

Section A6.2

Annex Point IIA6.2

IUCLID 5.0/21

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(21), Cellular and molecular metabolism of copper

analysed by agarose gel electrophoresis following restriction digestion with *EcoRI*. The largest inserts were subcloned and the nucleotide sequence determined by dideoxy chain termination. To isolate genomic clones encoding HAH1 (human ATX homologue 1), a ³²P-labelled full-length *HAH1* cDNA was used to screen 800,000 recombinants of a human genomic library. Isolated phage DNA was characterised by restriction enzyme digestion and nucleotide sequence determination.

RNA, Southern Blot Analysis and Chromosomal Localisation: RNA blot analysis was performed using nitrocellulose membranes containing poly(A)⁺ RNA from different human tissues. RNA was isolated by dissolution of human tissues in guanidinium isothiocyanate, followed by CsCl gradient centrifugation. RNA samples were subjected to agarose-formaldehyde gel electrophoresis, transferred to nylon membranes, and analysed using a ³²P-labelled *HAH1* cRNA. Human genomic DNA was isolated from peripheral blood leucocytes, digested with restriction enzymes, electrophoresed on 0.8% agarose gels, transferred to nylon membranes and analysed using a ³²P-labelled *HAH1* cDNA probe. A bacteriophage clone encompassing the *HAH1* gene was labelled with digoxigenin dUTP by nick translation. Labelled probe was hybridised to normal metaphase chromosomes from phytohemagglutinin-stimulated lymphocytes in 50% formamide, 10% dextran sulphate, and 2 x SSC. Specific hybridisation signals were detected by incubation with fluorescein-conjugated anti-digoxigenin antibodies, followed by counterstaining of slides with 4'-diaminidino-2-phenylindole. A total of 57 specifically labelled metaphase cells were analysed, and localisation was confirmed using a probe known to localise to 5q21.

Complementation experiments: To express *HAH1* in *S. cerevisiae*, the coding region encompassing nucleotides 114-318 was amplified by polymerase chain reaction and directionally subcloned into the *EcoRI* and *BamHI* sites of pSM703. The resulting construct pHAH703 was verified by nucleotide sequencing and transformed into KS107 (*sod1*⁻) and SL215 (*atx1A*) strains. The empty CEN pRS413 and 2 μ pSM703 vectors, and the CEN p413-A1 and 2 μ pRS-A1 plasmids containing a functional copy of the *ATX1* gene were transformed into these same strains. To test for reversal of lysine auxotrophy, cells were grown in air on synthetic dextrose (SD) plates with or without lysine for 3 days at 30°C. Iron-dependent growth was determined on SD complete media buffered with 50 mM Na-2-(N-morpholino)ethanesulphonic acid (MES), pH 6.1, and 3 μM ferrozine with or without 350 μM ferrous ammonium sulphate for 5 days at 30°C.

HoloFet3p Biosynthesis: To examine the biosynthesis of holoFet3p (copper incorporation into Fet3p), saturated cultures were used to inoculate 100 ml of yeast nitrogen base culture medium without copper, iron, and dextrose, supplemented with amino acids, 2% glucose, 50 mM Na-MES, pH 6.1, and 100 μM ferrozine. All stock solutions were treated with Chelex-100. After growth to A₆₀₀ of 0.4, cells were labelled for 2 h with 2.5 μCi/ml ⁶⁴Cu. Cells were washed extensively in 150 mM NaCl, 25 mM Tris-HCl pH 7.5, and an aliquot of cells was used to determine cell-associated radioactivity. Vigorous shaking with glass beads for 1 hour lysed cells, and protein extracts of crude membrane fractions were prepared. Samples (100 μg) were analyzed on 7.5% nonreducing SDS-PAGE without heat denaturation followed by direct autoradiography of the gels. For Western blot analysis of Fet3p, crude membrane extracts were separated by SDS-PAGE and transferred to nitrocellulose by semidry transfer. Following blocking in 5% non-fat

X

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dry milk, 0.1% Tween 20, 0.02% Nonidet P-40 in phosphate-buffered saline, membranes were incubated for 1 h with a 1:1000 dilution of an anti-peptide antiserum specific for Fet3p. Membranes were subsequently washed in phosphate-buffered saline containing 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, and the antibody-antigen complex was detected using enhanced chemiluminescence.

111.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

To search for a mammalian homologue of *ATX1*, a human liver cDNA library was screened and a cDNA clone was isolated, which encodes a protein with 47% amino acid identity to Atxlp including conservation of the MTCXGC copper-binding domain. RNA blot analysis using this cDNA identified an abundant 0.5-kilobase mRNA in all human tissues and cell lines examined. Southern blot analysis using this same clone indicated that the corresponding gene exists as a single copy in the haploid genome, and chromosomal localization by fluorescence *in situ* hybridization detected this locus at the interface between bands 5q32 and 5q33. Yeast strains lacking copper/zinc superoxide dismutase (*SOD1*) are sensitive to redox cycling agents and diogenin and are auxotrophic for lysine when grown in air, and expression of this human *ATX1* homologue (*HAH1*) in these strains restored growth on lysine-deficient media. Yeast strains lacking *ATX1* are deficient in high affinity iron uptake and expression of *HAH1* in these strains permits growth on iron-depleted media and results in restoration of copper incorporation into newly synthesized Fet3p. These results identify *HAH1* as a novel ubiquitously expressed protein, which may play an essential role in antioxidant defense and copper homeostasis in humans.

111.3 Conclusion

It has been demonstrated that *HAH1* is one of a series of human copper chaperone proteins involved in the transfer of copper from the cell surface to the intracellular targets. Specifically, *HAH1* is thought to deliver copper to a Cu-ATPase in the Golgi apparatus.

111.3.1 Reliability

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

2

111.3.2 Deficiencies

Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field.

No internationally accepted guidelines are available that address the objective of the research presented in this summary.

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

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

COMMENTS FROM ...

Date

Give date of comments submitted

Figure A6.2(21)-1

A

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1      ctgtcgccctgcacgggtgacccgggtgtgcgaggccttcattggccaggatccg

54  ggtggagaggctgctgtgatccgcgcgcacacccgcgcacacccgcgcctgctcagtc
    MetProLysHisGluPheSerValAspMetThrCysGlyGlyCysAlaGluAlaValSer
114  ATGCCGAAGCACCAGTTCTCTGTGGACATGACCTGTGGAGGCTGTGCTGAAGCTGTCTCT
    ArgValLeuAsnLysLeuGlyGlyValLysTyrAspIleAspLeuProAsnLysLysVal
174  CGGGTCTCAATAAGCTTGGAGGAGTTAAGTATGACATTGACCTGCCCAACAAGAAGGTC
    CysIleGluSerGluHisSerMetAspThrLeuLeuAlaThrLeuLysLysThrGlyLys
234  TGCATTGAATCTGAGCACAGCATGGACACTCTGCTTGCACCCCTGAAGAAAACAGGAAAG
    ThrValSerTyrLeuGlyLeuGlu---
294  ACTGTTTCTTACCTTGGCTTGGAGtagcagggggcctgggtccccacagccacaggatgga
354  ccaaggggggcaggatgctgatcctcccgctggcttcagacagacctgggacttggcag
414  tcatgcggggtgatggtgttcctgoggagacctcagttgtctatctctctcagcttc
474  cctgcataaatcaagctggttttgg

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B

	MTCXGC
<i>H. sap.</i>	MP--KH--EFSVDMTCGGCAEAVSRVLNKLQ-GV-KYD-
<i>S. cer.</i>	MAEIKH-YQFNVMTCSGCSGAVNKVLTKLEPDVSKID-
<i>C. ele.</i>	M---TQ-YVFEMGMITCNGCANAAARKVLGKLGEDKIKIDD
<i>A. tha.</i>	MA---QTVVLKVGMSQGGCVGAVKRVLGKME-GVESFD-

<i>H. sap.</i>	IDL-PNKKVCIESEH-SMDTLLATLKKTGKTV-SYLGLE
<i>S. cer.</i>	ISL-EKQLVDVYTTL-PYDFILEKIKKTGKEVRSQKQL
<i>C. ele.</i>	INV-ETKKITVTDDL-PASDVLEALKKTGKEI---KQL
<i>A. tha.</i>	IDIKEQK-VTVKGNVEPEEAVFQTVSKTGKKT-SY

FIG. 1. A, nucleotide sequence of *HAH1* cDNA clone and derived amino acid sequence of *HAH1* (nucleotides 114-318); polyadenylation consensus sequence is *underlined*. B, derived amino acid sequence of *HAH1* (*H. sap*) aligned with homologues identified in *S. cerevisiae* (*ATX1*, *S. cer.*), *Caenorhabditis elegans* (*C. ele.*), and *Arabidopsis thaliana* (*A. tha.*). Amino acids identical to *HAH1* are shown in **bold**. The copper-binding motif (MTCXGC) and the carboxyl-terminal lysine rich region (****) are indicated.

Figure A6.2(21)-2

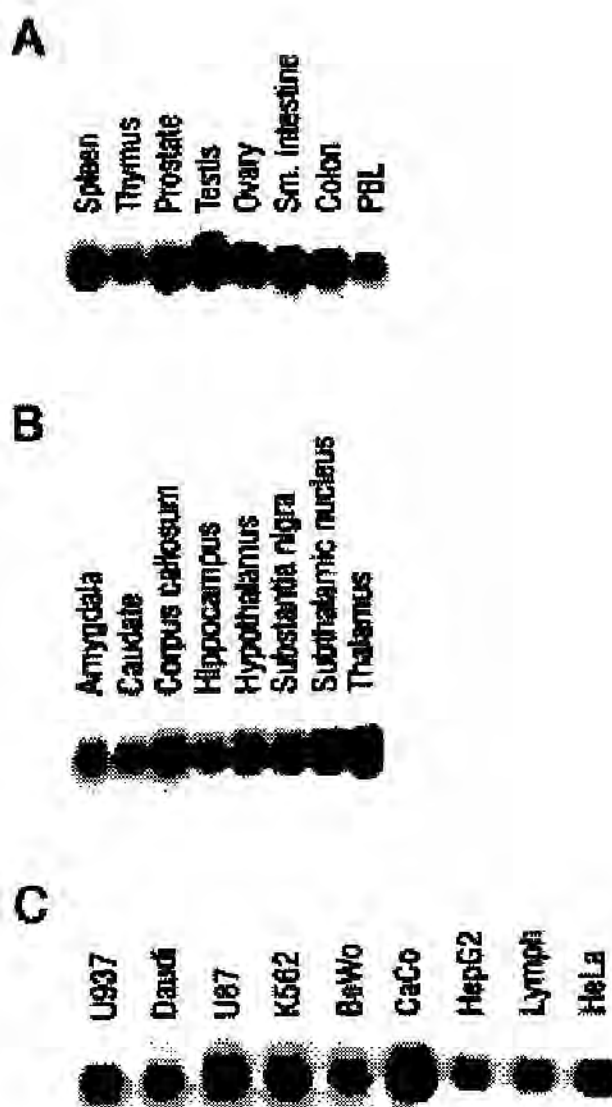


FIG. 2. Expression of the *HAH1* gene in human tissues and cell lines. RNA blot analysis of poly(A)⁺ RNA from human tissues (A), regions of the central nervous system (B), and human tissue-derived cell lines (C). 2 μ g of poly(A)⁺ RNA (A and B) or 15 μ g of total RNA (C) were subjected to electrophoresis, transferred to membranes, and hybridized with a ³²P-labeled *HAH1*-specific cRNA probe as described under "Experimental Procedures." Blots were exposed to film for 1 h at -70 °C.

Figure A6.2(21)-3

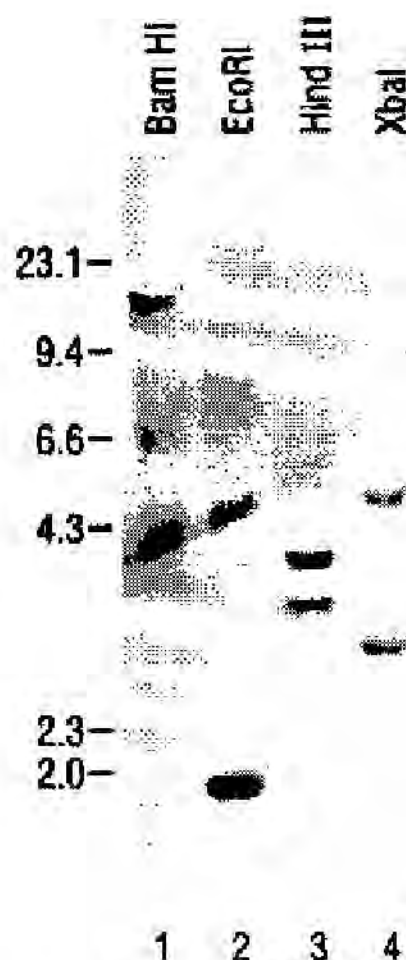


FIG. 3. Southern blot analysis of the *HAH1* gene. Human genomic DNA (10 μ g/lane) was digested with the restriction enzymes shown, subjected to electrophoresis, transferred to membranes, and hybridized with a ^{32} P-labeled *HAH1*-specific cDNA probe as described under "Experimental Procedures." Molecular size markers are shown on the left in kilobases. The blot was exposed to film at -70°C for 22 h.

Figure A6.2(21)-4



Figure A6.2(21)-5

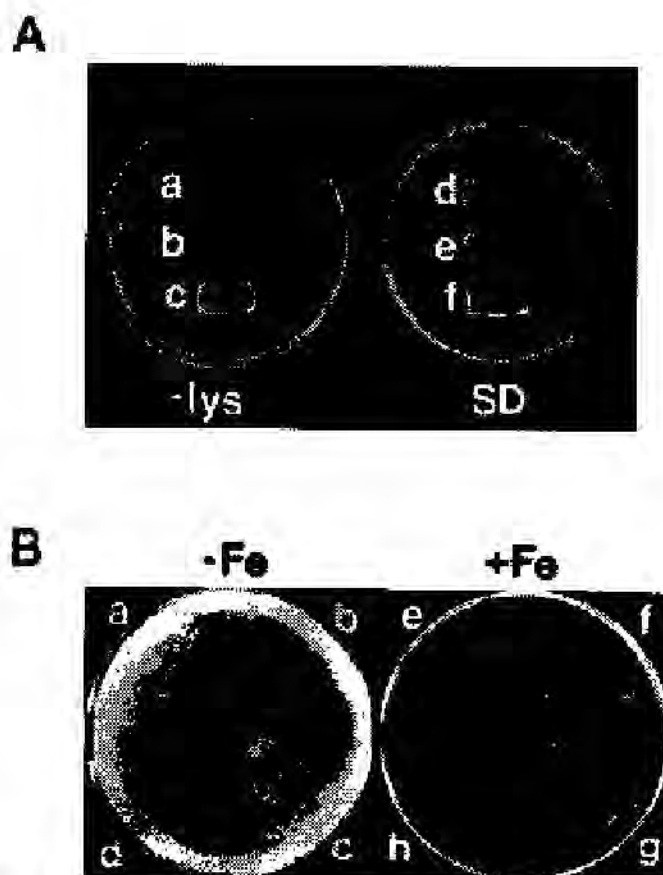


FIG. 5. *A*, complementation of aerobic lysine auxotrophy in *sod1Δ* strain KS107 grown on lysine-deficient (*a–c*) or SD complete media containing lysine (*d–f*) following transformation with pHAH-703 (*b* and *e*) or pRS-A1 (*c* and *f*). *B*, complementation of iron-deficient growth in *atx1Δ* strain SL215 grown on 3 μ M ferrozine (*a–d*) or 3 μ M ferrozine plus 350 μ M ferrous ammonium sulfate (*e–h*) following transformation with *ATX1* containing p413-A1 (*a* and *e*), control vector pRS413 (*b* and *f*), HAH1 containing pHAH 703 (*c* and *g*), or control vector pSM703 (*d* and *h*).

Figure A6.2(21)-6

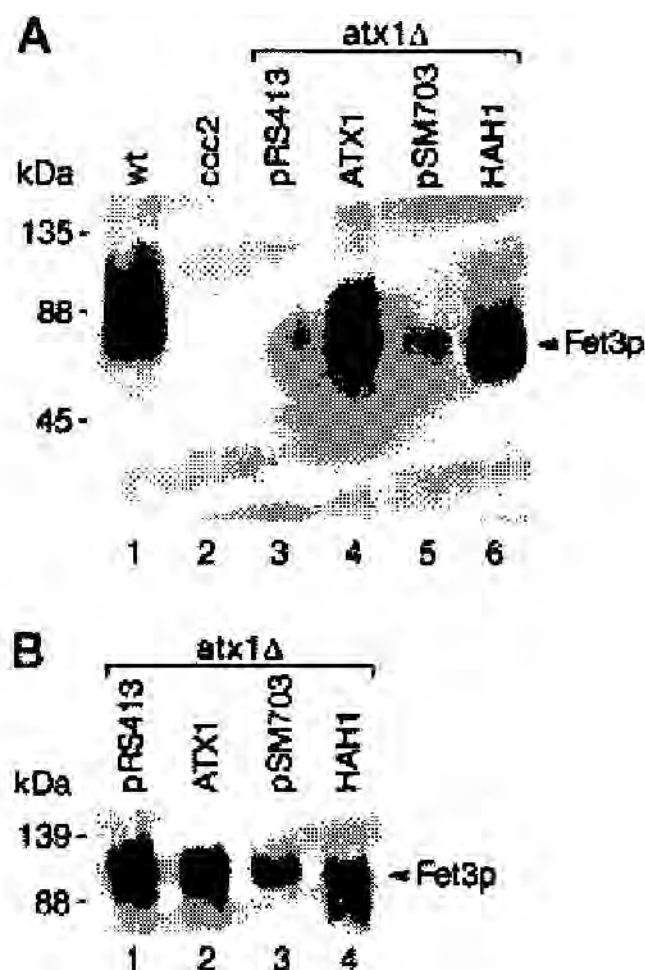


FIG. 6. *A*, biosynthesis of holoFet3p in wild type (lane 1), *ccc2Δ* mutant (lane 2), and *atx1Δ* null mutant (lanes 3–6) *S. cerevisiae* strains. *atx1Δ* strains were transformed with the indicated control vectors (lanes 3 and 5), p413-A1 (*Atx1*) (lane 4), or pHAH-703 (*HAH1*) (lane 6) prior to analysis as described under "Experimental Procedures." To identify holoFet3p, cells were pulse-labeled with ^{64}Cu , and membrane fractions analyzed by nonreducing SDS-PAGE, followed by copper autoradiography of gels as described under "Experimental Procedures." ^{64}Cu -labeled Fet3p is indicated by the arrow. *B*, Western blot analysis of Fet3p in *atx1Δ* strains transformed with control vectors (lanes 1 and 3), *ATX1* (lane 2), or *HAH1* (lane 4). Identical membrane fractions as in *A* were electrophoresed in 7.5% SDS-PAGE, transferred to nitrocellulose, incubated with a polyclonal antiserum to Fet3p, and developed by chemiluminescence as described under "Experimental Procedures."

