of gestation, although the small sample size, and the imprecise nature of the parameters measured must be taken into account.

Table A6.8.1- 5 Growth and development of 11.5 day old embryos

| Parameter | Controls | Copper loaded |
|---|---|---|
| Number of dams examined | 14 | 6 |
| Number of embryos examined | 56 | 95 |
| Number (%) of embryos showing: | | |
| Presence of heart beat | 56 (100) | 95 (100) |
| Presence of fused allantois | 56 (100) | 94 (99) |
| Normally closed anterior neuropore | 56 (100) | 92 (96) |
| Normally closed posterior neuropore | 53 (94) | 80 (84) |
| Presence of normal turning | 54 (96) | 87 (91) |
| Presence of forelimb buds | 56 (100 | 95 (100) |
| Presence of normal optic vesicle | 56 (100) | 92 (96) |
| Presence of normal otic vessel | 56 (100) | 93 (97) |
| Mean somite number | 23.48 | 22.03* |
| Mean crown-rump length in mm | 2.98 | 2.71* |
| Mean yolk sac diameter in mm | 4.56 | 3.98* |

* $P < 0.005$

The number of offspring per litter and the mean foetal weights of the treated animals were stated to be similar to controls. Similarly, external and visceral examination revealed no differences. Skeletal examination showed reduction in the number of ossified centres in almost all the ossification centres examined, which was significant generally in 21.5 day old foetuses but significant only in cervical vertebrae, caudal vertebrae and hindlimb phalanges in newborn pups. These ossification findings are generally considered transient, in that they reflect the stage of the ossification process, and it is significant that the incidence was much lower in the new-born pups than in the day 20.5 foetuses. It should be noted the presence or absence of an ossification centre is not the same

as the presence or absence of the feature itself; absence means that the feature has not yet ossified i.e. it is still cartilage. The differences may reflect maternal copper-calcium balance, leading to slightly reduced availability of calcium to the foetus.

Table A6.8.1- 6: Mean number of ossification centres in 21.5 day old foetuses and newborn pups

| Parameter | 21.5 day old foetuses | | Newborn pups | |
|---|---|---|---|---|
| | Control | Copper loaded | Control | Copper loaded |
| Number of dams examined | 7 | 14 | 7 | 14 |
| Number of foetuse/newborn pups examined | 42 | 88 | 40 | 96 |
| Cervical vertebrae | 2.64 | 1.78*** | 5.42 | 4.01** |
| Sternum | 6.00 | 5.92* | 6.00 | 5.93 |
| Metacarpals | 4.00 | 3.97 | 4.00 | 4.00 |
| Forelimb phalanges | 2.69 | 2.16** | 4.00 | 3.98 |
| Caudal vertebrae | 4.55 | 4.23** | 8.77 | 8.26** |
| Metatarsals | 4.43 | 4.07*** | 5.00 | 5.00 |
| Hindlimb phalanges | 0.83 | 0.20*** | 4.00 | 3.95* |

* $P < 0.025$

** $P < 0.01$

*** $P < 0.005$

**Conclusions:**
Copper loading elevated the copper content of the liver and kidneys of the treated dams by a factor of 10, and induced liver and kidney changes similar to those seen in longer-term studies at high levels of administration, in excess of MTD, but did not affect the general reproductive performance of the animals at mating and during gestation. Slight retardation in growth and differentiation in the 11.5 day old embryos were noted but these had disappeared by the time of birth. Skeletal abnormalities, characterised by retardation of ossification, were observed in the 21.5 day old foetuses but the newborn pups were less affected indicating that the retardation was a transient delay rather than a permanent change. Excessive dietary maternal copper intake did not elevate the hepatic or renal copper intake of the offspring, despite causing maternal liver and kidney changes.

**A6.8.1/07**

**Report:** Marois, M. and Buvet, M. (1972). Etude de l'action de l'ion cuivre sur la gestation de la ratte et de la lapine (Study on the effect of copper ions on the gestation of the rat and the rabbit) C.R. Seances Soc. Biol Fil. (Paris) **166**:1237-1240.

**Guidelines:** None stated.

**GLP:** No.

**Materials and methods:**
Timed-mated female rats were anaesthetised on day 7 (day sperm detected in vaginal smear defined as day 1 of gestation – most studies in rats define this as day 0) and pregnancy confirmed by exploratory laprotomy. This practice is considered disruptive. On days 7 to 10, animals were given subcutaneous injections of either 10 or 15 mg/kg bw/day copper as copper acetate, in distilled water, or 10 or 15 mg/kg bw/day copper acetate days 7 – 10 plus a subcutaneous injection of 10 mg progesterone days 7 –20, or a sub cutaneous injection of 10 mg progesterone days 7 –20 only. A

group of untreated rats acted as controls. Animals were sacrificed on day 21 (=day 20, using the definition of day 0 used in typical studies). Numbers of foetuses and resorptions were counted.

In a second study, timed mated female rabbits were injected with varying amounts of copper acetate on days 8 – 10 of pregnancy. Total doses ranged from 15.7 to 47 mg/animal, divided between the three days of dosing (bodyweight not stated, but assuming the female NZW rabbit is approximately 2.5 – 3kg, doses may have been of the order of 2 – 8 mg/kg bw/day). The purpose was to administer the same dose at the equivalent time of pregnancy as was given to rats in the first part of the study.

**Findings:**
Subcutaneous administration of copper acetate alone to rats during days 7 to 10 of pregnancy (defining day sperm detected as day 1) interrupted (ended) pregnancy in 3 of 6 females. The same doses of copper acetate with 10 mg progesterone showed 100% pregnancy rate. Intravenous administration of copper acetate to pregnant rabbits did not affect pregnancy outcome.

**Conclusions:**
In the rat, subcutaneous administration of copper acetate ended pregnancy in 3 of 6 rats, but administration of copper acetate plus progesterone did not adversely affect pregnancy. Intravenous administration to rabbits did not adversely affect pregnancy. The authors conclude that the effect in rats is due to central (i.e. CNS) control of pregnancy.

## Fertility and teratology in the mouse:

**A6.8.1/05 Doc.No. URA-97-08740-057 (see ref. A6.4.1/03)**

| | |
|---|---|
| **Report:** | Anonymous (1982): Joint FAO/WHO Expert Committee on food additives: Copper toxicological evaluation of certain food additives; WHO Food additives Series 17. |
| **Guidelines:** | None stated. |
| **GLP:** | No. |

**Materials and methods:**
Copper gluconate was administered orally to gravid Swiss mice from days 6 to 14 of the gestation period at dosages of 0, 0.1, 3 and 30 mg/kg bw/day.

The results cited in the reference are based on the following report, which is not available: de la Iglesia, F.W. et al. (1972b): Teratology and embryotoxicity study of W10219A (Copper gluconate) in mice, Warner-Lambert, Sheridan, Canada, 250-0655).

**Findings:**
The mean numbers of foetuses/litter, foetal viability and resorption sites in the treated groups were not significantly different from the control group. Average foetal weight and length were comparable among all groups. Weekly body weights and implantation data (corpora lutea, implantation sites, implantation loss) did not show any significant influence of copper at any level tested. The mean numbers of foetuses/litter, foetal viability and resorption sites in the treated groups were not significantly different from the control group. Average foetal weight and length were comparable among all groups.

**Conclusions:**
Under the conditions of this investigation, it was concluded that copper gluconate was neither embryotoxic nor teratogenic in the mouse.

**A6.8.1/08**

**Report:** Lecyk, M. (1980). Toxicity of CuSO4 in mice embryonic development. Dept. of Comparative Anatomy, Wroclaw University. *Zoologica Poloniae*, **28**, 101-105.

**Guidelines:** Not stated.

**GLP:** No.

**Materials and methods:**
Copper sulphate pentahydrate was administered to groups of male and female mice, strains C57BL and DBA, by admixing the solution with the diet at dose levels of 0, 500, 1,000, 1,500, 2,000, 3,000 and 4,000 ppm. The feed was granulated and dried before administering to the animals. The males and females were paired after one month of treatment and the day of mating (appearance of a vaginal plug) was designated Day 0 of gestation. On Day 19 of gestation the females were killed and foetuses (living and dead) were counted and weighed. One half of the foetuses in each group was examined for visceral abnormalities (Wilson technique) and the other half was cleared and stained with alizarin for skeletal examination.

**Findings:**
Although the paper does not give details of group size and pregnancy rate, from the numbers of pregnant females (particularly at 4000, 3000 ppm), pregnancy rate was not adversely affected by dietary administration of copper at up to 4000 ppm for one month prior to mating. In both strains of mice, there was no effect on the embryonic growth at the lower doses, 2,000 ppm and below. The authors claimed a slight stimulation indicated by lower % foetal mortality and slightly higher weights of the foetuses than the controls at doses up to 2000 ppm. A treatment-related effect was noted at higher levels, at 3,000 and 4,000 ppm, where decreased foetal weights and a higher mortality were recorded. It should be noted that mean litter size was smaller than normal for the mouse in all groups. Various development malformations were observed in both these groups in both strains, although there was no consistent pattern of type. Abnormalities classed by the authors as malformations at 3000 ppm (3 foetuses in total) were last lumbar vertebra included in sacrum (one foetus) and unilateral fused rib (two foetuses); at 4000 ppm, hernia of the thoracic wall, hydrocephalus and fusion of thoracic ribs and vertebrae, (each one foetus, two foetuses with encephalocoel and two foetuses with (last lumbar) hemivertebra as part of sacrum.

**Conclusions:**
Dietary administration of 3,000 and 4,000 ppm copper as sulphate (approximately equivalent to dose levels of 430 and 570 mg/kg bw/day, using the US FDA conversion factor of 7 for mice) caused an increase in foetal mortality, decreases in foetal weights and slight increase in incidence of malformations. It should be noted that the study did not measure maternal bodyweight gains, or maternal liver histology or copper content. The NOEL for foetal effects was 2,000 ppm (approximately 285 mg/kg bw/day).