112 REFERENCE

- 1.1 Reference** *Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).*
Amaravadi, R., Glerum, D.M. and Tzagoloff, A. (1997). Isolation of a cDNA encoding the human homolog of *COX17*, a yeast gene essential for mitochondrial copper recruitment. Hum Genet. **99**: 329-333 (published).
- 1.2 Data protection** No
(indicate if data protection is claimed)
- 1.2.1 Data owner Give name of company
Public domain
- 1.2.2 Criteria for data protection Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed

113 GUIDELINES AND QUALITY ASSURANCE

- 113.1 Guideline study** No. This was a non-regulatory study that describes the use of functional complementation to clone the human homolog of yeast *COX17*. *COX17* codes for a 69 amino acid cytosolic protein involved in the recruitment of Cu to mitochondria (null mutations in *COX17* elicit a loss of cytochrome oxidase due to a failure of mutants to complete assembly of the complex). No guidelines are available to address this objective. (If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines.xy")
- 113.2 GLP** No. This was a non-regulatory study.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)
- 113.3 Deviations** Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

114 MATERIALS AND METHODS

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

- 114.1 Test material** Refer to section 2.3
- 114.1.1 Lot/Batch number Not applicable
- 114.1.2 Specification Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

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114.1.2.1 n	Description	If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution) Not applicable
114.1.2.2	Purity	Give purity in % of active substance [REDACTED]
114.1.2.3	Stability	Describe stability of test material Not applicable
114.1.2.4 ling	Radiolabel	give structural location of radio labelling, give reason if not labelled Not applicable
114.2 Test Animals		Non-entry field
114.2.1 Species		<i>Saccharomyces cerevisiae</i>
114.2.2 Strain		<i>cox 17</i> null mutant.
114.2.3 Source		Not applicable
114.2.4 Sex		Not applicable
114.2.5 Age		Not applicable
114.3 Procedures		Non-entry field
114.3.1 Strains, transformation conditions and screen for functional complementation		The <i>cox17</i> null mutant (W303ACOX17) was transformed with a human cDNA expression library consisting of HeLa cell cDNA cloned into the <i>ADH1</i> expression cassette of the <i>Escherichia coli</i> /yeast shuttle vector pDB20. This vector also carries the URA3 gene for selection of transformants. Transformation mixtures were plated on minimal glucose medium containing all auxotrophic requirements of W303ACOX17 except uracil. Plates generally containing 1.0×10^4 – 1.5×10^4 uracil-independent transformants were replicated into YEPG media (3% glycerol, 2% ethanol, 1% yeast extract) and incubated at 30°C for up to 1 week.
114.3.2 Miscellaneous methods		Isolation of plasmid DNA from yeast, transformation of <i>E. coli</i> with recombinant plasmids, restriction enzyme mapping, and isolation and ligation of restriction fragments were all performed by standard methods. DNA sequencing was done using 5' end-labelled single-stranded restriction fragments.

115 RESULTS AND DISCUSSION

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

115.1 Results

Non-entry field

115.1.1 Complementation of a <i>cox17</i> null mutant by recombinant plasmids with human cDNAs	W303ACOX17 is a respiratory deficient mutant carrying a disrupted allele of <i>COX17</i> . Several large-scale transformations of this mutant with a yeast expression library constructed from HeLa cell cDNA yielded approximately 8×10^5 uracil-independent clones. Further screens revealed that 40 transformants had acquired the ability to grow on the nonfermentable substrate glycerol. In each case, the respiratory competent phenotype co-segregated with the uracil prototrophy, confirming that the transformed phenotype was plasmid-dependent. Plasmid DNA was isolated from 20 respiratory competent clones and
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115.1.2 The presence of a common sequence in plasmids capable of complementing the yeast *cox17* mutant

amplified in *E. coli*. Most of the complementing plasmids had cDNA inserts of a size (450 bp) identical to that of pG74H/T1 (**Figure A6.2(22)-1A**). Two plasmids (pG74H/T4 and pG74H/T11) had larger inserts of 1.1 kb and 0.85 kb. The latter two contain chimeric cDNAs, only part of which are related to the insert of pG74H/T1.

Based on its sequence, the cDNA insert recovered from pG74H/T1 had a length of 443 nucleotides (**Figure A6.2(22)-2**). Analysis of the DNA sequence revealed a single open reading frame. The predicted protein consisted of 62 amino acids with a calculated M_r of 6916. The primary structure of the protein predicted from the DNA sequence was homologous to yeast Cox17p, suggesting that the cloned cDNA codes for the human homolog.

Partial sequence analysis of the 850 bp fragment cloned in pG74H/T11 indicated the presence of the sequence from nt -53 to 292 reported in **Figure A.2(21)-2**, and another fragment with part of the cDNA for human reticulocalbin. The fusion of the two different sequences occurred at nt 292 of the inert pG74H/T1. The insert in pG74H/T4 was more complex, having been produced by the fusion of the cDNA present in pG74H/T1 to two other fragments, one originating from human mitochondrial DNA and the other coding for the ribosomal protein L32.

In the three plasmids studied, the presence of an identical reading frame whose encoded product is homologous to Cox17p, provided strong evidence that the restoration of respiration in the *cox17* mutant is due to complementation by the human equivalent of the yeast protein.

115.1.3 Growth properties of the *cox17* null mutant transformed with yeast and human *COX17*

The *cox17* mutant transformed with pG74H/T1 has a slower growth rate on YEPG than either wild-type yeast or the mutant transformed with the yeast COX17 gene (**Figure A6.2(22)-3**). The failure of the cDNA fragment in pG74H/T1, removed from the *ADH1* cassette and integrated in single copy (pG74H/ST7 in **Figure A6.2(22)-1B**) into the chromosomal DNA of the mutant, to confer growth on the respiratory substrate also argues that the human cDNA only partially complements the mutant. This could be due to less efficient expression of the human gene or because the human protein is only partially functional in the yeast. To distinguish between these two possibilities, a new plasmid was constructed in which the regions 5' and 3' of the coding sequence in pG74H/T1 were replaced by the sequences flanking the yeast gene (pG74H/ST5 in **Figure A6.2(22)-1B**). This construct is identical to pG74/ST8 (see **Figure A6.2(22)-1B**), except for the presence of the human coding sequence. Introduction of the plasmid into the *cox17* null mutant did not result in any noticeable improvement in growth on YEPG over that conferred by pG74H/T1 (not shown). The lack of effect of the yeast flanking sequences on complementation suggests that the explanation for the partial restoration of respiration lies in the properties of the protein itself. This interpretation is supported by the failure of the human cDNA (pG74H/T1) to complement the *cox17* mutant when integrated in single copy at the *LEU2* locus.

In contrast to pG74H/T1 and pG74H/T4, growth on YEPG of the transformant harbouring pG74H/T11 was almost as good as wild-type (**Figure A6.2(22)-3**). Since the inserts in pG74H/T1 and pG74H/T11 have different 5' and 3' flanking sequences, either sequence could affect expression of the human *COX17* gene. The 3' flanking region in pG74H/T11 containing the recombinant sequence coding for part of reticulocalbin was excluded from being important since its removal

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(pG74H/ST6 in **Figure A6.2(22)-1B**) did not alter the growth properties of the transformant. Replacement of the 3' flanking region of pG74H/T1 with the 3' region of pG74H/ST6 also did not affect growth of the transformant on glycerol (see pG74H/ST11 in **Figure A6.2(22)-1B**). To exclude an effect of plasmid copy number, pG74H/T11 was integrated at the *URA3* locus. The transformant harbouring the human gene with the shorter 5' leader in single copy was complemented for its respiratory defect, but grew more slowly than wild type or transformants containing the plasmid in multicopy. These results indicate that the absence of 33 nucleotides in the upstream region of pG74H/T11 is responsible for the improved growth in glycerol, probably as a result of more efficient expression of the protein.

115.1.4 Human and yeast Cox17p are highly homologous and contain a cysteine-rich domain also present in metallothioneins

Human and yeast Cox17p share 22 identities and 8 conservative substitutions (**Figure A6.2(22)-4**). A comparison of the Cox17p to human and mouse metallothionein reveals a short common domain. Two other conserved cysteines in Cox17p can be aligned with cysteines in the mammalian metallothioneins if two short gaps are allowed in the sequences. All five of the cysteines that are conserved between the metallothioneins and the Cox17ps are involved in binding Cu.

115.2 Discussion

Evidence is presented for the existence of a human gene whose encoded product functions in cytochrome oxidase assembly. This protein is homologous to a cytoplasmic protein of yeast that functions in delivery of Cu to mitochondria. A HeLa cell cDNA encoding the human Cox17p homolog was selected from an expression library by functional complementation of a yeast *cox17* null mutant. The protein's primary structure, deduced from the nucleotide sequence of the cDNA clone, displayed a high degree of sequence similarity to yeast Cox17p. The existence of a human Cox17p homolog, and its ability to substitute *in vivo* for the yeast protein, supports the idea that Cox17p is likely to be present in all mammalian cells in which cytochrome oxidase is the terminal oxidase of the respiratory chain.

Even though human Cox17p is capable of complementing the defect in the yeast *cox17* null mutant, the growth properties of transformants harbouring different constructs suggest that the human Cox17p is less efficient than the yeast protein in the heterologous environment. This is supported by 1) the slow growth rate on glycerol transformants harbouring pG74H/T1 (single or multi-copy) and 2) a similar slow growth on glycerol of the *cox17* mutant transformed with a plasmid containing the human coding sequence flanked by the yeast 5' and 3' untranslated regions (UTRs). The same plasmid with the yeast coding sequence elicits wild-type growth on glycerol. In addition, growth differences of transformants harbouring two complementing plasmids with the same coding region but different flanking regions (pG74H/T1 and pG74H/ST6) demonstrate that noncoding human sequences, particularly in the 5'UTR, can affect expression of the human protein, and hence may also contribute to the observed growth phenotypes.

The human and yeast Cox17ps share features with metallothioneins. Both Cox17p and metallothionein are low-molecular-weight proteins with a high molar content of cysteine residues. Both also contain a short conserved domain with the sequence KxCCxC. Metallothioneins protect cells from toxic metals by means of their high binding potential for Cu, Cd and other heavy metals. The ability of high exogenous Cu to rescue *cox17* null mutants argues against a metal-scavenging role for

Cox17p, but favours a role in the delivery of Cu to mitochondria.

116 APPLICANT'S SUMMARY AND CONCLUSION

116.1 Materials and methods

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

A study that describes the use of functional complementation to clone the human homolog of yeast COX17 was carried out. COX17 codes for a 69 amino acid cytosolic protein involved in the recruitment of Cu to mitochondria (null mutations in COX17 elicit a loss of cytochrome oxidase due to a failure of mutants to complete assembly of the complex). The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP. The following techniques were used:

Strains, transformation conditions and screen for functional complementation: The *cox17* null mutant (W303ACOX17) was transformed with a human cDNA expression library consisting of HeLa cell cDNA cloned into the *ADH1* expression cassette of the *Escherichia coli*/yeast shuttle vector pDB20. This vector also carries the *URA3* gene for selection of transformants. Transformation mixtures were plated on minimal glucose medium containing all auxotrophic requirements of W303ACOX17 except uracil. Plates generally containing $1.0 \times 10^4 - 1.5 \times 10^4$ uracil-independent transformants were replicated into YEPG media and incubated at 30°C for up to 1 week.

Miscellaneous methods: Isolation of plasmid DNA from yeast, transformation of *E. coli* with recombinant plasmids, restriction enzyme mapping, and isolation and ligation of restriction fragments were all performed by standard methods. DNA sequencing was done using 5' end-labelled single-stranded restriction fragments.

116.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

*Complementation of a *cox17* null mutant by recombinant plasmids with human cDNAs :* W303ACOX17 is a respiratory deficient mutant carrying a disrupted allele of *COX17*. Large-scale transformations of this mutant with a yeast expression library constructed from HeLa cell cDNA yielded approximately 8×10^5 uracil-independent clones. Further screens revealed that 40 transformants had acquired the ability to grow on nonfermentable substrate. In each case, the respiratory competent phenotype co-segregated with the uracil prototrophy, confirming that the transformed phenotype was plasmid-dependent.

Plasmid DNA was isolated from 20 respiratory competent clones and was amplified in *E. coli*. Most of the complementing plasmids had cDNA inserts of a size (450 bp) identical to that of pG74H/T1. Two other plasmids (pG74H/T4 and pG74H/T11) had larger inserts of 1.1 kb and 0.85 kb, respectively. These contained chimeric cDNAs, only part of which are related to the insert of pG74H/T1.

*The presence of a common sequence in plasmids capable of complementing the yeast *cox17* mutant:* The cDNA insert recovered from pG74H/T1 had a length of 443 nucleotides. Analysis of the DNA sequence revealed a single open reading frame. The predicted protein consisted of 62 amino acids with an M_r of 6916. The predicted structure of the protein was homologous to yeast Cox17p, suggesting that the cloned cDNA coded for the human homolog. Partial analysis of the

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fragments cloned in pG74H/T11 and pG74H/T1 also revealed the presence of this sequence. An identical reading frame in all 3 plasmids, whose encoded product was homologous to Cox17p, provided evidence that restoration of respiration in the *cox17* mutant was due to complementation by the human equivalent of the yeast protein.

Growth properties of the cox17 null mutant transformed with yeast and human COX17: The *cox17* mutant transformed with pG74H/T1 had a slower growth rate on YEPG than either wild-type yeast or the mutant transformed with the yeast COX17 gene. Failure of the cDNA fragment in pG74H/T1 to confer growth when integrated into chromosomal DNA of the mutant also argues that the human cDNA only partially complements the mutant. To investigate this, a new plasmid was constructed in which the 5' and 3' regions of the coding sequence in pG74H/T1 were replaced by the sequences flanking the yeast gene. Introduction of this plasmid into the *cox17* null mutant did not result in any noticeable improvement in growth on YEPG. This lack of effect suggests that the explanation for the partial restoration of respiration lies in the properties of the protein itself. This interpretation is supported by the failure of the human cDNA to complement the *cox17* mutant when integrated in single copy at the *LEU2* locus.

In contrast to pG74H/T1 and pG74H/T4, growth on YEPG of the transformant harbouring pG74H/T11 was almost as good as wild-type. Since the inserts in pG74H/T1 and pG74H/T11 have different 5' and 3' flanking sequences, either sequence could affect expression of the human *COX17* gene. The 3' flanking region in pG74H/T11 (pG74H/ST6) was excluded from being important, since removal did not alter the growth properties of the transformant. Replacement of the 3' flanking region of pG74H/T1 with the 3' region of pG74H/ST6 also did not affect growth on glycerol. To exclude an effect of plasmid copy number, pG74H/T11 was integrated at the *URA3* locus. The transformant harbouring the human gene with the shorter 5' leader in single copy was complemented for its respiratory defect, but grew more slowly than wild type or transformants containing multiple copies of the plasmid. These results indicate that the absence of 33 nucleotides in the upstream region of pG74H/T11 is responsible for the improved growth in glycerol, probably as a result of more efficient protein expression.

Human and yeast Cox17p are highly homologous and contain a cysteine-rich domain also present in metallothioneins: Human and yeast Cox17p share 22 identities and 8 conservative substitutions. A comparison of Cox17p to human metallothionein reveals a short common domain. Two other conserved cysteines in Cox17p can be aligned with cysteines in the mammalian metallothioneins if two short gaps are allowed in the sequences. All 5 of the cysteines that are conserved between the metallothioneins and the Cox17ps are involved in binding Cu. Metallothioneins protect cells from toxic metals by means of their high binding potential for Cu, Cd and other heavy metals. The ability of high exogenous Cu to rescue *cox17* null mutants argues against a metal-scavenging role for Cox17p, but favours a role in the delivery of Cu to mitochondria.