Table A6.8.1- 7 Mouse embryonic development

| | Dose level (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **500** | **1000** | **1500** | **2000** | **3000** | **4000** |
| **C57BL mice** | | | | | | | |
| Number of pregnant females | 21 | 10 | 18 | 7 | 10 | 22 | 18 |
| Number of live foetuses (%) | 65 (83.1) | 46 (89.2) | 81 (86.5) | 31 (87.1) | 42 (78.6) | 55 (72.8) | 35 (71.5) |
| Number of dead foetuses (%) | 11 (16.9) | 5 (10.8) | 11 (13.5) | 4 (12.9) | 9 (21.4) | 15 (27.2) | 10 (28.5) |
| Mean litter size | 3.09 | 4.60 | 4.50 | 4.42 | 4.20 | 2.50 | 1.94 |
| Mean foetal weight (g) | 1.10 | 1.35 | 1.22 | 1.14 | 1.25 | 1.00 | 0.99 |
| Abnormal foetuses (%) | - | - | - | - | - | 1 (1.8) | 3 (8.5) |
| **DBA mice** | | | | | | | |
| Number of pregnant females | 17 | 10 | 10 | 14 | 10 | 18 | 20 |
| Number of live foetuses (%) | 76 (84.3) | 54 (90.8) | 51 (88.3) | 58 (82.8) | 41 (83.0) | 56 (75.0) | 45 (70.4) |
| Number of dead foetuses (%) | 12 (15.7) | 5 (9.2) | 6 (11.7) | 10 (17.2) | 7 (17.0) | 14 (25.0) | 16 (29.6) |
| Mean litter size | 4.47 | 5.40 | 5.10 | 4.14 | 4.10 | 3.11 | 2.70 |
| Mean foetal weight (g) | 0.96 | 1.24 | 1.19 | 1.17 | 1.13 | 1.11 | 1.09 |
| Abnormal foetuses (%) | - | - | - | - | - | 2 (3.7) | 4 (7.4) |

**Post-implantation embryo development in the mouse *in vivo* and *in vitro*:**

**A6.8.1/09**

**Report:** O'Shea, K.S. and Kaufman, M.H. (1979). Influence of copper on the early post-implantation mouse embryo: an in vivo and in vitro study. Wilhelm Roux's Archives 186,297-308.

**Guidelines:** None stated.

**GLP:** No.

**Materials and methods:**

Groups of six timed-mated female CFLP mice were injected intravenously (tail vein) with copper sulphate on day 7 (early or late in the day), day 8 or day 9 of pregnancy, at 0.08 mg Cu/mouse (equivalent to approximately 4 mg Cu/kg bw) and sacrificed on day 10. Day 1 of pregnancy was the day the vaginal plug was detected. Embryos from untreated females were explanted on day 9 and cultured in vitro in various concentrations of copper sulphate. Day 7 is the early egg cylinder stage of development, day 8 is advanced egg cylinder to primitive streak stage, and day 9 is the early somite stage. On day 10, females were killed and uterine contents were examined. Implantations were either subjected to protein analysis or scanning electron microscopy. Additional females were injected on day 8 and examined on day 12.

The in vitro phase of the study involved standard explantation techniques on the morning of day 9. Explanted embryos were cultured for 36 hours in solutions containing either 0.332, 1.60 or 3.2 µg copper/ml.

**Findings:**
Injection early on day 7 produced no viable embryos; all implantations were classed as resorptions. Injection late on day 7 showed four litters where all implants were resorbed, and two litters containing only 'unturned' embryos. 'Unturned' embryos at day 10 represents a 12 to 24 hour delay in development. Injection on day 8 was associated with neural tube and heart defects in 81/92 embryos, and injection on day 9 resulted in a low incidence of anomalies (4/65 embryos). Examination on day 12 of the additional two females injected on day 8 showed a high proportion of foetuses (12/23) with cranial anomalies. In vitro culture showed similar neural tube and heart defects to those seen in vivo.

**Conclusions:**
Intravenous injection of copper sulphate at critical stages of embryonic development resulted in either embryolethality, a delay in development or neural tube/heart abnormalities. Direct exposure of explanted embryos resulted in the same defects, indicating that it is exposure of the foetus to unbound copper that results in foetal abnormalities. The study also showed that the 'window' for developmental exposure was very narrow. The type and incidence of foetal abnormalities seen with intravenous injection of copper are not relevant to exposure to copper salts from oral administration, as data show that oral administration results in bound copper (Section 6.2).

**Post-implantation embryo development in hamster:**

**A6.8.1/10**

**Report:** Ferm, V.H. and Hanlon, D.P. (1974). Toxicity of copper salts in hamster embryonic development. Biology of Reproduction **11**, 97-101.

**Guidelines:** None stated.

**GLP:** No

**Materials and methods:**
Timed-mated golden hamsters were obtained, and were injected intravenously (lingual vein, under pentobarbitone anaesthesia) with copper salt solutions on day 8 of pregnancy. The day following evening of mating was defined as day 1 of pregnancy. The animals were injected with copper sulphate solution, or copper citrate complex (made by adding copper chloride to citric acid, adjusted for pH with sodium hydroxide). Dose levels of copper as sulphate were 2.13, 4.25, 7.50 or 10.0 mg Cu/kg bw, and of copper citrate complex 0.25-1.50, 1.80, 2.20, or 4.9 mg Cu/kg bw. Controls were similarly injected with demineralised water. All animals were sacrificed on day 12 or 13 of pregnancy, and the uteri assessed for resorptions, live implants and macroscopically abnormal foetuses.

A second series of experiments were performed by injecting six timed-mated hamsters as described above with 2.5 mgCu/kg copper citrate complex plus $^{64}$Cu as nitrate. These animals were sacrificed 24 hours after injection, on day 9. Tissue samples were obtained and the sample counts compared to those of a standard solution to compensate for the decreases in radioactivity resulting from the short half-life (12.8 hours) of $^{64}$Cu.

**Findings:**
The majority of animals were treated at low doses of both copper sulphate and citrate. Smaller numbers of animals were treated at higher doses. Doses of 10.0 mgCu/kg as sulphate and 4.0 mgCu/kg as citrate were lethal in all cases. The higher non-lethal doses resulted in higher resorption rates, although the very small numbers of females used should be noted. Intravenous administration was associated with increased incidence of foetal malformation, particularly in females treated with copper citrate complex. It should be noted that the highest non-lethal dose of copper citrate complex tested, 2.2 mgCu/kg, showed a markedly higher incidence of malformations (35%) than the females treated with copper sulphate at 2.13 mgCu/kg (6%). It should also be noted that doses of 7.5 and 4.25 mgCu/kg as sulphate were tolerated, a dose of 4.0 mgCu/kg copper citrate complex was lethal. Given that free copper rapidly binds to blood constituents such as albumin, histidine and transcuprein before being transported to the liver (Section A6.2), the results of this experiment indicate that higher doses of free copper ion (as sulphate) injected intravenously are rapidly bound and effectively removed, whereas the citrate complex appears to be much more toxic, both to the adult (causing death at doses that were not lethal in animals given copper as sulphate) and the foetus. The citrate complex maybe more widely distributed in the body, where the copper may have more direct toxic effects. The study demonstrates that even direct

introduction to the blood of large amounts of free ion are less toxic than insoluble complexes, indicating that the bloodstream can act to bind and effectively remove any free ion introduced. The study is not strictly relevant to oral administration of copper salts, in that dosing was by intravenous injection. However, the study serves to demonstrate that free ions in the blood are less toxic than insoluble complexes, presumably because the free ion is rapidly bound and effectively removed. The study also shows that the dose-response curve for intravenous injection is steeper than for oral administration. The higher tolerance of copper sulphate and the lower incidence of malformed embryos compared to copper citrate is shown in the table below.

Table A6.8.1- 8: Lethality and uterine data after injection of copper sulphate or copper citrate

| Dose level mgCu/kg | Number of females | Number of gestation sacs (implantations) | Number (%) of resorptions | Number (%) of live foetuses | Number (%) of abnormal embryos |
|---|---|---|---|---|---|
| | | | | | |
| Control | 10 | 125 | 10 (8) | 115 (92) | 0 (0) |
| Copper sulphate | | | | | |
| 2.13 | 16 | 210 | 55 (26) | 155 (74) | 12 (6) |
| 4.25 | 3 | 49 | 42 (86) | 7 (14) | 4 (8) |
| 7.50 | 3 | 30 | 22 (74) | 0(0) | - |
| 10.0 | 2 | Maternally lethal | | | |
| Copper citrate | | | | | |
| 0.25-1.50 | 13 | 172 | 29 (16) | 143 (83) | 4 (2) |
| 1.80 | 6 | 81 | 33 (41) | 48 (59) | 14 (17) |
| 2.20 | 8 | 99 | 34 (34) | 65 (66) | 35 (35) |
| 4.00 | 2 | Maternally lethal | | | |

**Findings:** Foetal abnormalities included tail defects, thoracic wall hernias, including ectopic heart, neural tube defects including spina bifida, craniorachischisis, exencephaly, as well as microphthalmia, hydrocephalus and abdominal wall defect.

Intravenous administration of copper, particularly as a citrate complex, induced foetal malformations, particularly of the vertebral column (tail), thorax and neural tube. These doses were administered at a critical period of organogenesis, at near lethal levels. The timing of the administration is particularly crucial, in that the doses were administered to embryos that were robust enough to survive, but that the embryonic liver cells had not started producing sufficient albumin to bind and remove the excess copper ion from the foetal circulation. The authors state that the timing of the injection is critical to the induction of abnormalities. It is unfortunate that the paper does not describe clinical observations or other maternal data such as bodyweight gains or food intake, such that a maternal MTD could be derived. It is also unfortunate that the data used to show that the embryo is most vulnerable at day 8 were not given in the paper.

The second part of the paper described the administration of a single intravenous dose of 2.5 mgCu/kg copper citrate complex spiked with $^{64}Cu$ as nitrate to six pregnant females. The paper notes that the error in counting radioactivity for the embryos could have been as high as ± 20%.

The authors conclude that the placenta is permeable to copper during the critical period or organogenesis, consistent with the notion that copper teratogenicity is due to a direct effect of the metal ion on specific embryonic sites.