116.3 Conclusion

The *COX17* gene of *S. cerevisiae* codes for a cytoplasmic protein essential for the expression of functional cytochrome oxidase. To determine if Cox17p is present in human cells, a yeast strain carrying a null mutation of *COX17* was transformed with a human cDNA expression library. All the respiratory competent clones obtained from

Section A6.2**Annex Point IIA6.2****IUCLID 5.0/22****Metabolism in mammals***Specify section no., heading and species as appropriate***A6.2(22), Cellular and molecular metabolism of copper**

	the transformations carried a common cDNA sequence with a reading frame predicting a product homologous to yeast Cox17p. The cloning of a mammalian <i>COX17</i> homolog suggests that the encoded product is likely to function in copper recruitment in eucaryotic cells in general.
116.3.1 Reliability	<i>Based on the assessment of materials and methods include appropriate reliability indicator 0, 1, 2, 3, or 4</i> 2
116.3.2 Deficiencies	Yes This study was not conducted and/or reported in strict compliance with the principles of GLP, and experimental details have been omitted on a number of occasions. However, this does not necessarily compromise the validity of the data presented, or the author's interpretation of those data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed publication, and has therefore been subject to the prior scrutiny of experts in the field. In addition, this report has been included in a number of expert reviews of copper toxicokinetics. No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary. Overall, this is an adequately-presented study, and its findings are considered to make a valuable contribution to the 'weight of evidence' approach that has been adopted for the purposes of the current review of copper toxicokinetics. A reliability indicator of 2 has been assigned on this basis. <i>(If yes, discuss the impact of deficiencies and implications on results. If relevant, justify acceptability of study.)</i>

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE**Date****Materials and Methods****Results and discussion****Conclusion****Reliability****Acceptability****Remarks****COMMENTS FROM ...****Date***Give date of comments submitted*

Figure A6.2(22)-1

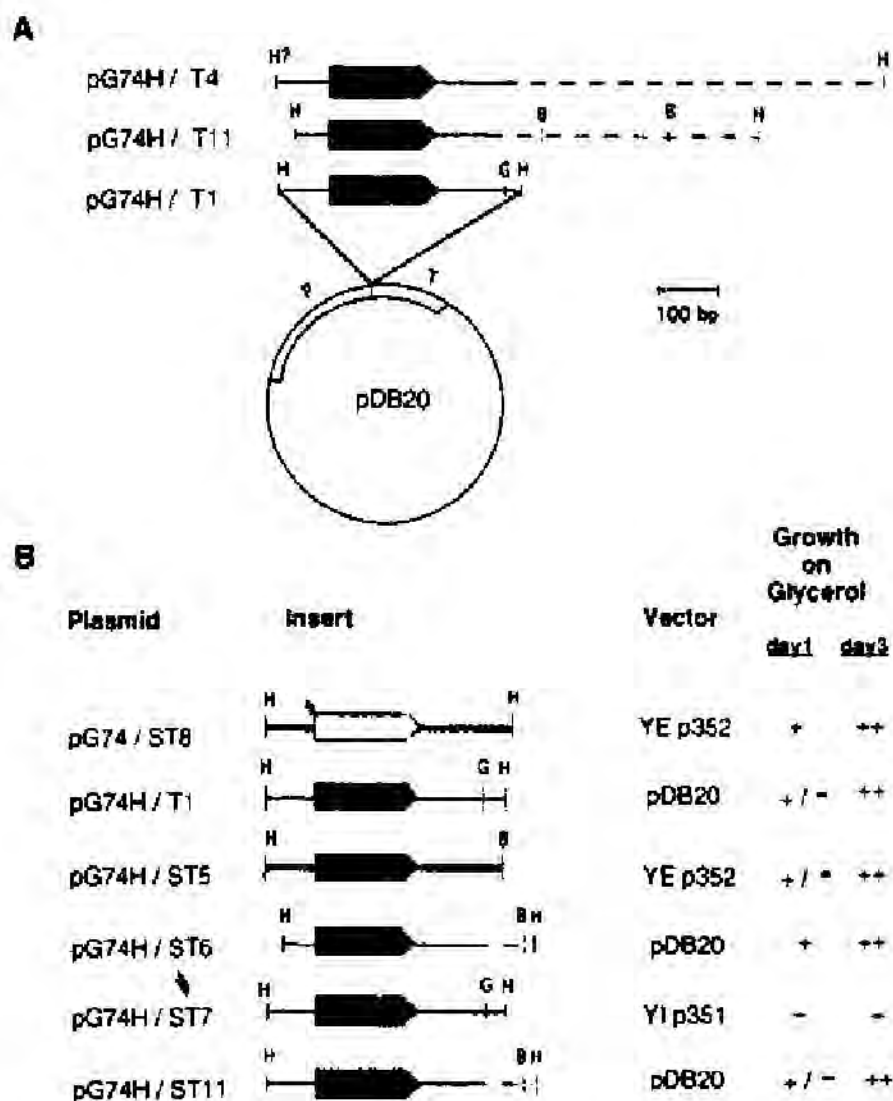


Fig. 1A,B Restriction maps of human cDNA clones and of derivative plasmids. **A** The locations of the *Hind*III (*H*), *Bam*HI (*B*), and *Bgl*II (*G*) sites in the cDNA inserts of pG74H/T1, T4, and T11 are shown above the pDB20 vector. The *dashed lines* in the inserts of pG74H/T4 and T11 correspond to sequences derived from other cDNAs. The *question mark* denotes an approximate location of the upstream *Hind*III site in pG74H/T4. The reading frame coding for the human Cox17p is indicated by the *solid arrow*. *Open arcs* in pDB20 represent the promoter (*P*) and terminator (*T*) regions of *ADHI*. **B** Complementation of the *cox17* null mutant by different constructs containing human and yeast sequences. YEp352 is a multicopy plasmid with *URA3* as the selective marker and Y1p351 is an integrative plasmid with the yeast *LEU2* gene (Hill et al. 1986). *Minus signs* indicate absence of visible growth on YEPG, *+/-* indicate poor growth, and *++* indicate good growth. The *open bar* and *arrow* denote yeast coding and flanking regions, and the *solid line* and *arrow* represent the corresponding human sequences