Table A6.8.1- 9: Foetal abnormality data after injection of copper sulphate or copper citrate

| Foetal abnormality | Number of abnormalities at dose level (mgCu/kg) as | | | | | |
|---|---|---|---|---|---|---|
| | Control | Copper sulphate | | Copper citrate complex | | |
| | 0 | 2.13 | 4.25 | 0.25-1.50 | 1.80 | 2.20 |
| **Number of live embryos** | **105** | **155** | **7** | **143** | **48** | **65** |
| Tail defect | | | | 2 | 13 | 25 |
| Abnormal spinal curve | | | 1 | | | |
| Thoracic wall defect/hernia | | 5 | | | | 6 |
| Abdominal hernia | | | 1 | | | |
| Abdominal wall defect | | | | | | 1 |
| Craniorachischisis | | | | 1 | | |
| Spina bifida | | 2 | | | | |
| Exencephaly | | | 1 | | | |
| Encephalocoel | | 4 | | | | |
| Hydrocephalus | | | 1 | | | |
| Meningocoel | | | | | 1 | |
| Microphthalmia | | 1 | | 1 | | 2 |
| Facial cleft | | | | | | 1 |
| Total abnormal foetuses | 0 | 12 | 4 | 4 | 14 | 35 |

Table A6.8.1- 10: Levels of $^{64}$Cu in tissues following injection of $^{64}$Cu citrate complex

| Micrograms of $^{64}$Cu/g tissue | | | | |
|---|---|---|---|---|
| **Maternal blood** | **Maternal liver** | **Uterus** | **Placenta** | **Embryo** |
| 0.55 | 12.8 | 0.53 | 1.47 | 0.81 |

**Conclusions**:
Copper administered intravenously was lethal to adult hamsters after a single dose at 10.0 mgCu/kg as copper sulphate and 4.0 mgCu/kg as copper citrate complex. Lower doses induced foetal malformations, particularly of the tail, thoracic wall and neural tube/skull. Copper as sulphate was markedly less toxic than the citrate complex, indicating that free copper in the blood was rapidly bound and transferred to the liver, whereas the citrate complex was not bound, and was able to act directly. Copper was shown to cross the placenta when administered intravenously as citrate complex, and may act at specific sites in the embryo. This study does not reflect the situation following oral dosing, where absorbed copper does not affect blood plasma levels.

**A6.8.1/11**

**Report:** DiCarlo Jr., F.J. (1979). Copper-induced heart malformations in hamsters. Experientia 35/6 827-828.

**Guidelines:** Not stated

**GLP:** No.

**Materials and methods:**
Study followed earlier work by Ferm and Hanlon. Timed mated golden hamsters were injected intra peritoneally (i.p) with 2.7 mg/kg bw copper citrate complex on day 8 of pregnancy. The day following the evening of mating was defined as day 1 of pregnancy. Animals were sacrificed on day 12 or 13. Embryos were placed in 0.9% saline and

examined. Live embryos were checked for malformations. Embryos showing external malformations were embedded in paraffin wax and sectioned using a microtome through the thorax, and examined for cardiac anomalies with a light microscope. Where an embryo was found to have cardiac malformations, littermates were also similarly sectioned and examined. A group of control females were injected with deionised water. Embryos from control litters were selected randomly, sectioned and examined.

**Findings:**
Maternal survival was not affected by a single i.p. dose at 2.7 mg/kg bw.. There were no data on bodyweight gain or clinical observations post-dosing. Previous work has shown that a single i.p. dose of 4 mg/kg bw is lethal to pregnant female hamsters. Approximately 15% of embryos exposed to copper citrate showed oedema, and 5% showed heart defects. Only oedematous embryos showed heart defects. Incidence of other malformations was not reported: the study was an investigation of heart defects only. Control embryos showed normal development.

Table A6.8.1- 11: Incidence of embryonic oedema and heart defects following injection of copper citrate

| Group | Number of hamsters | Number of implants | Number (%) of: | | | Number of hearts examined |
|---|---|---|---|---|---|---|
| | | | Live embryos | Live embryos with oedema | Live embryos with heart defects | |
| De-ionised water i.p. | 12 | 150 | 145(97) | 0(0) | 0(0) | 50 |
| Copper citrate i.p. | 17 | 215 | 144(67) | 21(15) | 7(5) | 34 |

The heart defects were specific: pulmonary hypoplasia and double-outlet right ventricle with associated ventricular septal defect. This condition is known in humans, and dogs, where it is under genetic control. The authors propose that the artificial induction of the condition by i.p. injection of copper citrate in hamsters may be a useful experimental model, presumably for exploring the pathogenesis of a genetic condition in humans.

**Conclusions:**
Intra peritoneal injection of copper citrate complex on day 8 of pregnancy induces specific cardiac malformations in hamsters. The author proposes the technique as an experimental model for exploring this condition, which may arise genetically in dogs and humans. Intra peritoneal injection and the use of relatively large amounts of the citrate complex allows direct exposure of the developing embryo to copper, because the route and form of copper used by-pass the natural control mechanisms. This should not be related to dietary exposure to proteins containing copper, or to simple copper salts, where the natural copper homeostatic mechanisms of the body bind the copper and effectively remove it from circulation.