Figure A6.2(22)-2

```

-86          5' - CCGGAAGTGA CTGCGGACGAATCGGCCTTTGCCBAG
-50 GCTGGCATAGATTGGCTGTCTCCGCTCATAGCTCCTTTTGGCGCGAAAG

      Met Pro Gly Leu Val Asp Ser Asn Pro Ala Pro Pro
+1   ATG CCG GGT CTG GTT GAC TCA AAC CCT GCC CCG CCT

      Glu Ser Glu Glu Lys Lys Pro Leu Lys Pro Cys Cys
+37  GAG TCT CAG GAG AAG AAG CCG CTG AAG CCC TGC TGC

      Ala Cys Pro Glu Thr Lys Lys Ala Arg Asp Ala Cys
+73  GCT TGC CCG GAG ACC AAG AAG GCG CGC GAT GCG TGT

      Ile Ile Glu Lys Gly Glu Glu His Cys Gly His Leu
+109 ATC ATC GAG AAA GGA GAA GAA CAC TGT GGA CAT CTA

      Ile Glu Ala His Lys Glu Cys Met Arg Ala Leu Gly
+145 ATT GAG GCC CAC AAG GAA TGC ATG AGA GCC CTA GGA

      Phe Lys Ile OP
+181 TTT AAA ATA TGA AATGGTGGTCTGCTGTGTGAATAAATAATTCCT

+226 GAAGAATGAAGAAGATTAAATTTGGGAGTTCTTTGACGAACTTTGATATG

+276 TGGAAAAAGTATTTATAATTTATTGTGAAGAAGAAAGTAAAATATTACTAG

+326 TGGAAAGATCTTCAAAAAAAAAAAAAAAAAAAAAA

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Fig. 2 Nucleotide sequence of the cDNA insert of pG74H/T1. Only the sequence of the coding strand is shown. The open reading frame proposed to code for human Cox17p is translated and is shown above the DNA sequence. A possible polyadenylation signal has been *underlined*. The sequence has been deposited in GenBank under accession number L77701

Figure A6.2(22)-3

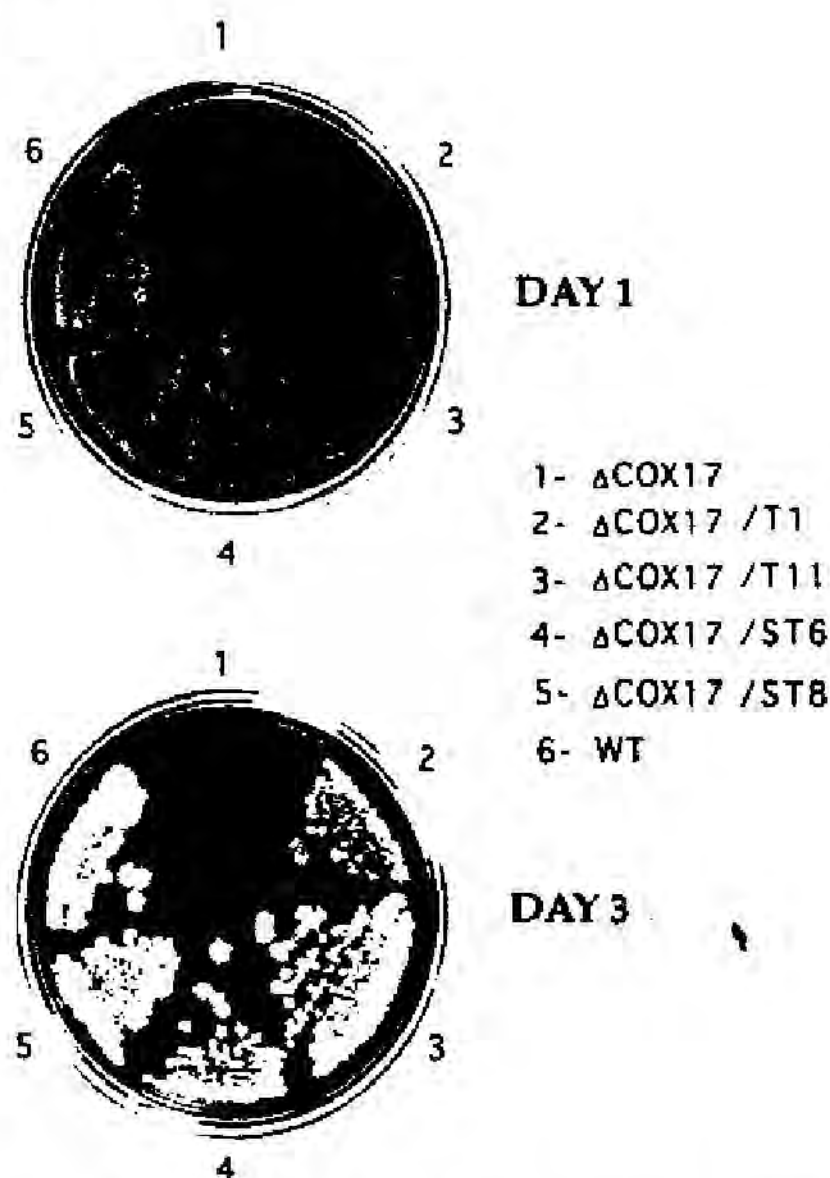


Fig. 3 Growth of the *cox17* null mutant harboring plasmids coding for the yeast and human Cox17p. The mutant, wild-type, and transformant strains were replica-plated from rich glucose medium (YPD) to rich glycerol medium (YEPG). Growth on the YEPG plate was scored after incubation at 30°C for 1 day (*upper*) and 3 days (*lower*). The following strains were streaked on: *sectors* 1 the *cox17* null mutant W303DCOX17. 2 mutant transformed with pG74H/T1; 3 mutant transformed with pG74H/T11; 4 mutant transformed with pG74H/ST6; 5 mutant transformed with pG74/ST8; and 6 wild-type W303-1B

Figure A6.2(22)-4

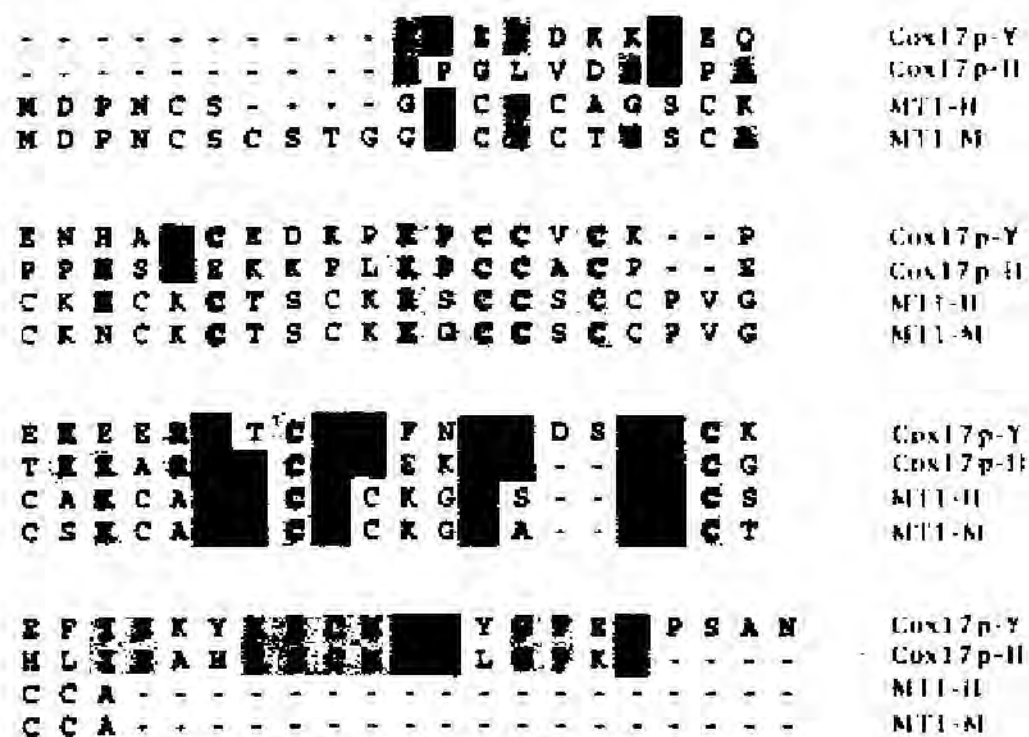


Fig. 4 Homology of human and yeast Cox17p. Human (*H*) and yeast (*Y*; Glerum et al. 1996) Cox17p's, and the human (MT1-H; Schmidt et al. 1985) and mouse (MT1-M; Glanville et al. 1981) metallothionein sequences were aligned by the motif recognition algorithm of Vingron and Argos (1991). Identical residues and conserved substitutions present only in the two Cox17p sequences are highlighted by the *lighter* and *darker boxes*, respectively. Also highlighted are identical and conserved residues present in at least one Cox17p and one of the metallothionein sequences. Wherever possible, identical or conserved residues across all four species have been marked

117 REFERENCE

- 1.1 Reference** *Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages) If necessary, copy field and enter other reference(s).* X
Culotta, V.C., Klomp, J.S., Casareno, R.L.B., Krems, B. And Gitlin, J.D. (1997). The Copper Chaperone for Superoxide Dismutase. The Journal of Biological chemistry. **272 (38)**: 23469 - 23472 (published).
- 1.2 Data protection** No
(indicate if data protection is claimed)
- 1.2.1 Data owner Give name of company
Public domain
- 1.2.2 Criteria for data protection Choose one of the following criteria (see also TNsG on Product Evaluation) and delete the others: No data protection claimed

118 GUIDELINES AND QUALITY ASSURANCE

- 118.1 Guideline study** No. This was a non-regulatory study to confirm the insertion of copper into superoxide dismutase 1 (SOD1) by a specific metal carrier or "copper chaperone" identified as *Saccharomyces cerevisiae* LYS7 and human CCS (copper chaperone for SOD). No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")
- 118.2 GLP** No. This was a non-regulatory study.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)
- 118.3 Deviations** Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

119 MATERIALS AND METHODS

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

- 119.1 Test material** Refer to section 2.3 X
- 119.1.1 Lot/Batch number Not applicable
- 119.1.2 Specification Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

Section A6.2**Annex Point IIA6.2**

IUCLID: 5.0/23

Metabolism in mammals*Specify section no., heading and species as appropriate***A6.2(23), Cellular and molecular metabolism of copper**

119.1.2.1 n	Description	<i>If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)</i> Not applicable
119.1.2.2	Purity	<i>Give purity in % of active substance</i> [REDACTED]
119.1.2.3	Stability	<i>Describe stability of test material</i> Not applicable
119.1.2.4 ling	Radiolabel	<i>give structural location of radio labelling, give reason if not labelled</i> ⁶⁴ Cu
119.2 Test Animals		<i>Non-entry field</i>
119.2.1 Species		Not applicable
119.2.2 Strain		Not applicable
119.2.3 Source		Not applicable
119.2.4 Sex		Not applicable
119.2.5 Age		Not applicable
119.3 Procedures		<i>Non-entry field</i>
119.3.1 Cloning and RNA Analysis		Overlapping cDNA clones corresponding to expressed sequence tags (ESTs) homologous to LYS7 were sequenced using a fluorescent dye terminator cycle sequencing kit (Perkin-Elmer), followed by analysis on a Perkin-Elmer ABI 373A XL sequencer. A 1068 bp cDNA sequence was identified containing an 822-base pair open reading frame, which was termed Copper Chaperone for SOD (CCS). RNA blot analysis was performed using membranes containing poly(A)⁺ RNA from different human and foetal tissues or isolated from human cell lines. To construct the CCS expressing plasmid pSMCCS, the coding region of human CCS was amplified by PCR and inserted at the <i>EcoRI</i> and <i>BamHI</i> sites of pSM703, placing CCS under the control of <i>S. cerevisiae</i> <i>PGK1</i> (phosphoglycerol kinase) promoter. The pHAL7 plasmid expressing the epitope-tagged LYS7-HA protein was obtained by first amplifying LYS7 sequences –440 to the stop codon by PCR using a downstream primer that converted the termination codon to a <i>NdeI</i> site. This modified LYS7 fragment was then inserted at the <i>ApaI</i> and <i>NdeI</i> sites of the LEU2 CEN vector, pA2HA-cen-resulting in the in-frame fusion of LYS7 to two copies of the HA epitope from influenza virus. Construction of the <i>sod2Δ::URA3</i> plasmid pGSOD2 was as follows: <i>S. cerevisiae</i> SOD2 sequences –680 to –80 with respect to the start codon were amplified by PCR and directionally inserted at the <i>BamHI</i> and <i>HindIII</i> sites of pRS306, an <i>URA3</i> integrating vector. SOD2 sequences +560 to +1700 were then directionally inserted in this plasmid at <i>SpeI</i> and <i>BamHI</i> sites. The resultant pGSOD2 plasmid was linearised by digestion with <i>BamHI</i> and used to delete the chromosomal SOD2 gene of strain 1783 by a one-step gene deletion, creating strain JS002.

Section A6.2

Annex Point IIA6.2

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Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(23), Cellular and molecular metabolism of copper

119.3.2 SOD Activity Gels For analysis of SOD1 activity by NBT (nitro blue tetrazolium), cells were grown in 50 ml YPD medium to an A_{600} of 1.0, were harvested and washed once in cold H_2O , and the resultant cell pellet re-suspended in 0.5 ml of a lysis buffer containing 10 mM sodium phosphate, 1.0 mM EDTA, 0.1% Triton, 20 μ g/ml leupeptin, 10 μ g/ml pepstatin and 1.0 mM phenylmethylsulfonyl fluoride. Cells were then broken by glass bead homogenisation and glycerol added at a final concentration of 5%. 20 μ g of the resultant cell extract were applied directly without boiling to a nondenaturing 12% polyacrylamide gel. Following electrophoresis, the gel was stained in a solution containing 50 mM potassium phosphate (pH 7.8), 275 μ g/ml nitro blue tetrazolium, 65 μ g/ml riboflavin and 3.2 μ l/ml Temed. After 45 minutes incubation in the dark, the blue NBT stain for superoxide (O_2^-) was developed by exposure to light. Staining was absent at sites of O_2^- scavenging.

119.3.3 Immunodetection of SOD1 and LYS7-HA Western blot analyses were carried out. A polyclonal rabbit anti-yeast SOD1 serum and a polyclonal PMA1 antibody from rabbit were used at 1:5000 and 1:2000 dilutions, respectively, and were detected using as secondary antibody, anti-rabbit donkey IgG at a 1:10,000 dilution. LYS-HA was detected using a monoclonal anti-HA antibody from mouse diluted 1:10,000 and as secondary antibody, sheep anti-mouse IgG. All three secondary antibodies were conjugated to horseradish peroxidase and the Hybond ECL kit was used for detection.

Indirect immunofluorescence microscopy utilised strain AA255 transformed with pHAL7. Yeast cells were grown to an A_{600} of 1.0 in minimal medium and were prepared for immunofluorescence microscopy. Incubation with mouse anti-HA diluted 1:1000 proceeded for 2 h, and detection of LYS-HA was carried out with a FITC labelling kit. Nucleic acids were stained by DAPI.

119.3.4 Metabolic labelling of SOD1 For metabolic labelling studies, yeast strains were labelled with 200 μ Ci of ^{64}Cu . Cell pellets were re-suspended in 0.5 ml of a lysis buffer supplemented with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin, and pepstatin and subjected to glass bead homogenisation for 30 minutes at 4°C. Cytosolic and membrane fractions were obtained by centrifugation at 15000 rpm at 4°C for 30 minutes. Equal amounts of cytosolic protein were analysed on 10% nondenaturing, non-reducing polyacrylamide gels, followed by autoradiography for 1 hour to detect holoSOD1. Incorporation of ^{64}Cu into FET3 was then analysed.

120 RESULTS AND DISCUSSION

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

120.1 Results

A yeast genetic approach was used to identify the copper chaperone for SOD1. It was predicted that a yeast mutant for the SOD1 copper chaperone would lack SOD1 activity and would exhibit the same phenotypes as a *sod11s* null mutant. Yeast cells lacking SOD1 are known to be auxotrophic for methionine and lysine when grown in air. The *lys7* complementation group of *S.cerevisiae* is also auxotrophic for both lysine and methionine. As with *sod11s* mutants, the dependence of *lys71s* cells for lysine and methionine is only observed in the presence of oxygen (Figure A6.2(23)-1), suggesting that SOD1 and LYS7 may be functionally related. Multiple human ESTs homologous to LYS7 were identified, and sequence analysis revealed the complete open reading frame of the human LYS7 homologue (Figure A6.2(23)-2A),

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termed CCS (copper chaperone for SOD1). Human CCS is coded by a gene which is ubiquitously expressed in all human tissues and cell types examined (**Figure A6.2(23)-2B**). CCS also exhibits notable homology to SOD1. To test whether CCS is a functional homologue of LYS7 or SOD, the CCS open reading frame was placed under the control of the yeast constitutive *PGK1* promoter and was used to transform *sod1 Δ* and *lys7 Δ* yeast. The expressed CCS gene failed to complement a *sod1 Δ* mutation, and no SOD activity was associated with CCS (data not presented). In comparison, the same CCS clone fully restored the aerobic growth of *lys7 Δ* mutants on medium lacking lysine and methionine (**Figure A6.2(23)-1**), demonstrating that human CCS is a functional homologue to yeast LYS7.

To test whether LYS7 was essential for SOD1 activity, SOD activity was examined in *lys7 Δ* cell lysates run on non-reducing polyacrylamide gels and exposed to nitro blue tetrazolium. As shown in **Figure A6.2(23)-3A**, the *lys7 Δ* mutant mimicked a *sod1 Δ* mutant and displayed no SOD1 activity, but exhibited normal levels of O₂-scavenging by SOD2. By western blot analysis of these same lysates, *lys7 Δ* mutants contained wild type levels of SOD1 protein (**Figure A6.2(23)-3B**), demonstrating that the SOD1 polypeptide in *lys7 Δ* mutants is inactive for O₂-scavenging.

To directly determine if the lack of SOD1 activity observed in *lys7 Δ* mutants was due to a defect in copper incorporation into SOD1, yeast strains were metabolically labelled with ⁶⁴Cu *in vivo*. In this analysis, wild type cells exhibited a single ⁶⁴Cu-labelled protein corresponding to SOD1 (**Figure A6.2(23)-3C**). Notably, *lys7 Δ* mutants were devoid of ⁶⁴Cu-labelled SOD1, and this defect was complemented by an episomal plasmid harbouring human CCS (**Figure A6.2(23)-3C**). It was therefore apparent that LYS7 was essential for copper incorporation into SOD1, and this function is conserved in the human homologue, CCS.

The sub-cellular localisation of LYS7 was examined using a HA epitope-tagged protein. When expressed on a low-copy centromeric vector, LYS7-HA fully complemented a *lys7 Δ* mutation and was observed to migrate as a single band on SDS gels (**Figure A6.2(23)-4A**). Extracts from cells expressing LYS-HA were resolved into soluble and membrane-associated components, and as expected for a soluble protein, LYS-HA co-fractionated with cytosolic SOD1 to the supernatant, but was absent in the membrane fraction containing the proton ATPase (**Figure A6.2(23)-4A**). When examined by immunofluorescence microscopy, LYS7-HA exhibited diffuse staining throughout the cytosol and was largely excluded from the nucleus. LYS7 is therefore cytosolic, as is the case for its recipient of copper delivery, SOD1.

LYS7 is highly specific for SOD1. For example, it does not participate in the delivery of copper to the excretory pathway, as *lys7 Δ* cells were found to be fully functional for incorporating ⁶⁴Cu into FET3 (**Figure A6.2(23)-3D**). Furthermore, over-expression of another copper chaperone, ATX1, could not rescue the SOD1 copper labelling defect of *lys7 Δ* mutants (**Figure A6.2(23)-3C**). Hence, distinct pathways of copper trafficking are mediated by ATX1 and LYS7. It is considered that the trafficking pathways are likely to involve protein-protein interaction between the metal chaperone and its copper-requiring recipient.

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Annex Point IIA6.2

IUCLID: 5.0/23

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(23), Cellular and molecular metabolism of copper

120.2 Discussion

The results of this study demonstrate that copper incorporation into SOD1 in intact cells is absolutely dependent on the presence of LYS7/CCS. It is assumed that the presence of multiple copper binding factors in an intact cell limits available copper, thus creating the need for a SOD1-specific chaperone, as well as chaperones for other copper proteins such as cytochrome oxidase in the mitochondria and the copper-transporting ATPases in the secretory pathway.

121.1 Materials and methods

121 APPLICANT'S SUMMARY AND CONCLUSION

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

A study was carried out to confirm the insertion of copper into superoxide dismutase 1 (SOD1) by a specific metal carrier or "copper chaperone" identified as *Saccharomyces cerevisiae* LYS7 and human CCS (copper chaperone for SOD). The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP. The following techniques were used: *Cloning and RNA Analysis*: cDNA clones corresponding to expressed sequence tags (ESTs) homologous to LYS7 were sequenced using a fluorescent dye terminator cycle sequencing kit and analysed. A 1068 bp sequence (termed CCS) was identified that contained an 822 bp open reading frame. RNA blot analysis was carried out using membranes containing poly(A)⁺ RNA from human and foetal tissues or cell lines.

To construct the CCS expressing plasmid pSMCCS, the coding region of human CCS was amplified by PCR and inserted into pSM703, placing CCS under control of *S. cerevisiae* *PGK1* (phosphoglycerol kinase) promoter. The pHAL7 plasmid expressing the epitope-tagged LYS7-HA protein was obtained by amplifying LYS7 sequences -440 to the stop codon by PCR. This fragment was then inserted into the LEU2 CEN vector, pA2HA-cen, resulting in the in-frame fusion of LYS7 to two copies of the HA epitope from influenza virus. To construct the *sod2A::URA3* plasmid pGSOD2, *S. cerevisiae* SOD2 sequences -680 to -80 were amplified by PCR and inserted into pRS306, an *URA3* integrating vector. SOD2 sequences +560 to +1700 were then inserted in this plasmid. The resulting plasmid was linearised and used to delete the chromosomal SOD2 gene of strain 1783, creating strain JS002.

SOD Activity Gels: For analysis of SOD1 activity by NBT (nitro blue tetrazolium), cells were grown in YPD medium, harvested, washed in water and the pellet re-suspended in a lysis buffer. Cells were homogenised and glycerol added at a final concentration of 5%. The extract was applied directly to a nondenaturing polyacrylamide gel. Following electrophoresis, staining solution was added and the gel was incubated in the dark. The NBT stain for superoxide was developed by exposure to light. Staining was absent at sites of O₂⁻ scavenging.

Immunodetection of SOD1 and LYS7-HA: Western blot analyses were carried out. A polyclonal rabbit anti-yeast SOD1 serum and a polyclonal PMA1 antibody from rabbit were detected using anti-rabbit donkey IgG as secondary antibody. LYS-HA was detected using a monoclonal anti-HA antibody from mouse and sheep anti-mouse IgG as secondary antibody. All three secondary antibodies were conjugated to horseradish peroxidase and a kit was used for detection.

Indirect immunofluorescence microscopy utilised strain AA255 transformed with pHAL7. Yeast cells were grown in minimal medium

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Annex Point IIA6.2

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Specify section no., heading and species as appropriate

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121.2 Results and discussion

and prepared for immunofluorescence microscopy. After incubation with mouse anti-HA, detection of LYS-HA was carried out with a FITC labelling kit. Nucleic acids were stained by DAPI.

Metabolic labelling of SOD1: Yeast strains were labelled with 200 μ Ci of ^{64} Cu. Cell pellets were resuspended in lysis buffer and homogenised. Cytosolic and membrane fractions were obtained by centrifugation. Equal amounts of cytosolic protein were analysed on nondenaturing, non-reducing polyacrylamide gels, followed by autoradiography to detect holoSOD1. Incorporation of ^{64} Cu into FET3 was then analysed.

Summarize relevant results; discuss dose-response relationship.