## Section A6.8.2 Two generations reproduction study

**Annex Point IIA6.8.2** **Rat**

| | | | Official use only |
|---|---|---|---|
| | | **111 REFERENCE** | |
| **111.1** | **Reference** | **A6.8.2/01:** | |
| | | [redacted] 2005): Copper sulphate pentahydrate – multigeneration reproduction study in rats. [redacted]. Report no.: DuPont-14226, July 27, 2005 (unpublished). | |
| **111.2** | **Data protection** | Yes | |
| 111.2.1 | Data owner | EU Anti-Fouling Copper Task Force | |
| 111.2.2 | Companies with letter of access | Spiess-Urania | |
| 111.2.3 | Criteria for data protection | Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I. | |
| | | **112 GUIDELINES AND QUALITY ASSURANCE** | |
| **112.1** | **Guideline study** | Yes | |
| | | US EPA OPPTS 870.3800 (1998); OECD 416 (2001) | |
| **112.2** | **GLP** | Yes | |
| **112.3** | **Deviations** | None | X |
| | | **113 MATERIALS AND METHODS** | X |
| **113.1** | **Test material** | Copper sulphate pentahydrate | |
| 113.1.1 | Lot/Batch number | 17919TA (Sigma-Aldrich) | |
| 113.1.2 | Specification | Not stated | |
| 113.1.3 | Purity | 101.0% (ICP/MS) | |
| 113.1.4 | Description | Blue crystalline solid | |
| 113.1.5 | Stability | The test substance appeared to be stable under the conditions of the study, no evidence of instability was observed. | |
| **113.2** | **Test animals** | | |
| 113.2.1 | Species | Rat | |
| 113.2.2 | Strain | Crl:CD(SD)IGS BR | |
| 113.2.3 | Source | [redacted] | |
| 113.2.4 | Sex | Female and male | |
| 113.2.5 | Age/weight at study initiation | Age: ca. 56 days<br>Body weight: ca. 262–332 g (males)<br>166–231 g (females) | |
| 113.2.6 | Number of animals per group | 30 males and 30 females. | |
| 113.2.7 | Mating | Each female was placed with a single non-sibling male from the same dose level. | |

## Section A6.8.2 Two generations reproduction study

**Annex Point IIA6.8.2** **Rat**

| | | | |
|---|---|---|---|
| 113.2.8 | Duration of mating | Until evidence of copulation was observed or until 2 weeks had elapsed. | |
| 113.2.9 | Deviations from standard protocol | None | |
| 113.2.10 | Control animals | Yes | |
| **113.3** | **Administration/ Exposure** | Oral | |
| 113.3.1 | Animal assignment to dosage groups | See Table A6.8.2- 2. | |
| 113.3.2 | Duration of exposure before mating | At least 70 days before mating. | |
| 113.3.3 | Duration of exposure in general, P, F1, F2 | From beginning of the study until sacrifice of parent, F1, or F2-generation. | X |
| 113.3.4 | Type | In the food. | |
| 113.3.5 | Concentration | 100, 500, 1000 or 1500 ppm, corresponding to 1.53–2.65, 7.7–13.3, 15.2–26.7 and 23.6–43.8 mg/kg bw/day (mean achieved dose levels). | X |
| 113.3.6 | Vehicle | None | |
| 113.3.7 | Concentration in vehicle | Not applicable | |
| 113.3.8 | Total volume applied | Not applicable | |
| 113.3.9 | Controls | Untreated diet (containing nutritionally adequate levels of copper). | |
| **113.4** | **Examinations** | | |
| 113.4.1 | Clinical signs | Yes (daily) | |
| 113.4.2 | Body weight | Yes (weekly) | |
| 113.4.3 | Food consumption | Yes (weekly) | |
| 113.4.4 | Oestrus cycle | Yes | |
| 113.4.5 | Sperm parameters | Testis weight | |
| | | Epididymis weight | |
| | | Sperm motility | |
| | | Sperm morphology | |
| | | Sperm count per cauda epididymis or per gram cauda epididymis | |
| | | Spermatid count per testis or per gram testis | |