Yeast cells lacking SOD1 are auxotrophic for methionine and lysine when grown in air, as is the *lys7* complementation group of *S. cerevisiae*. This similarity between *sod1 Δ* and *lys7 Δ* mutants suggests that SOD1 and LYS7 may be functionally related. Multiple human ESTs homologous to LYS7 were identified, and analysis revealed the complete open reading frame of the human LYS7 homologue, CCS. CCS is coded by a gene expressed in all human tissues and cell types examined. It also exhibits notable homology to SOD1. To test whether CCS is a functional homologue of LYS7 or SOD, the CCS open reading frame was placed under the control of the yeast *PGK1* promoter and was used to transform *sod1 Δ* and *lys7 Δ* yeast. The expressed CCS gene failed to complement a *sod1 Δ* mutation, and no SOD activity was associated with CCS. However, the CCS clone restored aerobic growth of *lys7 Δ* mutants on medium lacking lysine and methionine, showing that human CCS is a functional homologue to yeast LYS7.

To test whether LYS7 was essential for SOD1 activity, activity was examined in *lys7 Δ* cell lysates run on non-reducing polyacrylamide gels and exposed to NBT. The *lys7 Δ* mutant mimicked a *sod1 Δ* mutant and displayed no SOD1 activity, but exhibited normal levels of O₂-scavenging by SOD2. By western blot analysis of these lysates, *lys7 Δ* mutants contained wild type levels of SOD1 protein, showing that the SOD1 polypeptide in *lys7 Δ* mutants is inactive for O₂-scavenging. To determine if the lack of SOD1 activity in *lys7 Δ* mutants was due to a defect in Cu incorporation into SOD1, yeast strains were labelled with ^{64} Cu *in vivo*. Wild type cells had a ^{64} Cu-labelled protein corresponding to SOD1. However, *lys7 Δ* mutants were devoid of ^{64} Cu-labelled SOD1; a defect was complemented by a plasmid harbouring human CCS. It was therefore apparent that LYS7 was essential for Cu incorporation into SOD1, and this function is conserved in the human homologue, CCS.

Localisation of LYS7 within cells was examined using a HA epitope-tagged protein. It was found that LYS7-HA fully complemented *lys7 Δ* mutation and migrated as a single band on SDS gels. Extracts from cells expressing LYS-HA were resolved into soluble and membrane-associated components. It was found that LYS-HA co-fractionated with cytosolic SOD1 to the supernatant, but was absent in the membrane fraction containing the proton ATPase. Using immunofluorescence microscopy, LYS7-HA showed diffuse staining throughout the cytosol and was largely excluded from the nucleus. LYS7 is therefore cytosolic, as is the case for its recipient of Cu delivery, SOD1.

LYS7 was highly specific for SOD1. It does not participate in delivery of Cu to the excretory pathway, as *lys7 Δ* cells were fully functional for incorporation of ^{64} Cu into FET3. Furthermore, over-expression on another Cu chaperone (ATX1) could not rescue the SOD1 Cu-labelling

Section A6.2**Annex Point IIA6.2****IUCLID: 5.0/23****Metabolism in mammals***Specify section no., heading and species as appropriate***A6.2(23), Cellular and molecular metabolism of copper****121.3 Conclusion**

defect of *lys7* mutants. Hence, distinct pathways of Cu trafficking are mediated by ATX1 and LYS7. It is considered that the pathways are likely to involve protein-protein interaction between the metal chaperone and its Cu-requiring recipient.

The results of this study demonstrate that Cu incorporation into SOD1 in intact cells is absolutely dependent on the presence of LYS7/CCS.

121.3.1 Reliability

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

2

121.3.2 Deficiencies

Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP, and experimental details have been omitted on a number of occasions. However, this does not necessarily compromise the validity of the data presented, or the author's interpretation of those data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed publication, and has therefore been subject to the prior scrutiny of experts in the field. In addition, this report has been included in a number of expert reviews of copper toxicokinetics.

No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary.

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

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE**Date**

[REDACTED]

Materials and Methods

- [REDACTED]

Results and discussion

[REDACTED]

Conclusion

[REDACTED]

Reliability

[REDACTED]

Acceptability

[REDACTED]

Remarks

[REDACTED]

Section A6.2
Annex Point IIA6.2
IUCLID: 5.0/23

Metabolism in mammals
Specify section no., heading and species as appropriate
A6.2(23), Cellular and molecular metabolism of copper

Date

COMMENTS FROM ...
Give date of comments submitted

Figure A6.2(23)-1

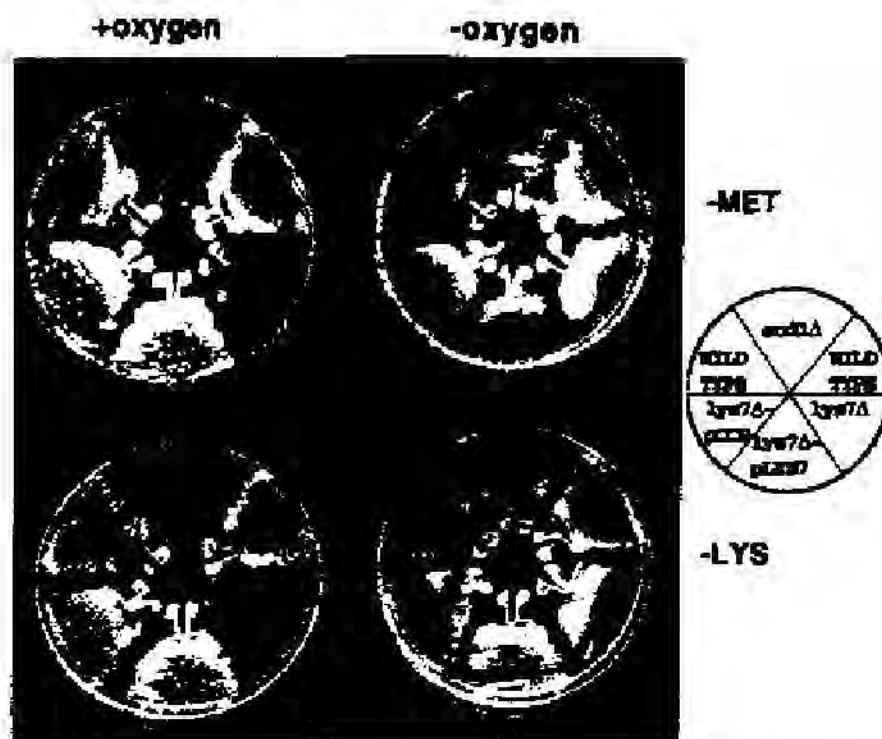


FIG. 1. Oxygen-dependent lysine and methionine auxotrophy of *lys7Δ* mutants. The indicated strains of yeast were plated onto complete synthetic dextrose medium (22) lacking either ("–*LYS*") or methionine ("–*MET*") and were incubated for 3 days at 30 °C either in air ("+oxygen") or in a BBL anaerobic culture jar ("–oxygen"). Strains utilized (shown in clockwise): wild type = 1783 (20); *sod1Δ* = KS105 (1783 *sod1Δ::TRP1*) (20); wild type = SY1699 (29); *lys7Δ* = SY2950 (SY1699 *lys7Δ::LEU2*) (29); *lys7Δ*-pLYS7 and *lys7Δ*-pCCS = SY2950 transformed with the pSL2502 plasmid harboring *S. cerevisiae* *LYS7* (29) and with pSMCCS-expressing human CCS from the yeast *PGK1* promoter, respectively.

Figure A6.2(23)-2

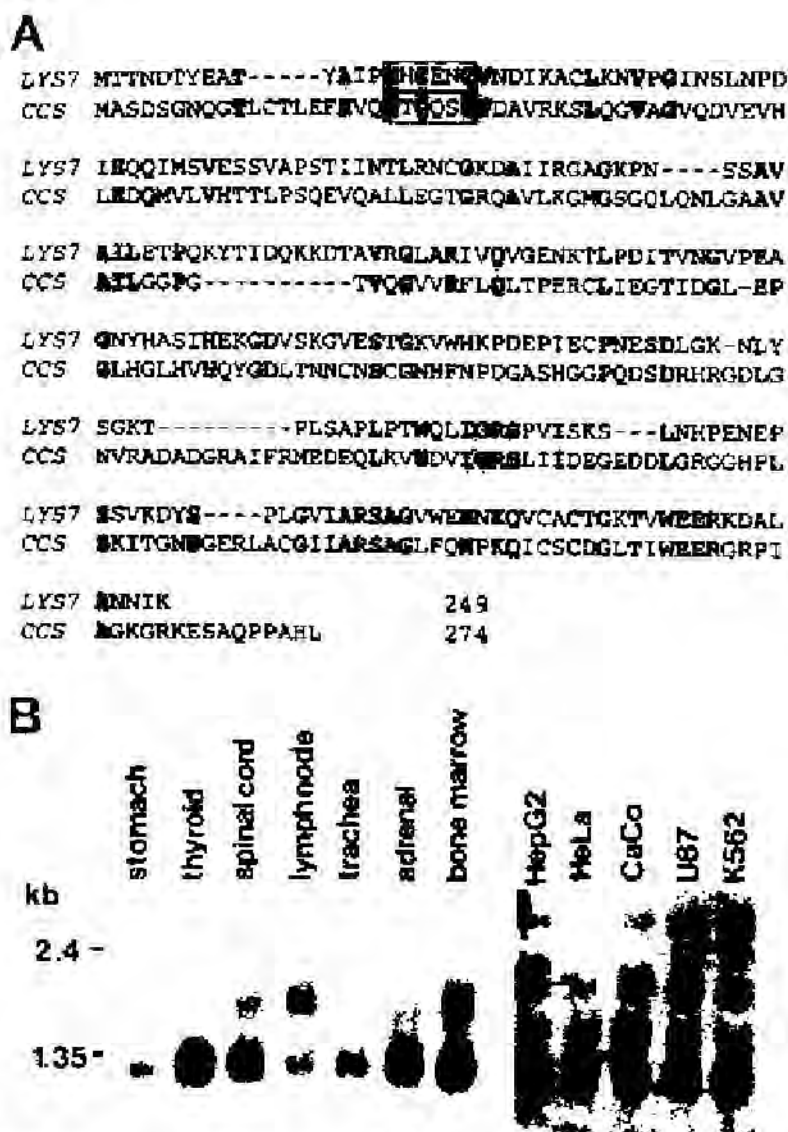


FIG. 2. *A*, sequence alignment of *S. cerevisiae* LYS7 and human CCS. The deduced amino acid sequence of CCS was aligned with LYS7; regions of amino acid identity are indicated by *shading*, and the putative copper binding motifs are shown in the *boxed region*. *B*, expression of the human CCS gene. RNA from the indicated tissues or cell lines was hybridized using a ^{32}P -labeled CCS-specific cDNA probe and exposed to film for 24 h. This analysis revealed a major CCS-specific transcript of 1.2 kilobases as well as multiple higher molecular weight species in all tissues and cell lines as shown. Identical transcripts were observed in RNA samples from 31 additional human adult and fetal tissues, including multiple regions of the central nervous system. Results are representative of three independent blots.

Figure A6.2(23)-3

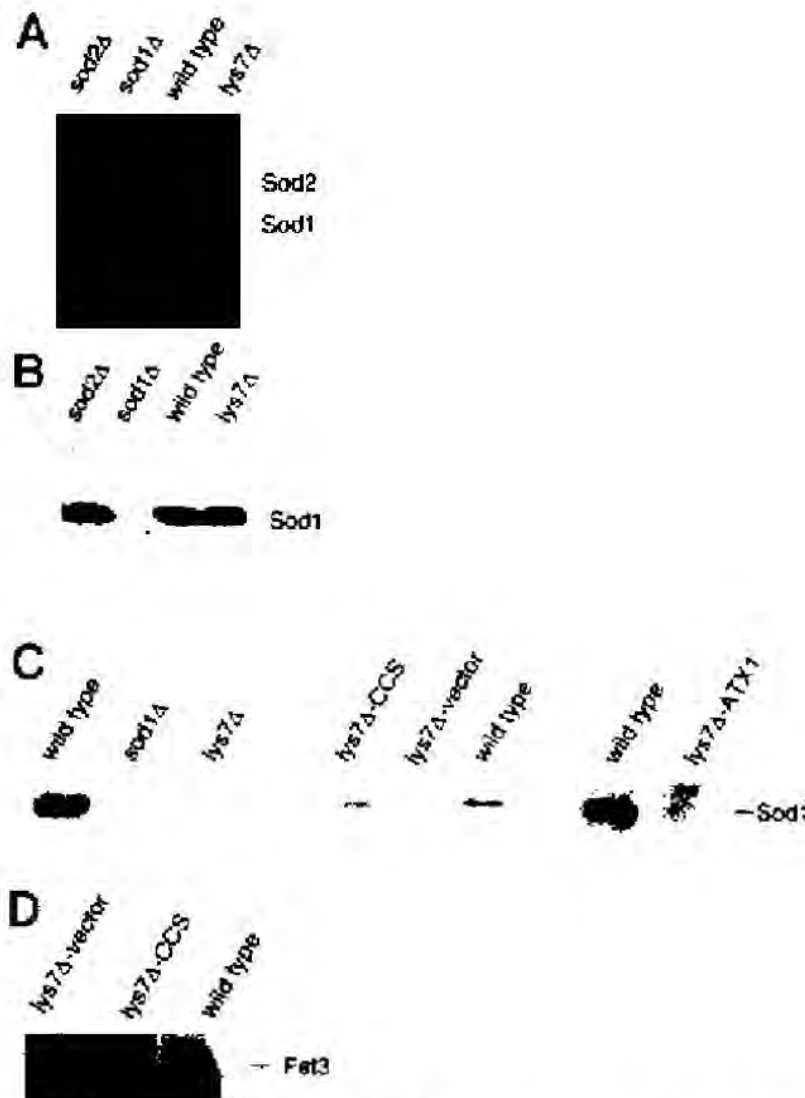


FIG. 3. LYS7, but not ATX1, is required for copper insertion into SOD1. *A* and *B*, lysates from the indicated strains were subjected to electrophoresis on a 12% polyacrylamide gel under nondenaturing, nonreducing conditions (*A*) or in the presence of SDS (*B*). *A*, assay for SOD activity was conducted using NBT. Positions of the mitochondrial manganese containing SOD2 and the copper zinc SOD1 are indicated. *B*, Western blot analysis of extracts using an antibody directed against *S. cerevisiae* SOD1. Strains utilized: *sod2Δ* = JS002 (see *A*); *sod1Δ* = KS006 (20); wild type = SY1699 (29); *lys7Δ* = SY2950 (29). Results are representative of four independent lysates examined. *C*, autoradiograph of ⁶⁴Cu-labeled SOD1 in cytosolic fractions from the indicated yeast strains following nondenaturing, nonreducing polyacrylamide gel electrophoresis. *lys7Δ* mutants are shown transformed with vector alone or plasmids containing either CCS or ATX1. *D*, biosynthesis of holoFET3 in crude membrane fractions from these same ⁶⁴Cu-labeled strains. Results are representative of three independent experimental trials, and the apparent increase in FET3 labeling in cells expressing CCS is consistent.

Figure A6.2(23)-4

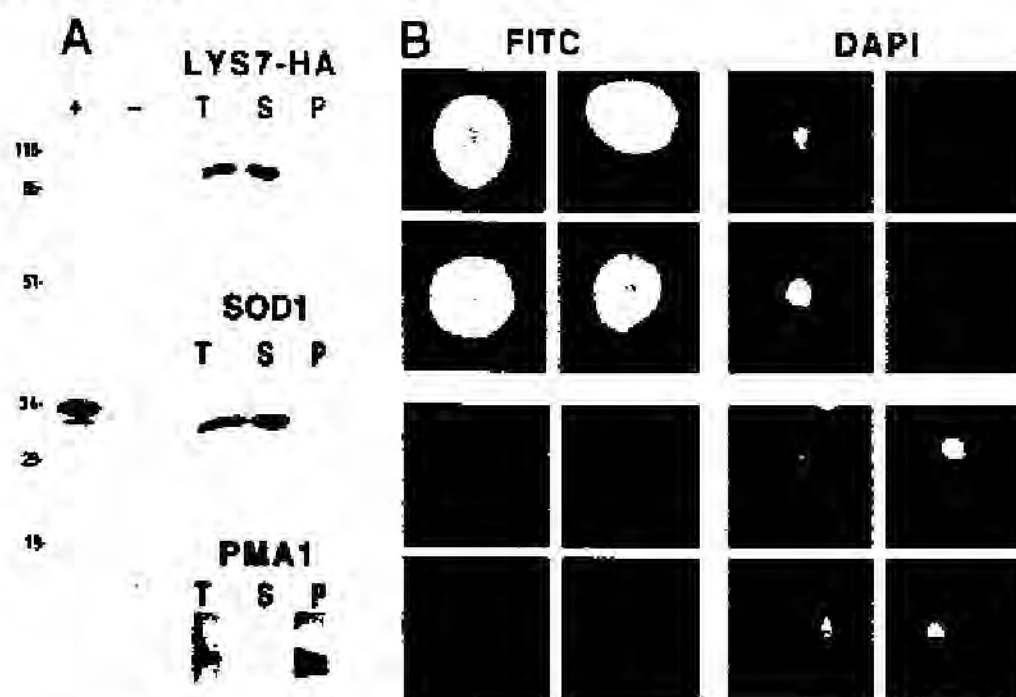


FIG. 4. LYS7 is a soluble cytosolic protein. *A*, total cell extracts prepared from "-" = untransformed strain AA255 (25) and "+" = AA255 expressing LYS7-HA from plasmid pHAL7 were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot. *Left*, sizes in kilodaltons and positions of molecular weight standards run in parallel are indicated. *Right*, extracts were subjected to centrifugation at $100,000 \times g$ as described (17), and the resultant supernatants ("S"), pellets ("P"), or total extracts ("T") were analyzed by Western blot, using where indicated, antibodies directed against either SOD1 ("SOD1"), HA ("LYS7-HA"), or the plasma membrane PMA1 proton ATPase ("PMA1"). *B*, strain AA255 transformed with the pLYS7-HA plasmid pHAL7 (*top*) or untransformed cells (*bottom*) was subjected to fluorescence microscopy (magnification $\times 1000$). "FITC" = LYS7-HA visualized with murine anti-HA primary antibody and a secondary antibody conjugated to FITC. "DAPI" = nucleic acids visualized with DAPI. Results are representative of approximately 200 stained cells examined.

122 REFERENCE

- 1.1 Reference** *Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)*
If necessary, copy field and enter other reference(s).
van Berge Henegouwen, G.P., Tangedahl, T.N., Hofmann, A.F., Northfield, T.C. La Russo, N.F. and McCall, J.T., (1977). Biliary Secretion of Copper in Healthy Man. Quantitation by an intestinal perfusion technique. *Gastroenterology*, **72**:1228-1231 (published).
- 1.2 Data protection** No
(indicate if data protection is claimed)
- 1.2.1 Data owner *Give name of company*
Public domain
- 1.2.2
- 1.2.3 Criteria for data protection Choose one of the following criteria (see also TNSG on Product Evaluation) and delete the others: No data protection claimed

123 GUIDELINES AND QUALITY ASSURANCE

- 123.1 Guideline study** No. This was a non-regulatory study carried out in human volunteers. The experimental protocol was approved by the Mayo Clinic Committee on Human Studies and by the Human Studies Committee of the Medical College of Virginia. This study was conducted to quantify daily copper secretion in man. No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")
- 123.2 GLP** No. This was a non-regulatory study carried out in human volunteers.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)
- 123.3 Deviations** No. Not applicable to non-guideline studies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

124 MATERIALS AND METHODS

- In some fields the values indicated in the EC or OECD test guidelines are given as default values. Adopt, change or delete these default values as appropriate.*
- 124.1 Test material** Samples of biliary secretions were analysed for copper content.
- 124.1.1 Lot/Batch number Not applicable.
- 124.1.2 Specification Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

Section A6.2**Metabolism in mammals****Annex Point IIA6.2**

Specify section no., heading and species as appropriate

IUCLID: 5.0/24**A6.2(24), Excretion of copper**

124.1.2.1 n	Description	<i>If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)</i> Not applicable.
124.1.2.2	Purity	<i>Give purity in % of active substance</i> [REDACTED]
124.1.2.3	Stability	<i>Describe stability of test material</i> Not applicable.
124.1.2.4 ling	Radiolabel	<i>give structural location of radio labelling, give reason if not labelled</i> not applicable to a study of this type. Non-entry field

124.2 Test Subjects

124.2.1	Species	Human volunteers
124.2.2	Strain	Not applicable
124.2.3	Source	Not applicable
124.2.4	Sex	Not stated.
124.2.5	Age/weight/height at study initiation	<i>Young adults recommended</i> Not stated.

124.3 Procedures*(fill in respective route in the following, delete other routes)*

124.3.1	Quantitation of biliary copper excretion – perfusion method.	Non-entry field. Biliary copper secretion was measured in 14 persons in the following groups: 5 healthy volunteers; 5 patients with radiolucent gallstones in visualising gall-bladders. These patients were examined on two occasions, during one of which they were receiving cheno-deoxychloric acid to induce gallstone dissolution; 4 patients with type IIA hyperlipoproteinemia not receiving medication. All subjects were admitted to a metabolic Unit for 6 days. A repeating diet of solid food, providing 30 cal per kg body weight, was fed over this period. Copper concentration of the diet was measured by atomic absorption spectroscopy after the sample had been dried, ground and dissolved in concentrated nitric acid. Secretion studies were carried out on the 6th day. A triple lumen tube was passed nasally and positioned under fluoroscopic control so that the perfusion site was situated in the second part of the duodenum (25 cm apart) and the aspiration site was situated at the ligament of Treitz. A third reinfusion port was situated 20 cm beyond the ligament of Treitz; a gastric tube was passed perorally under fluoroscopic control for introduction of the meals; during the secretion studies, three equicaloric liquid meals were fed. The gastric tube also permitted the checking of samples for duodenal reflux. Duodenal perfusion with polyethylene glycol 4,000 (PEG), a non-absorbable marker, and measurements of biliary output of bile acids were carried out and the hourly biliary output of copper was calculated as follows: $\text{Copper output/hour} = (\text{Copper}_a \times \text{PEG}_p \times \text{Vol}_p) / \text{PEG}_a$ Where: Copper_a = the concentration of copper in the aspirate;
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Section A6.2

Annex Point IIA6.2

IUCLID: 5.0/24

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(24), Excretion of copper

	PEG_p and PEG_a = are the concentration of PEG in the perfusate and aspirate, respectively; Vol_p = the hourly perfusion rate. This figure was divided by body weight in kilograms to obtain the output per kilogram of body weight. These calculations assume that all dietary copper was absorbed proximal to the aspirating site and that no absorption of biliary copper occurred in the mixing segment. In each individual study, biliary output of copper was corrected for the copper content of the perfusate which was less than 1% of the measured output. No correction was made for the copper content of the liquid meals, which averaged 0.05 mg/meal; the total copper intake in the liquid meals was about 10% of the measured biliary copper output. Copper analyses in the samples were done by atomic absorption spectroscopy.
124.3.2 Quantitation of biliary copper excretion – Baldwin T-tube method.	Four 24 hour collections of bile were obtained from 3 patients with a Baldwin T-tube, who had undergone cholecystectomy several months previously. A complete collection of bile was ensured by inflating the balloon in the distal limb of the T-tube. Liver function tests were normal at the time of the bile collection.
124.3.3 Balance studies in hyperproteinemia patients.	Complete copper balance studies were done in 4 patients. The faecal output of copper was calculated and corrected for irregular colonic emptying using the non-absorbable marker, chromium sesquioxide (Cr₂O₃). The chromium sesquioxide was administered for 1 week before admission and throughout the 4-day balance period at a dose of 500 mg, three times a day. All stools were collected and pooled at the end of each 24-hour period. Aqueous homogenates were prepared, and an aliquot of the homogenate was used for copper and chromium determination by atomic absorption procedure. For the chromium determinations, dried samples were dissolved in nitric acid and oxidised by addition of concentrated perchloric acid after complete dissolution had been obtained. They were then heated until dense perchloric acid fumes appeared, and heating was continued for an additional 20 minutes.

125 RESULTS AND DISCUSSION

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

125.1 Results

Non-entry field

125.1.1 Biliary copper output	Daily biliary output ($\pm$ SD) was 24.6 ± 12.8 μg/kg bodyweight, and was not significantly different in any group tested (Table A6.2(24)-1). The 24 hour output from the three T-tube patients was within the range observed in healthy subjects using the perfusion technique.
125.1.2 Copper balance	In the 4 patients in whom complete balance studies were carried out, biliary excretion was not different from faecal output, which in turn did not differ from dietary intake (Table A6.2(24)-2).
125.1.3 Pattern of biliary copper output	Output for normal subjects was similar during the day when meals were eaten from 8 AM to 8 PM (16.1 ± 9.2 μg/kg) (mean $\pm$ SD) and during overnight fast from 8 PM to 8 AM (14.0 ± 6.0 μg/kg). There was no correlation between the hourly rate of copper output and the hourly rate of bile acid output, nor was there any correlation between daily copper output and daily bile acid output. Typical 24-hour patterns of copper and bile acid outputs are shown in Figure A6.2(24)-1.

Section A6.2**Annex Point IIA6.2**

IUCLID: 5.0/24

Metabolism in mammals*Specify section no., heading and species as appropriate***A6.2(24), Excretion of copper****125.2 Discussion**

Non-entry field

125.2.1 Copper metabolism in healthy humans

The results of this study indicate that dietary intake, biliary secretion and faecal excretion are similar, thereby providing support for the view that copper balance is maintained by biliary secretion and subsequent faecal excretion of copper.

125.2.2 Accuracy of methodology

There are two potential sources of error in the copper output data of the perfusion study. First, copper which was secreted in bile may have been absorbed in the mixing segment. Second, dietary copper may not have been completely absorbed proximal to the collecting site at the ligament of Treitz. These potential errors are opposing, in that absorption of copper in the mixing segment would result in an underestimate, whereas a contribution of dietary copper to the copper in the aspirate would result in an overestimate. The magnitude of the first potential error should be small, given that values for biliary secretion and faecal excretion are identical. This is consistent with the view that intestinal absorption of copper secreted in bile is negligible in healthy humans. The magnitude of the second potential error must also be small, given that dietary intake was only 10% of the estimated outputs. These sources of error are not present in the values obtained in the 24 hour collections from the bile fistula patients; in fact, these values are in good agreement with those obtained by the perfusion technique. In conclusion, it is considered that the figures obtained from this study represent an accurate estimate of biliary copper secretion.

126.1 Materials and methods**126 APPLICANT'S SUMMARY AND CONCLUSION**

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

A study was conducted to quantify daily Cu secretion in man. The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP. The following techniques were used:

Quantitation of biliary copper excretion – perfusion method: Biliary Cu secretion was measured in 14 persons in the following 3 groups: 5 healthy volunteers; 5 patients with radiolucent gallstones; 4 patients with type IIA hyperlipoproteinemia. The Cu content of the diet fed for 6 days prior to the start of the secretion study was measured by AAS. On the 6th day, a triple lumen tube was passed nasally and positioned so that the perfusion site was in the second part of the duodenum and the aspiration site was at the ligament of Treitz. A third re-infusion port was placed beyond the ligament of Treitz. During secretion studies, 3 liquid meals were fed via a gastric tube. Duodenal perfusion with a non-absorbable marker (PEG) was carried out hourly. Biliary output of bile acids was measured and Cu output calculated. It was assumed that all dietary Cu was absorbed proximal to the aspirating site, and that there was no absorption of biliary Cu in the mixing segment. Biliary output of Cu was corrected for Cu content of the perfusate. No correction was made for Cu content of liquid meals, which represented about 10% of measured biliary output. Cu analyses were by AAS.

Quantitation of biliary copper excretion – Baldwin T-tube method: Four 24 hour complete collections of bile were obtained using a Baldwin T-tube from 3 patients who had undergone cholecystectomy several months previously.

Section A6.2**Annex Point IIA6.2**

IUCLID: 5.0/24

Metabolism in mammals*Specify section no., heading and species as appropriate***A6.2(24), Excretion of copper****126.2 Results and discussion**

Balance studies in hyperproteinemia patients: Complete Cu balance studies were done in 4 patients. The faecal Cu output was calculated and corrected for irregular colonic emptying using a non-absorbable marker (chromium sesquioxide). The marker was administered for 1 week before admission and throughout the 4-day balance period. Stools were collected and pooled at the end of each 24-hour period. Cu and Cr content was determined by AAS.

Summarize relevant results; discuss dose-response relationship.

Biliary copper output: Daily biliary output ($\pm$ SD) was 24.6 ± 12.8 $\mu\text{g/kg}$ bodyweight, and was not significantly different in any group tested. The 24 hour output from the three T-tube patients was within the range observed using the perfusion technique.

Copper balance: In the 4 patients in whom complete balance studies were carried out, biliary excretion was not different from faecal output, which in turn did not differ from dietary intake. This finding suggests that intestinal absorption of CU secreted in bile is negligible.

Pattern of biliary copper output Mean output ($\pm$ SD) was similar during the day when meals were eaten from 8 AM to 8 PM (16.1 ± 9.2 $\mu\text{g/kg}$) and during overnight fast from 8 PM to 8 AM (14.0 ± 6.0 $\mu\text{g/kg}$). There was no correlation between the hourly rate of Cu output and the hourly rate of bile acid output, nor was there any correlation between daily Cu output and daily bile acid output.

126.3 Conclusion

The results of this study indicate that dietary intake, biliary secretion and faecal excretion are similar, thereby suggesting that copper balance is maintained primarily by biliary secretion and subsequent faecal excretion of copper which has been absorbed in the stomach and proximal jejunum. Mean copper excretion is estimated to be in the order of 1.7 mg Cu/day (24.6 ± 12.8 $\mu\text{g Cu/kg}$ bodyweight)

126.3.1 Reliability

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

2

126.3.2 Deficiencies

Yes

This study was not conducted and/or reported in strict compliance with the principles of GLP. However, this does not compromise the validity of the data generated, or the author's interpretation of that data, given that the study was not carried out for regulatory purposes. Furthermore, the research was published in a peer-reviewed journal, and has therefore been subject to the prior scrutiny of experts in the field. In addition this report has been included in a number of expert reviews of copper toxicokinetics.

No internationally accepted guidelines are available that specifically address the objective of the research presented in this summary.

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

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

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/24

A6.2(24), Excretion of copper

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

COMMENTS FROM ...

Date

Give date of comments submitted

TABLE 1. *Daily biliary copper output in all subjects: duodenal perfusion technique^a*

Normal subjects			Gallstone patients			Hyperlipoproteinemia patients			Bile fistula patients		
Subject	Output	Patient	No medication, output	Chemical treatment, output	Patient	Output	Patient	Output			
	$\mu\text{g/kg}$		$\mu\text{g/kg}$	$\mu\text{g/kg}$		$\mu\text{g/kg}$		$\mu\text{g/kg}$			
A	53.0	F	40.4	2.41	K	0.82	P ₁	1.07			
B	49.0	G	14.6	1.14	L	1.22	P ₂	1.64			
C	19.2	H	9.0	0.54	M	2.47	R	2.61			
D	18.3	I	41.6	3.34	N	1.43	S	5.01			
E	27.3	J	19.1	1.31							
Mean	33.4		24.9	1.75		1.49		2.88			
$\pm$ SD	± 16.5		± 16.1	± 1.11		± 0.70					

^a Mean ($n = 19$) of all perfusion studies: $24.6 \mu\text{g per kg-day} \pm 12.6 (1.64 \pm 0.80 \text{ mg per day})$.^b Two 24-hr collections were obtained for patient P. The mean of the two values was used to calculate the mean of all collections.

TABLE 2. *Balance studies in hyperlipoproteinemia patients*^a

Copper balance	Subject				Mean ($\pm$ SD) (of 4 subjects)
	1	2	3	4	
<i>$\mu\text{g/kg BW/24 hr}^b$</i>					
Dietary intake ^c	17.14 (± 4.4)	19.39 (± 5.6)	18.11 (± 6.1)	20.09 (± 5.6)	18.68 (± 5.4)
Biliary output ^d	13.83	20.02	29.0	16.39	19.82 (± 6.63)
Fecal output ^e	17.33 (± 3.2)	20.25 (± 7.9)	18.24 (± 7.3)	20.76 (± 6.6)	19.14 (± 7.3)

^a Values are means ($\pm$ SD).^b BW, body weight.^c During the 4 days before study (days 1 to 4).^d On study day (day 6).^e Mean ($\pm$ SD), during the 4 days before study (days 1 to 4).

Figure A6.2(24)-1

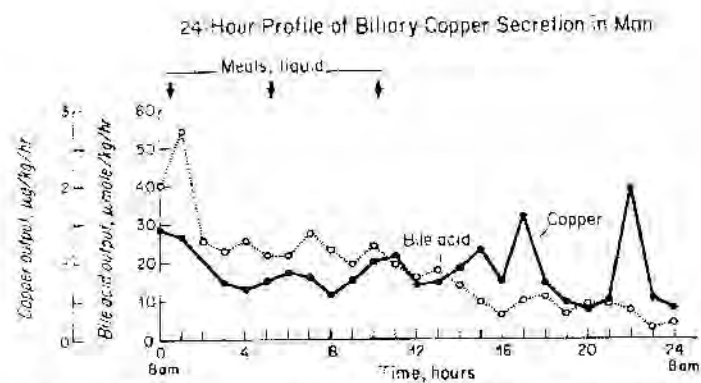


FIG. 1. Hourly biliary output of copper and bile acids in a normal volunteer. Bile acid output is higher during the day in agreement with previous studies,^{13, 14} whereas copper output does not diminish during overnight fasting.

127 REFERENCE

127.1 Reference

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

Aoyagi, S. and Baker, D.H. (1993). Bioavailability of Copper in Analytical-Grade and Feed Grade Inorganic Copper Sources when Fed to Provide Copper at Levels Below the Chick's Requirement. Poultry Science. **72**: 1075-1083 (published).

127.2 Data protection

No
(indicate if data protection is claimed)

127.2.1 Data owner

Give name of company
Public domain

127.2.2 Criteria for data protection

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

128 GUIDELINES AND QUALITY ASSURANCE

128.1 Guideline study

No. This was a non-regulatory study carried out in chicks to establish a sensitive bioassay system for assessing Cu bioavailability at Cu levels below the chick's Cu requirement. After a suitable criterion was found, relative Cu bioavailability in analytical grade (AG) Cu₂O, AG-CuO, AG-CuCO₃.Cu(OH)₂, AG-CuCl, food grade (FG) CuSO₄.5H₂O and FG-CuO was established relative to the Cu provided in AG-CuSO₄.5H₂O. No guidelines are available to address these objectives. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*

128.2 GLP

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

128.3 Deviations

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

129 MATERIALS AND METHODS

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

129.1 Test material

Cu²⁺ as Copper sulphate (CuSO₄.5H₂O), analytical grade.
Cu²⁺ as Copper sulphate (CuSO₄.5H₂O), food grade.
Cu²⁺ as Cupric oxide (CuO), food grade.
Cu²⁺ as Cuprous oxide (Cu₂O), analytical grade.
Cu²⁺ as Basic copper carbonate (CuCO₃.Cu(OH)₂), analytical grade.
Cu²⁺ as Copper chloride (CuCl), analytical grade.

Section A6.2**Metabolism in mammals****Annex Point IIA6.2***Specify section no., heading and species as appropriate***IUCLID: 5.0/25****A6.2(25), Bioavailability of copper**

129.1.1	Lot/Batch number	Not available
129.1.2	Specification	Deviating from specification given in section 2 as follows (describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):
129.1.2.1	Description	If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution) Refer to section 3.1.
129.1.2.2	Purity	Give purity in % of active substance [REDACTED]
129.1.2.3	Stability	Describe stability of test material Not available
129.1.2.4	Radiolabelling	give structural location of radio labelling, give reason if not labelled Not deemed necessary for the purposes of this study.
129.2	Test Animals	Non-entry field
129.2.1	Species	Chicks
129.2.2	Strain	Cross of New Hampshire males and Columbian females.
129.2.3	Source	Not stated.
129.2.4	Sex	Male
129.2.5	Age/weight/height at study initiation	Young adults recommended Age: The study covered the period 0 - 23 days post-hatching. Weight: Weight gain was monitored throughout the study.
129.2.6	Number of animals per group	Give number Specify, if there are differences for example for treatment and recovery groups Four replicate groups of 5 chicks were fed each of the 9 experimental diets.
129.2.7	Controls	Yes
129.3	Administration/Exposure	(fill in respective route in the following, delete other routes) Oral administration of the test substances in the diet.
129.3.1	Duration of treatment	Up to 23 days post-hatching.
129.4	Procedures	Non-entry field
129.4.1	Experiment 1	This experiment was conducted to confirm the sensitivity of the bioassay system. During the first 7 days post-hatching, half of the chicks were fed a casein-soy protein concentrate basal diet containing 0.56 mg Cu/kg and the other half were fed the basal diet fortified with 8 mg Cu/kg from AG-CuSO ₄ .5H ₂ O. After overnight withdrawal of feed, chicks from each pre-test group were weighed and assigned randomly to one of 7 levels of Cu supplementation (0, 1, 2, 4, 8, 12 and 16 mg Cu/kg from AG-CuSO ₄ .5H ₂ O) at day 8 post-hatching. Three replicate groups of five chicks were assigned to each of the 14 dietary treatments. The test was carried out between 8 and 23 days post-hatching. Distilled-deionised water and diets were provided <i>ad-libitum</i> .

Section A6.2**Annex Point IIA6.2**

IUCLID: 5.0/25

Metabolism in mammals*Specify section no., heading and species as appropriate***A6.2(25), Bioavailability of copper**

At assay termination, chicks and feeders were weighed, after which all chicks were bled. They were subsequently killed by CO₂ suffocation, and their livers, gall bladder, hock joint tendons were quantitatively removed. All samples were pooled by replicate. Blood was assayed for haemoglobin and haematocrit, and the plasma portion was analysed for Cu concentration. Liver and gall bladder samples were freeze-dried, weighed, and ashed with HNO₃. Copper concentration was measured by flame atomic absorption spectrophotometry. Tendon samples were freeze-dried and subjected to 24-hour HCl (6 mol/l) hydrolysis, followed by lysine analysis by ion-exchange chromatography.

To obtain information on effects of chick age on Cu storage in the gall bladder, 3 replicates of 5 chicks were killed at hatching, and 3 separate replicates of 5 chicks were killed at 8 days post-hatching following feeding of the two pre-test diets. Blood, liver, tendon, and gall bladder were analysed as described above.

129.4.2 Experiment 2

Relative bioavailability of Cu from AG-Cu₂O and AG-CuO were compared with AG-CuSO₄·5H₂O. Crossbred male chicks were fed the Cu-deficient basal diet during the first 7 days post-hatching, after which they were used in a slope-ratio assay using bile Cu concentration as the response parameter. After an overnight deprivation of feed, 105 chicks were weighed and assigned randomly to experimental groups at day 8 post-hatching. Three replicate groups of 5 chicks were fed each of the 7 experimental diets from 8 to 22 days post-hatching. Graded levels (0, 0.5 and 1 mg/kg) of supplemental Cu from AG-CuSO₄·5H₂O were used to generate a straight-line standard curve. Two test compounds were also supplemented in the basal diet to provide graded levels of Cu: AG-Cu₂O, 0.5 and 1 mg Cu/kg; AG-CuO, 1 and 2 mg Cu/kg. Other aspects of the feeding study were as described for experiment 1 (section 3.4.1).

129.4.3 Experiment 3

The same procedure was used as in Experiment 2 (section 3.4.2), except that the test compounds evaluated were FG-CuSO₄·5H₂O and FG-CuO.

129.4.4 Experiment 4

The same procedure was used as in Experiment 2 (section 3.4.2), except that the test compounds were AG-CuCO₃·Cu(OH)₂ and AG-CuCl.

129.4.5 Statistical analysis

Analysis of variance and regression analyses were conducted using the General Linear Models (GLM) procedure of SAS software. Copper bioavailability was determined relative to an AG-CuSO₄·5H₂O set at 100% by common-intercept multiple linear regression (slope-ratio) of bile Cu regressed on supplemental Cu intake. Additional orthogonal and non-orthogonal contrasts were also performed, where appropriate.

130 RESULTS AND DISCUSSION

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

130.1 Experiment 1

Effects of supplemental copper on criteria of copper status are shown in **Table A6.2(25)-1**.

At the end of the 8-day pre-test period, body weight, haematocrit and haemoglobin values were not different between Cu-deficient and Cu-adequate chicks. The same was true for tendon lysine concentration. Concentration of Cu in plasma, gall bladder and liver, however, was reduced in Cu-deficient chicks, suggesting some degree of Cu depletion as a result of feeding 0.56 mg Cu/kg diet during the pre-test period.

At the end of the 15-day test period, weight gain, feed intake,

haematocrit, haemoglobin, tendon lysine concentration and plasma Cu did not show dose-dependent linear responses to supplemental Cu, regardless of Cu level fed during the 8-day pre-test period. Liver Cu concentration, however, increased linearly ($P < 0.05$) in chicks fed supplemental Cu doses between 0 and 2 mg/kg. Gall bladder Cu of chicks pre-tested on the Cu-deficient diet increased linearly ($P < 0.01$) as Cu supplementation increased to 2 mg/kg. Thereafter, an apparent plateau was reached. The linear response in gall bladder Cu represented a better fit in chicks pretested on the Cu-deficient diet than in those pretested on the Cu-adequate diet, although Cu concentration in gall bladder of chicks on the Cu-adequate diet was greater ($P < 0.01$) at the end of the experiment for chicks fed 1 mg Cu/kg than for those fed no supplemental Cu during the test period.

Gall bladder Cu at hatching was 41.0 µg/g, but it decreased to 3.80 µg/g after chicks were pretested for 8 days on the Cu-deficient diet. In contrast, after 8 days of pretesting on the Cu-adequate diet, gall bladder Cu was 57.7 µg/g, 15 times greater ($P < 0.01$) than that observed in chicks pretested on the Cu-deficient diet.

Based upon the results of Experiment 1, it was considered that bile Cu of chicks fed 0.56 mg Cu/kg during the 8 day pretest period followed by levels between 0 and 1 mg Cu/kg during the 15-day test period would allow Cu bioavailability quantification in different Cu sources.

130.2 Experiment 2

None of the experimental treatments in Experiment 2 affected weight gain or feed intake ($P > 0.05$). In this comparison of analytical grade Cu sources, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased bile Cu ($P < 0.01$), but CuO did not (**Table A6.2(25)-2**). Orthogonal contrasts were used to separate treatments ($df = 6$) into the following components: basal versus Cu supplementation (1df), Cu source (2 df), Cu level (1df), and Cu source by Cu level (2 df). This confirmed the results of Experiment 1, in that bile Cu increased as Cu supplementation from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased. However, there were differences among sources. The $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O increased bile Cu more than CuO. Also the higher level of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O (1 mg/kg) increased bile Cu more ($P < 0.01$) than the lower level (0.5 mg/kg).

There was a significant ($P < 0.05$) interaction between Cu source and Cu level (**Table A6.2(25)-2**). The higher level of Cu supplementation increased bile Cu much more when $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O were fed than when CuO was fed. The CuO, in fact, did not increase bile Cu ($P > 0.05$). Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake was performed only for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O . The ratio of regression coefficients (i.e. slopes) revealed that Cu_2O had an efficacy value of 97.9%, which was not different ($P > 0.05$) from the standard ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), which was set at 100%.

130.3 Experiment 3

None of the experimental treatments in Experiment 3 affected weight gain or feed intake ($P > 0.05$). It was found in this comparison of the most commonly used FG inorganic Cu sources against AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ that the FG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased bile Cu as much as AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($P < 0.01$), but that FG-CuO did not (**Table A6.2(25)- 3**). Bile Cu data were treated in the same manner as in Experiment 2. There was a significant ($P < 0.05$) interaction between Cu source and Cu level. The FG-CuO behaved much like AG-CuO, in that it did not increase bile Cu ($P > 0.05$), suggesting that the bioavailability of Cu in

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FG-CuO is zero. Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake was performed, without using data points for FG-CuO. The ratio of regression coefficients revealed that FG-CuSO₄.5H₂O had an efficacy value of 93.5%, which was not significantly different ($P>0.05$) from the standard AG-CuSO₄.5H₂O. It was concluded that FG versus AG made no difference to the bioavailability of Cu from the salt. Furthermore, the data obtained for CuO suggest that the majority of FG copper oxide must be CuO rather than Cu₂O.

130.4 Experiment 4

Weight gain and feed intake increased ($P<0.01$) as Cu was supplemented from all 3 AG sources used: CuSO₄.5H₂O, CuCO₃.Cu(OH)₂ and CuCl (**Table A6.2(25)-4**). However, dose-dependent responses due to Cu supplementation were more pronounced and linear for bile Cu data than for weight gain. Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake revealed that AG-CuCO₃.Cu(OH)₂ and AG-CuCl had efficacy values of 112.9 and 142.5% ($P<0.05$), respectively, when the standard (AG-CuSO₄.5H₂O) was set at 100%.