## Section A6.8.2 Two generations reproduction study

**Annex Point IIA6.8.2** **Rat**

| | | | |
|---|---|---|---|
| 113.4.6 | Offspring | Number and sex of pups | |
| | | Stillbirths | |
| | | Gross anomalies | |
| | | Live births | |
| | | Abnormal behaviour and appearance | |
| | | Weight gain | |
| | | F1 female rats designated for mating: vaginal patency | |
| | | F1 male rats designated for mating: preputial separation | |
| 113.4.7 | Organ weights P, F1 | Uterus (with oviducts and cervix); ovaries; testes; epididymides; right cauda epididymis; prostate; seminal vesicles; brain; liver; kidneys; spleen; pituitary; thyroid; adrenal glands. | |
| 113.4.8 | Organ weights, F1, F2 weanlings | Liver, brain, spleen, thymus. | |
| 113.4.9 | Histopathology P, F1 | Vagina; cervix; uterus; ovaries; testis; epididymis; seminal vesicle; prostate; coagulating gland; liver; brain and gross observations (if applicable) in control and high dose animals. | |
| | | Primordial and growing ovarian follicles were counted for the first 10 lactating F1 females (surviving to scheduled sacrifice) from control and high dose groups. | |
| 113.4.10 | Histopathology, F1 not selected for mating, F2 | Liver, brain and gross observations (if applicable) from control and high dose groups. | |
| **113.5** | **Further remarks** | F1 and F2 litters were culled to 4 pups/sex/litter (if possible) on postnatal day 4. | |
| | | Plasma, brain and liver samples from P and F1 adults and F1 and F2 weanlings were analysed for concentrations of copper, zinc, manganese and iron. | |
| | | **114 RESULTS** | X |
| **114.1** | **Effects** | | |
| 114.1.1 | Parent males | No treatment-related effects were observed. | |
| 114.1.2 | Parent females | At 1500 ppm, absolute and mean spleen weights were decreased (9%) compared to the control (Table A6.8.2- 3). Only the decrease in mean relative spleen weight was statistically significant. | |
| 114.1.3 | F1 males | No treatment-related effects were observed. | |
| 114.1.4 | F1 females | No treatment-related effects were observed. | |
| | | The mean age at vaginal opening at 1500 ppm was significantly increased (33.6 days) compared to the control group (32.1 days). However, the delay was only small (1.5 days) and was within the laboratories historical control range. Thus, this delay in vaginal opening was not considered test substance related. | |

**Section A6.8.2** **Two generations reproduction study**

**Annex Point IIA6.8.2** **Rat**

| | | | |
|---|---|---|---|
| 114.1.5 | F1 weanlings | Male and female F1 weanlings of the high dose group showed a small decrease in absolute (9%) and relative (10–11%) mean spleen weights when compared to the controls, which was not statistically significant (Table A6.8.2- 3). | |
| 114.1.6 | F2 weanlings | Decreased absolute (10–15%) and relative (10–15%) mean spleen weights were observed in high dose males and females (Table A6.8.2-3). Except for the male mean absolute decrease (10%), these differences were statistically significant. | |
| **114.2** | **Other** | | X |
| 114.2.1 | Copper content in the diet | The mean concentration of copper in the control diet was 13.7 ppm, while the range of targeted concentrations added to the diet was 25–382 ppm (equivalent to 100–1500 ppm copper sulphate). | |
| 114.2.2 | Tissue metal concentrations | Increased liver copper concentrations were found in F1 males, and F1 and F2 male and female weanlings at 1000 and 1500 ppm and in P and F1 females at 1500 ppm. The brain copper concentration was increased in F1 females, and F1 and F2 male weanlings at 1500 ppm. Decreased liver iron concentrations were detected in high dose P females. The concentration of plasma iron was decreased in F2 male and female weanlings at 1500 ppm. | |
| | | **115 APPLICANT'S SUMMARY AND CONCLUSION** | |
| **115.1** | **Materials and methods** | Groups of 30 male and 30 female rats in each generation were treated with concentrations of 0, 100, 500, 1000 or 1500 ppm of Copper sulphate pentahydrate mixed into the diet throughout the entire experimental period up to weaning of F2 litters (pre-mating, mating, gestation and rearing). The two generation reproduction study was carried out in full compliance with OECD guideline 416 (2001). | |

**Section A6.8.2** **Two generations reproduction study**

**Annex Point IIA6.8.2** **Rat**

| | | | |
|---|---|---|---|
| **115.2** | **Results and discussion** | There were no effects at any concentration on the following parameters:<br>- mortality and clinical signs of toxicity in P1 and F1 adults<br>- body weights, weight gain, food consumption, food efficiency in P1 and F1 adults.<br>At 1 500 ppm, effects considered to be related to copper sulphate treatment included decreased weight of spleen in F1 and F2 weanlings, increased liver copper concentration in P1 females, F1 males and females and F1 and F2 male and female weanlings, increased plasma copper concentration in F1 males, increased brain copper concentration in F1 females and F1 and F2 male weanlings, decreased liver iron concentration in P1 males and females, decreased plasma iron concentration in F1 and F2 male and female weanlings. However, these are not considered to represent adverse effects.<br>At 1,000 ppm, effects considered to be related to copper sulphate treatment included increased liver copper concentration in F1 males and F1 and F2 male and female weanlings, decreased plasma iron concentration in F1 male and female weanlings, and increased liver zinc concentration in F1 male and female weanlings. However, these are not considered to represent adverse effects.<br>For a discussion of effects on reproductive function, see section 5.3 below. | X |

**Section A6.8.2** **Two generations reproduction study**

**Annex Point IIA6.8.2** **Rat**

**115.3 Conclusion**

Copper sulphate may be considered to be void of reproductive toxicity, since there were no effects considered to be related to copper sulphate treatment on the following parameters at any concentration:

- sperm and oestrous cycle parameters in P1 and F1 adults
- 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 reproductive organs, thyroid gland, brain, liver, spleen, adrenal glands, kidneys and pituitary in P1 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 reproductive organs in P1 and F1 adults
- microscopic observations in liver and brain in P1 and F1 adults and F1 and F2 weanlings

Therefore, under the conditions of this study, the no-observed-effect level for reproductive toxicity was established at the highest concentration tested (i.e. 1500 ppm). The NOAEL for P and F1 rats and F1 and F2 offspring during lactation was 1000 ppm, based on reduced spleen weight in P adult females and F1 and F2 male and female weanlings at 1500 ppm. The dietary concentration of 1000 ppm was equivalent to mean dietary intakes of copper in a range of 15.2 to 23.5 mg/kg bw/day for male rats during pre-mating and 17.0 to 26.7 mg/kg bw/day for female rats during pre-mating and gestation.