The Cu bioavailability estimate for AG-CuCO₃.Cu(OH)₂ was not different from the standard ($P>0.05$). The Cu bioavailability estimate of AG-CuCl was higher than the standard.

130.5 Toxic effects, clinical signs

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

130.6 Recovery of labelled compound

state percentage
Not stated.

131 APPLICANT'S SUMMARY AND CONCLUSION**131.1 Materials and methods**

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

A study was carried out to investigate the relative bioavailability of copper in analytical and feed-grade inorganic copper sources fed to chicks. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Young chicks were fed a casein-soy protein concentrate basal diet (0.56 mg Cu/kg) containing added Cu (0, 0.5, 1.0 mg/kg) from analytical grade (AG) CuSO₄.5H₂O, Cu₂O, CuO, CuCO₃.Cu(OH)₂, CuCl and also from feed-grade (FG) CuSO₄.5H₂O and CuO.

Experiment 1: For 7 days post-hatching, half the chicks were fed a basal diet containing 0.56 mg Cu/kg and the other half were fed the basal diet fortified with 8 mg Cu/kg from AG-CuSO₄.5H₂O. At 8 days old, chicks from each group were weighed and assigned to one of 7 levels of Cu supplementation (0, 1, 2, 4, 8, 12 and 16 mg Cu/kg from AG-CuSO₄.5H₂O). Three replicate groups of 5 chicks were assigned to each of the 14 dietary treatments. The test was carried out between 8 and 23 days post-hatching. Water and diets were provided *ad-libitum*.

At assay termination, chicks and feeders were weighed, after which all chicks were bled and killed by CO₂ suffocation. Liver, gall bladder, and tendon from the hock joint were quantitatively removed. All samples were pooled by replicate. Blood was assayed for haemoglobin and haematocrit, and plasma was analysed for Cu concentration. Liver

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and gall bladder samples were freeze-dried, weighed, and ashed with HNO_3 . Copper concentration was measured by flame AAS. Tendon samples were freeze-dried and hydrolysed for 24 hours in HCl , followed by lysine analysis by ion-exchange chromatography.

To obtain information on effects of chick age on Cu storage in the gall bladder, 3 replicates of 5 chicks were killed at hatching, and 3 separate replicates of 5 chicks were killed at 8 days post-hatching following feeding of the two pre-test diets. Blood, liver, tendon, and gall bladder were analysed as described above.

Experiment 2: Bioavailability of Cu from AG- Cu_2O and AG-CuO was compared with that of AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Crossbred male chicks were fed Cu-deficient basal diet for 7 days post-hatching, after which they were used in a slope-ratio assay using bile Cu concentration as the response parameter. On day 8, 105 chicks were weighed and assigned randomly to experimental groups. Three replicate groups of 5 chicks were fed the 7 experimental diets from 8 to 22 days. Graded levels (0, 0.5 and 1 mg/kg) of supplemental Cu from AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ were used to generate a straight-line standard curve. Two test compounds were also supplemented in the basal diet to provide graded levels of Cu: AG- Cu_2O , 0.5 and 1 mg Cu/kg; AG-CuO, 1 and 2 mg Cu/kg. Other aspects of the study were as described for experiment 1.

Experiment 3: The same procedure was used as in Experiment 2 except that the test compounds fed were FG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and FG-CuO.

Experiment 4: The same procedure was used as in Experiment 2, except that the test compounds fed were AG- CuCO_3 , $\text{Cu}(\text{OH})_2$ and AG-CuCl.

131.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Experiment 1: At the end of the pre-test period, no difference was seen in tendon lysine concentration, body weight, haematocrit or haemoglobin in Cu-deficient and Cu-adequate chicks. However, Cu in plasma, gall bladder and liver was reduced in Cu-deficient chicks, suggesting depletion as a result of feeding 0.56 mg Cu/kg diet.

At the end of the 15-day test period (days 8 – 23), weight gain, feed intake, haematocrit, haemoglobin, tendon lysine concentration and plasma Cu did not show dose-dependent linear responses to supplemental Cu, regardless of the level fed during the pretest period. However, liver Cu concentration increased linearly in chicks fed supplemental Cu doses of 0 - 2 mg/kg. Gall bladder Cu of chicks pretested on Cu-deficient diet increased linearly as Cu supplementation increased to 2 mg/kg. Thereafter, an apparent plateau was reached.

Gall bladder Cu at hatching was 41.0 $\mu\text{g/g}$, but decreased to 3.80 $\mu\text{g/g}$ after chicks were pretested on Cu-deficient diet. In contrast, after pretesting on the Cu-adequate diet, gall bladder Cu was 57.7 $\mu\text{g/g}$, 15 times greater than that observed in chicks pretested on the deficient diet.

Based upon these results, it was considered that bile Cu of chicks fed 0.56 mg Cu/kg during a pretest period, followed by levels of 0 - 1 mg Cu/kg during the 14-day test period would allow Cu bioavailability quantification in different Cu sources.

Experiment 2: None of the experimental treatments affected weight gain or feed intake. In this comparison of analytical grade Cu sources, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased bile Cu, but CuO did not. Orthogonal contrasts were used to separate treatments into the following components: basal

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versus Cu supplementation, Cu source, Cu level, and Cu source by Cu level. This confirmed the results of Experiment 1, in that bile Cu increased as Cu supplementation from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased. However, there were differences among sources. The $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O increased bile Cu more than CuO. Also the higher level of supplemental Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O (1 mg/kg) increased bile Cu more than the lower level (0.5 mg/kg).

There was a significant interaction between Cu source and Cu level. The higher level of Cu supplementation increased bile Cu much more when $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O were fed than when CuO was fed. The CuO, in fact, did not increase bile Cu. Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake was performed only for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and Cu_2O . The ratio of regression coefficients (i.e. slopes) revealed that Cu_2O had an efficacy value of 97.9%, which was not different from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (set at 100%).

Experiment 3: None of the treatments in this experiment affected weight gain or feed intake. In this comparison of FG inorganic Cu sources against AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, it was found that the FG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ increased bile Cu as much as AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, but that FG-CuO did not. Bile Cu data were treated in the same manner as in Experiment 2. There was a significant interaction between Cu source and Cu level. The FG-CuO behaved much like AG-CuO, in that it did not increase bile Cu, suggesting that the bioavailability of Cu in FG-CuO is zero. Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake was performed, without using data points for FG-CuO. The ratio of regression coefficients revealed that FG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ had an efficacy value of 93.5%, which was not significantly different from AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (set at 100%).

It was concluded that FG versus AG made no difference to the bioavailability of Cu from the salt. Furthermore, the data obtained for CuO suggest that the majority of FG copper oxide must be CuO rather than Cu_2O .

Experiment 4: Weight gain and feed intake increased as Cu was supplemented from all 3 AG sources used ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ and CuCl). However, dose-dependent responses due to Cu supplementation were more pronounced and linear for bile Cu than for weight gain. Common-intercept multiple linear regression of bile Cu regressed on supplemental Cu intake showed that AG- $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ and AG-CuCl had efficacy values of 112.9 and 142.5%, respectively, when AG- $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (set at 100%).

The Cu bioavailability estimate for AG- $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ was not different from the standard. The Cu bioavailability estimate of AG-CuCl was higher than the standard.

131.3 Conclusion

The bioavailability of Cu from CuO was considered to be essentially zero. There was no significant difference between the bioavailability of Cu derived from Cu_2O (97.9%), $\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$ (112.9%) and that of the standard ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (100%). The Cu bioavailability estimate of CuCl was higher than that of the standard (142.5%).

131.3.1 Reliability

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

2

131.3.2 Deficiencies

Yes

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

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

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE**Date****Materials and Methods****Results and discussion****Conclusion****Reliability****Acceptability****Remarks****COMMENTS FROM ...****Date***Give date of comments submitted*

TABLE A6.2(25)-1. Effects of supplemental copper on criteria of copper status, Experiment 11

Supplemental Cu ² Pretest ³	Test	Weight gain	Feed intake	Hematocrit	Haemoglobin	Tendon Lys ⁴	Gall bladder Cu ^{4,5}		Plasma Cu	Liver Cu ^{6b}
----- (mg/kg)			-- (g) -----	(%)	(µg/100 mL)	(µg)	CUg/g)	(wg)	(wg/1-)	(ug/g)
0	0	156	296	28.5	10.2	1.01	4.3	.20	127	13.7
0	1	180	325	29.7	9.9	1.04	18.0	1.16	193	15.8
0	2	162	302	28.8	10.1	1.05	32.7	2.16	220	16.7
0	4	168	320	28.2	9.7	1.06	29.2	1.52	133	17.1
0	8	165	309	29.2	9.6	1.02	27.2	1.47	173	17.0
0	12	162	312	29.5	13.0	1.03	23.3	.94	120	16.8
0	16	174	316	29.5	9.9	1.03	22.7	1.09	89	17.9
8	0	186	341	29.0	9.7	1.04	6.5	.33	NA ⁷	15.1
8	1	166	331	29.8	12.0	1.10	26.3	1.59	127	17.3
8	2	167	333	29.2	9.9	1.04	20.0	.85	160	17.4
8	4	173	327	30.3	10.4	1.06	26.5	1.33	200	17.8
8	8	182	332	30.7	12.3	1.01	22.5	1.52	130	17.1
8	12	173	318	29.0	9.8	NA ⁷	NA ⁷	NA ⁷	127	17.7
8	16	170	329	29.8	9.9	1.06	24.1	1.05	127	17.8
Pooled SEM		8	9	7	1.2	.07	2.9	.22	18	.8

¹ Data represent mean values per chick of triplicate groups of five chicks from 8 to 23 days posthatching; average initial weight on Day 8 was 84 g (birds pretested from day 0 to 8 on 0.56 mg Cu/kg) and 88 g (birds pretested from day 0 to 8 on 8.56 mg Cu/kg).

² Cu was supplied from analytical-grade CuSO₄·5H₂O to the basal diet containing 0.56 mg Cu/kg.

³ At the end of the 8-day pretest, birds fed 0.56 and 8.56 mg Cu/kg had, respectively, haematocrits of 19.4 and 20.3%, haemoglobins of 8.3 and 9.2 g/100 mL, tendon lysine values of 1.06 and 1.14%, gallbladder Cu of 3.8 and 57.5 µg/g (0.14 and 2.32 µg total Cu) (P < 0.01), plasma Cu of 47 and 147 µg/L (P < 0.01) and liver Cu of 14.3 and 19.6 µg/g (P < 0.01).

⁴ Expressed on a dry tissue basis.

⁵ Gall bladder Cu (both concentration and total Cu) at Day 23 posthatching increased linearly (P < 0.01) between 0 and 2 mg/kg supplemental Cu for birds pretested on 0.56 mg Cu/kg; for birds pretested on 8.56 mg Cu/kg, gall bladder Cu at day 23 was greater (P < 0.01) at 1 mg/kg supplemental Cu than at 0 mg/kg supplemental Cu.

⁶ Liver Cu at day 23 posthatching increased linearly (P < 0.05) in birds fed between 0 and 2 mg/kg supplemental Cu during the test period.

⁷ Sample lost and not available for analysis.

Table A6.2(25)-2. Effects of supplemental copper from analytical-grade sources on bile copper concentration, Experiment 2¹

Supplemental Cu ²						
Level	Source	Weight Gain	Feed Intake	Supplemental Cu intake	Bile Cu ³	RBV ⁴
(mg/kg)			(g)	(µg)	(µg/L)	(%)
0		228	348	0	400	
.5	CuSO ₄ ·5H ₂ O	243	355	177	984	
1	CuSO ₄ ·5H ₂ O	245	366	366	3,373	100
.5	Cu ₂ O	236	347	174	1,187	
1	Cu ₂ O	241	349	349	3,053	97.9
1	CuO	245	359	359	677 ⁵	
2	CuO	237	348	695	735 ⁵	
	Pooled SEM	8	10	10	210	

¹ Data represent mean values per chick of triplicate groups of five chicks from 8 to 22 days post hatching; average initial weight 84g.

² The basal diet contained .56mg Cu/kg, and this diet was fed during the 8-day pretest period.

³ Multiple linear regression of bile Cu as function of supplemental Cu intake for CuSO₄·5H₂O (X₁) and Cu₂O (X₂) was $Y = 27 + 8.41 (\pm 1.12) X_1 + 8.23 (\pm 1.30) X_2$, $R^2 = .83$.

⁴ Relative bioavailability (RBV) determined by multiple linear regression and slope-ratio methodology.

⁵ CuO was not different ($P > .05$) from 0 supplemental Cu.

Table A6.2(25)-3. Effects of supplemental copper from feed-grade sources on bile copper concentration, Experiment 3¹

Supplemental Cu ²						
Level	Source	Weight Gain	Feed intake	Supplemental Cu intake	Bile Cu ³	RBV ⁴
(mg/kg)			(g)	(µg)	(µg/L)	(%)
0		158	242	0	387	
.5	AG CuSO ₄ ·5H ₂ O	164	247	123	1,387	
1	AG CuSO ₄ ·5H ₂ O	174	253	253	2,720	100
.5	FG CuSO ₄ ·5H ₂ O	172	252	126	1,173	
1	FG CuSO ₄ ·5H ₂ O	168	244	244	2,560	93.5
1	FG CuO	165	251	251	787 ⁵	
2	FG CuO	156	252	504	760 ⁵	
	Pooled SEM	5	6	5	186	

¹ Data represent mean values per chick of triplicate groups of five chicks from 8 to 22 days post hatching average initial weight was 58g.

² The basal diet contained .56mg Cu/kg, and this diet was fed during the 8 day pretest period.

Abbreviation used: AG = analytical-grade; FG = feed grade.

³ Multiple linear regression of bile Cu as function of supplemental Cu intake for analytical-grade CuSO₄·5H₂O (X₁) and feed-grade CuSO₄·5H₂O (X₂) was $Y = 259 + 9.60 (\pm 1.17) X_1 + 8.98 (\pm 1.20) X_2$, $R^2 = .87$.

⁴ Relative bioavailability (RBV) determined by multiple linear regression and slope-ratio methodology.

⁵ CuO was not different ($P > .05$) from supplemental Cu.

Table A6.2(25)-4. Effects of supplemental copper from analytical-grade copper sulfate, cupric carbonate basic or cuprous chloride on bile copper concentration, Experiment 4¹

Supplemental Cu ²						
Level	Source	Weight gain ³	Feed intake ³	Supplemental Cu intake	Bile Cu ³	RBV ⁴
(mg/kg)			(g)	(µg)	(µg/L)	(%)
0		170	278	0	195	
.5	CuSO ₄ .5H ₂ O	198	290	145	1,251	
1	CuSO ₄ .5H ₂ O	211	308	308	2,769	100
.5	CuCO ₃ .Cu(OH) ₂	212	310	155	1,300	
1	CuCO ₃ .Cu(OH) ₂	223	311	311	2,990	112.9
.5	CuCl	205	302	151	1,807	
1	CuCl	219	317	317	3,835	142.5
	Pooled SEM	8	9	8	380	

¹ Data represent mean values per chick of quadruplicate groups of four chicks from 8 to 22 days post hatchings; average initial weight was 77 g.

² The basal diet contained .56 mg Cu/kg, and this diet was fed during the 8-day pretest period.

³ Linear ($P < .01$) response to supplemental Cu from all three sources. Multiple linear regression of bile Cu as function of supplemental Cu intake for analytical-grade CuSO₄.5H₂O (X_1), analytical-grade CuCO₃.Cu(OH)₂ (X_2) and analytical-grade CuCl (X_3) was $Y = 61 + 8.55 (\pm 1.58) X_1 + 9.65 (\pm 1.56) X_2 + 12.17 (\pm 1.53) X_3$, $R^2 = .74$.

⁴ Relative bioavailability (RBV) determined by multiple linear regression and slope- ratio methodology. CuCl was greater ($P < .05$) than CuSO₄.5H₂O.

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Official
use only

132 REFERENCE

132.1 Reference

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

Baker, D.H., Odle, J., Funk, M.A. and Wieland, T.M. (1991). Research Note: Bioavailability of Copper in Cupric Oxide, Cuprous Oxide, and in a Copper-Lysine Complex. *Poultry Science*. **70**: 177-179 (published).

132.2 Data protection

No
(indicate if data protection is claimed)

132.2.1 Data owner

Give name of company

Public domain

132.2.2 Criteria for data protection

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

133 GUIDELINES AND QUALITY ASSURANCE

133.1 Guideline study

No. This was a non-regulatory study carried out in chicks to assess relative Cu bioavailability in analytical-grade CuO and Cu₂O and in feed-grade Cu-lysine relative to a CuSO₄.5H₂O standard. Accumulation of Cu in the liver was used as the primary criterion of Cu bioavailability. No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

133.2 GLP

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

133.3 Deviations

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

134 MATERIALS AND METHODS

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

134.1 Test material

Cu²⁺ as Copper sulphate (CuSO₄.5H₂O), reagent grade.

Cu²⁺ as Cupric oxide (CuO), reagent grade.

Cu²⁺ as Cuprous oxide (Cu₂O), reagent grade.

Cu²⁺ as Cu-lysine complex.

134.1.1 Lot/Batch number

Not available

134.1.2 Specification

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

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Specify section no., heading and species as appropriate

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134.1.2.1	Description	<i>If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)</i> Aqueous solution
134.1.2.2	Purity	<i>Give purity in % of active substance</i> [REDACTED]
134.1.2.3	Stability	<i>Describe stability of test material</i> Not available
134.1.2.4	Radiolabelling	<i>give structural location of radio labelling, give reason if not labelled</i> Not deemed necessary for the purposes of this study.
134.2 Test Animals		Non-entry field
134.2.1	Species	Chicks
134.2.2	Strain	Cross of New Hampshire males and Columbian females.
134.2.3	Source	Not stated.
134.2.4	Sex	Male
134.2.5	Age/weight/height at study initiation	<i>Young adults recommended</i> Age: The study covered the period 8 – 22 days posthatching. Weight: Average initial weight was 75 g.
134.2.6	Number of animals per group	<i>Give number</i> <i>Specify, if there are differences for example for treatment and recovery groups</i> Four replicate groups of 5 chicks were fed each of the 9 experimental diets.
134.2.7	Controls	Yes
134.3 Administration/ Exposure		<i>(fill in respective route in the following, delete other routes)</i> Oral administration of the test substance in the diet.
134.3.1	Duration of treatment	The total duration of treatment was 14 days.