The results of this study with Copper sulphate may be extrapolated without restriction to other less soluble Copper salts such as Copper hydroxide or Copper carbonate, since the sulphate may be seen as the most soluble and therefore bioavailable form, and any such extrapolation from the soluble sulphate may therefore be considered to represent a conservative basis for any toxicological assessment.

115.3.1 LOAEC

| | |
|---|---|
| Parent animals | 1500 ppm<br>(decreased spleen weights in females) |

| Section A6.8.2 | Two generations reproduction study | |
|---|---|---|
| Annex Point IIA6.8.2 | Rat | |
| F1-generation | 1500 ppm<br>(decreased spleen weights in high dose male and female weanlings) | |
| F2-generation | 1500 ppm<br>(decreased spleen weights in high dose male and female weanlings) | |
| 115.3.2 NOAEC | | |
| Parent animals | 1000 ppm | |
| F1-generation | 1000 ppm | |
| F2-generation | 1000 ppm | |
| 115.3.3 Reliability | 1 | |
| 115.3.4 Deficiencies | No | |

| Evaluation by Competent Authorities | |
|---|---|
| Use separate "evaluation boxes" to provide transparency as to the comments and views submitted | |
| | EVALUATION BY RAPPORTEUR MEMBER STATE (*) |
| **Date** | April 2006 |
| **Guidelines and quality assurance** | Deviation (2.3):<br>The following minor deviations occurred from the requirements of OECD guideline No. 416 (adopted 22nd January 2001):<br>• Animal rooms were maintained at a temperature of 18-26°C instead of the test guideline recommended 19-25°C.<br>• Testicular histopathological examinations are not fully described.<br>These deviations are not considered to have affected the scientific integrity, or outcome of this study. |

**Section A6.8.2** **Two generations reproduction study**

**Annex Point IIA6.8.2** **Rat**

**Materials and Methods**

**The materials and methods section should be more detailed. All the sections are filled but too much briefly. Essential information is reported but method is not explained.**

• Duration of exposure in general, P, F1, F2 (3.3.3):

Treatment Schedule

P1 and F1: 70 days before mating until sacrifice

Sacrifice Schedule

| Animals | Gen. | Schedule |
|---|---|---|
| Adult Males | P1<br>F1 | Test days 109-113<br>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) |

• Concentration (3.3.4):
100, 500, 1000 or 1500 ppm, corresponding to
1.53–2.65, 7.7–13.3, 15.2–26.7 and 23.6–43.8 mg**Cu**/kg bw/d

**Results and discussion**

**The results and discussion section should be more detailed. All the sections are filled but too much briefly. Essential information is presented but variations in different parameters should be reported (4.1-4.2):**

• Mean Body Weights and Body Weight Gains (4.1):

No test substance-related effects on body weight or body weight gain were observed at any dose level (in P1 and F1 males and females).

In parent males and females and F1 females, 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 (4.1):

There were no test substance-related effects on food consumption or food efficiency in P1 & F1 males and females 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.

• Estrous Cycle Parameters (4.1):
There were no test substance-related effects on the mean percent days in estrus, diestrus, or proestrus, or mean cycle length in either the P1 or F1 females 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 in both P1 and F1 females.

• Cause of Death P1 and F1 adult rats (4.1):
There were no test substance-related deaths in the study. Of the 120 P1 and 120 F1 adult males, one animal (Group I-O, Animal number 844) was sacrificed in extremis on day 14 because of a fractured nose. Of the 120 P1 and F1 females, one (Group VI-1, animal number 4664) was sacrificed in extremis on day 109 due to dystocia, one (Group VI-1, animal number 4722) was found dead on day 17 due to pyelonephritis, and one (Group VIII-1, animal number 4757) was sacrificed in extremis on day 119 for morbidity of undetermined cause.

• Gross findings (4.1):
There were no test substance-related gross observations in any of the adults, weanlings, or nursing pups in this study.
In P1 and F1 adult rats, all gross observations were consistent with normal background lesions in rats of this age and stock.
Gross observations in F1 and F2 weanlings occurred at low incidences, were randomly distributed across control and treatment groups, and/or were lesions common to rats of this stock and age.
Observations in F1 and F2 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.

• P1 and f1 adult reproductive failures (4.1):
The failure of 18 P1 (5, 1, 3, 5 and 4 in group II-0, IV-0, VI-0, VIII-0 and X-0, respectively) and 9F1 (2, 3, 4 in group II-1, VI-1 and VIII-1 respectively) adult pairs to produce litters was not related to test substance exposure. Gross and microscopic evaluation of the 27 pairs revealed a morphological explanation of their infertility in 3 P1 individuals (absence of recent corpora lutea in ovaries). The cause of reproductive failure in one F1 female was dystocia. The cause of the reproductive failure in the remaining 22 pairs was not determined.

• Tissue Metal Concentrations (4.2):

*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