134.3.2	Exposure scenario	Chicks were fed a 23% protein corn and soybean starter diet during the first 7 days post-hatching. On day 8, 180 chicks were weighed and allotted to experimental groups such that each group had a similar mean initial weight and weight distribution. Four replicate groups of 5 chicks were fed each of the 9 experimental diets from 8 to 22 days posthatching. Diets and water were provided <i>ad libitum</i> . The basal corn-soybean starter diet was fortified with 275 mg Cu/kg from reagent grade CuSO ₄ ·5H ₂ O. Together with the Cu from ingredients present in the corn and soybean diet (15 mg Cu/kg), this resulted in a Cu concentration in the basal diet of 290 mg/kg. Given that Cu accumulation in the liver is considered to be linear between 250 and 500 mg/kg, supplemental Cu levels subsequently selected for use were in this range. To the common basal diet, Cu was supplemented to provide either 75 or 150 mg Cu from CuSO ₄ ·5H ₂ O (25.1% Cu), Cu-lysine (7.5% Cu), Cu ₂ O (88.8% Cu) and CuO (79.9% Cu). Non-entry field.
134.4 Procedures		

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/26

A6.2(26), Bioavailability of copper

134.4.1 Sample processing and analysis

At the termination of the trial, all chicks were killed by cervical dislocation and their livers quantitatively removed. Livers were pooled by replicate, dried for 24 hours at 100°C, weighed, and then wet-ashed with HNO₃. Samples were assayed for Cu by atomic absorption spectrophotometry (AAS). Results were expressed both as µg Cu/g dry liver, and as total µg Cu per liver.

134.4.2 Statistical analysis

Statistical analysis was performed by ANOVA procedures appropriate for a completely randomised design. Orthogonal polynomials were used to separate treatments (8 df) into the following components: basal versus Cu supplementation (1 df), Cu source (3 df), Cu level (1 df), and Cu source by Cu level (3 df). Linear regression equations were also computed for CuSO₄·5H₂O, Cu-lysine, Cu₂O and CuO, total liver Cu (µg) being regressed on supplemental Cu intake (mg). The ratio of regression coefficients was calculated with CuSO₄·5H₂O taken as the standard and set at 100%, thus allowing estimation of quantitative efficacy of Cu-lysine, Cu₂O and CuO relative to CuSO₄·5H₂O.

135 RESULTS AND DISCUSSION

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

135.1 Results

None of the experimental Cu treatments affected weight gain or voluntary feed intake ($P > 0.05$), thus confirming that the range of Cu levels employed (290 to 440 mg Cu/kg) did not result in anorexia. All sources of supplemental Cu except CuO markedly increased Cu content of the liver (**Table A6.2(26)-1**).

135.2 Discussion

Liver Cu comparisons (main effects) between Cu₂O and CuSO₄·5H₂O and between Cu-lysine and CuSO₄·5H₂O indicated no significant differences ($P > 0.05$). As indicated by linear regression slope-ratio calculations, and relative to efficacy of the CuSO₄·5H₂O standard (100% relative efficacy assumed), Cu-lysine had an efficacy value of 115.5% ($P > 0.05$), Cu₂O had a value of 92.5% ($P > 0.05$), and CuO had a value of -1.7% ($P < 0.01$). The data in **Table A6.2(26)-1** together with the regression suggest that the bioavailability of Cu in CuO is not significantly different from zero.

135.3 Toxic effects, clinical signs

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

135.4 Recovery of labelled compound

state percentage
Not stated.

136 APPLICANT'S SUMMARY AND CONCLUSION

136.1 Materials and methods

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

A study was carried out in chicks to assess Cu bioavailability in CuO, Cu₂O and Cu-lysine relative to a CuSO₄·5H₂O standard. Accumulation of Cu in the liver was used as the primary criterion of Cu bioavailability. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP. Male chicks (a cross of New Hampshire males and Columbian females) were fed a starter diet containing 15 mg Cu/kg for the first 7 days post-hatching. On day 8, 180 chicks were weighed and allotted to

Section A6.2

Annex Point IIA6.2

IUCLID: 5.0/26

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(26), Bioavailability of copper

experimental groups such that each group had a similar mean initial weight and weight distribution. Four replicate groups of 5 chicks were fed each of 9 experimental diets from 8 to 22 days post-hatching. Diets and water were provided *ad libitum*.

The basal corn-soybean starter diet was fortified with 275 mg Cu/kg from reagent grade $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Together with Cu from ingredients present in the starter diet, this resulted in a Cu concentration in the basal diet of 290 mg/kg. The 8 diets containing supplementary copper were as follows: To the common basal diet, Cu was added to provide either 75 or 150 mg Cu from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Cu-lysine, Cu_2O and CuO.

At the termination of the trial, all chicks were killed by cervical dislocation and their livers quantitatively removed. Livers were pooled by replicate, dried for 24 hours at 100°C , weighed, and wet-ashed with HNO_3 . Samples were assayed for Cu by AAS. Results were expressed both as $\mu\text{g Cu/g dry liver}$, and as total $\mu\text{g Cu per liver}$.

Statistical analysis was performed by ANOVA procedures. Orthogonal polynomials were used to separate treatments into the following components: basal versus Cu supplementation, Cu source, Cu level, and Cu source by Cu level. Linear regression equations were also determined for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Cu-lysine, Cu_2O and CuO, with total liver Cu (μg) regressed on supplemental Cu intake (mg). The ratio of regression coefficients was calculated with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ taken as the standard and set at 100%, allowing estimation of quantitative efficacy of Cu-lysine, Cu_2O and CuO relative to $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

136.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

None of the experimental Cu treatments (290 to 440 mg Cu/kg) affected weight gain or voluntary feed intake. All sources of supplemental Cu except CuO markedly increased Cu content of the liver.

Liver Cu comparisons between Cu_2O and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and between Cu-lysine and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ indicated no significant differences at the dose levels tested. Relative to the efficacy of the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ standard, Cu-lysine had an efficacy value of 115.5%, Cu_2O had a value of 92.5%, and CuO had a value of -1.7%. The data suggest that the bioavailability of Cu in CuO is not significantly different from zero.

136.3 Conclusion

No significant differences were determined in the bioavailability of Cu derived from Cu_2O and that derived from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Similarly, there was no difference in the bioavailability of Copper from Cu-lysine relative to that from $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The bioavailability of Cu in CuO was not significantly different from zero.

136.3.1 Reliability

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

2

136.3.2 Deficiencies

Yes

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

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/26

A6.2(26), Bioavailability of copper

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

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

Materials and Methods

Results and discussion

Conclusion

Reliability

Acceptability

Remarks

COMMENTS FROM ...

Date

Give date of comments submitted

Copper Oxide

Table A6.2(26)-1. Effects of copper supplementation from various sources on copper desposition in the liver of chicks ¹

Supplemental Cu level ² (mg/kg)	Cu source	Weight Gain - (g) -	Feed Intake	Supplemental Cu intake ³ (mg)	Liver weight ⁴ (g)	Liver Cu ^{3,5,6} (µg/g)	Liver Cu ^{3,5,7} (µg)
0		29	47	0	2.17	39.5	85.8
75	CuSO ₄ ·5H ₂ O	27	40	30.0	2.11	96.5	203.6
150	CuSO ₄ ·5H ₂ O	27	38	58.9	2.10	186.4	392.3
75	Cu-lysine	22	38	29.8	2.25	99.7	224.4
150	Cu-lysine	22	31	58.7	2.19	199.4	435.9
75	Cu ₂ O	21	38	29.8	2.20	69.4	152.7
150	Cu ₂ O	21	40	61.4	2.20	173.5	381.8
75	CuO	28	32	29.4	2.15	39.8	85.5
150	CuO	28	37	59.6	2.09	38.4	80.4
Pooled SEM		4	5	0.6	0.06	19.6	40.0

¹ Data represented quadruplicate groups of five chicks from 8 to 22 days post-hatching, average initial weight was 75g.

² The basal corn and soybean meal diet contained 290 mg Cu/kg (15 mg/kg from corn and soybean diet plus 275 mg/kg from CuSO₄·5H₂O).

³ Cu level effect (P<.01).

⁴ Dry weight of intact liver.

⁵ Cu source effect (P<.01).

⁶ Cu source by Cu level (P<.05).

⁷ Regression of total liver Cu micrograms as function of supplemental Cu intake milligrams from CuSO₄·5H₂O (X₁), Cu lysine (X₂), Cu₂O (X₃) and CuO (X₄) were $Y = 72.7 + 5.22 (\pm .96SE) X_1$, $r^2 = .75$; $Y = 71.7 + 6.03 (\pm .86) X_2$, $r^2 = .83$; $Y = 59.8 + 4.83 (\pm 1.02) X_3$, $r^2 = .69$; $Y = 86.0 - 0.9 (\pm .30) X_4$, $r^2 = .01$.

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/27

A6.2(27), Bioavailability of copper

Official
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137 REFERENCE

137.1 Reference

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

Buescher, R.G., Griffin, S.A. and Bell, M.C. (1961). Copper Availability to Swine from Cu⁶⁴ Labelled Inorganic Compounds. *Journal of Animal Science*, **20**: 529-531 (published).

137.2 Data protection

No
(indicate if data protection is claimed)

137.2.1 Data owner

Give name of company
 Public domain

137.2.2 Criteria for data protection

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

138 GUIDELINES AND QUALITY ASSURANCE

138.1 Guideline study

No. This was a non-regulatory study carried out to determine the relative availability in swine of copper from different sources, using Cu⁶⁴ labelled cupric carbonate, cupric oxide and cupric sulphate. No guidelines are available to address this objective.
(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")

138.2 GLP

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

138.3 Deviations

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

139 MATERIALS AND METHODS

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

139.1 Test material

Cu²⁺ as Copper sulphate (cupric).
 Cu²⁺ as Copper oxide (cupric). Cu²⁺
 as Copper carbonate (cupric).

139.1.1 Lot/Batch number

Not available

139.1.2 Specification

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

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/27

A6.2(27), Bioavailability of copper

139.1.2.1 n	Description	If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution) See section 3.1
139.1.2.2	Purity	Give purity in % of active substance [REDACTED]
139.1.2.3	Stability	Describe stability of test material Not available
139.1.2.4 lling	Radiolabeling	give structural location of radio labelling, give reason if not labelled All copper salts utilised in this study incorporated Cu ⁶⁴ Non-entry field

139.2 Test Animals

139.2.1 Species	Pigs
139.2.2 Strain	Crossbred.
139.2.3 Source	Not stated.
139.2.4 Sex	Male (neutered)
139.2.5 Age/weight at study initiation	Young adults recommended Weight: Average weight at the beginning of the study was 56 lb.
139.2.6 Number of animals per group	Nine animals in three groups.
139.2.7 Controls	Yes

X

139.3 Administration/
Exposure(fill in respective route in the following, delete other routes)
Oral administration by gelatin capsules.

139.3.1 Duration of treatment	Post-dose sampling was carried out for a period of 96 hours.
----------------------------------	--

139.4 Procedure

Fifteen animals averaging 35 lb in weight were placed in individual pens and given feed and water *ad libitum*. After 17 days, 9 of these animals averaging 56 lb in weight were allotted to 3 groups according to litter and weight and placed in metabolism units for a 4-day adjustment and preliminary period. Each pig was dosed with 2 millicuries (mc) of Cu⁶⁴ by stomach tube using gelatin capsules containing either copper sulphate, copper carbonate or copper oxide, with each dose containing an elemental equivalent of 10 mg. At the termination of the metabolism study, the pigs were placed in individual pens for a 6-day rest and then returned to the metabolism units for a second trial. The same procedure was followed for the second trial, except that a different source of copper was given to the respective pigs.

The basal diet given to the animals contained 15 ppm Cu. Average daily gain, feed consumption and feed per pound of gain were measured during all phases of the experiment.

Blood samples were taken at 1, 2, 4, 8, 16, 24, 36, 48, 72 and 96 hrs post-dosing. The first urine and faeces samples were collected at 4 hrs; subsequent sampling followed the same schedule as blood sampling. 3 ml each of blood, plasma and urine and 3 g of faeces were counted directly in a well-type scintillation counter for Cu⁶⁴ activity.

Section A6.2

Annex Point IIA6.2

IUCLID: 5.0/27

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(27), Bioavailability of copper

140 RESULTS AND DISCUSSION

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

140.1 Results

Cu⁶⁴ concentration in blood and plasma, expressed as % of total dose, is shown in **Figure A6.2(27)-1**. The concentration of Cu⁶⁴ in both blood and plasma was similar for copper carbonate, oxide and sulphate, with the major portion of the Cu⁶⁴ being in the plasma. Eight hours post-dosing, the Cu⁶⁴ concentration in blood plasma began to plateau and remained relatively constant throughout the 96-hr study. As shown in **Figure A6.2(27)-1**, the average value of copper carbonate is somewhat higher than the sulphate or oxide through the first 16 hrs. However, due to large variations in Cu⁶⁴ activity during the first 5 sampling periods, these values were not significantly different. The average blood values at 16 hours post-dosing were 1.0, 0.9 and 0.8% and the standard deviations were 0.2, 0.1 and 0.2% of dose/litre for copper carbonate, sulphate and oxide, respectively.

Cumulative faecal and urinary excretions of Cu⁶⁴ are shown in **Figure A6.2(27)-1** and total excretions of Cu⁶⁴ are shown in **Table A6.2(27)-1**. There were no significant differences in Cu⁶⁴ faecal output, with copper carbonate being 62%, oxide 63% and sulphate 64% of the total administered dose. Approximately 30 – 35% of the dose was excreted via the faeces in the first 24 hrs. Cu⁶⁴ from copper carbonate excreted in the urine was higher (P<0.01) than from the sulphate or oxide. The total urinary Cu⁶⁴ from carbonate was 3.4% of the dose compared to 1.4% for sulphate and 1.0% for oxide. The increase in copper carbonate urinary excretion may be due to high absorption into the blood. Even though the blood values were not significantly different, it was considered that the increase in carbonate urinary output may be due to an increase over a threshold level of the kidney, and therefore the pigs excreted more Cu⁶⁴ from the carbonate compound than did the pigs which received copper sulphate or oxide.

The dietary level of copper used in this study was sufficient to promote good growth. The average daily gain for the entire experiment was 1.41 lb with a feed efficiency of 2.5 lb of feed per lb gain. While in the metabolism units the pigs gained well, averaging 1.14 lb per day with a feed efficiency of 3.0 lb of feed per lb gain.

140.2 Discussion

There were no significant differences found in blood or plasma concentration or total faecal output of Cu⁶⁴. There was a greater (P<0.01) amount of copper from copper carbonate excreted via the urine than from the oxide or sulphate. The availabilities of the three forms of inorganic copper compounds for swine were similar.

140.3 Toxic effects, clinical signs

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

5.5 Recovery of labelled compound

state percentage

Not applicable.

141 APPLICANT'S SUMMARY AND CONCLUSION

141.1 Materials and methods

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

Section A6.2

Annex Point IIA6.2

IUCLID: 5.0/27

Metabolism in mammals

Specify section no., heading and species as appropriate

A6.2(27), Bioavailability of copper

A study was carried out to determine the relative bioavailability in swine of copper from different sources, using Cu^{64} labelled cupric carbonate, cupric oxide and cupric sulphate. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Fifteen neutered male crossbred pigs, with an average weight of 35 lb, were placed in individual pens and given feed and water *ad libitum*. After 17 days, 9 of these animals, averaging 56 lb in weight, were allotted to 3 groups and placed in metabolism units for a 4-day adjustment period. Each pig was dosed with 2 mC of Cu^{64} by stomach tube using gelatin capsules containing either copper sulphate, copper carbonate or copper oxide, with each dose containing an elemental equivalent of 10 mg. At the termination of the metabolism study, the pigs were placed in individual pens for a 6-day rest and then returned to the metabolism units for a second trial. The same procedure was followed for the second trial, except that a different source of copper was given to the respective pigs.

The basal diet given to the animals contained 15 ppm Cu. Average daily gain, feed consumption and feed per pound of gain were measured during all phases of the experiment.

Blood samples were taken at 1, 2, 4, 8, 16, 24, 36, 48, 72 and 96 hrs post-dosing. The first urine and faeces samples were collected at 4 hrs; subsequent sampling followed the same schedule as blood sampling. 3 ml each of blood, plasma and urine and 3 g of faeces were counted directly in a well-type scintillation counter for Cu^{64} activity.

141.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

The concentration of Cu^{64} in both blood and plasma was similar for copper carbonate, oxide and sulphate, with the major portion of the Cu^{64} being in the plasma. Eight hours post-dosing, the Cu^{64} concentration in blood plasma began to plateau and remained relatively constant throughout the 96-hr study. The average value of copper carbonate was somewhat higher than the sulphate or oxide through the first 16 hrs. However, due to large variations in Cu^{64} activity during the first 5 sampling periods, these values were not significantly different. The average blood values at 16 hrs post-dosing were 1.0, 0.9 and 0.8% of dose/litre for copper carbonate, sulphate and oxide, respectively, with standard deviations of 0.2, 0.1 and 0.2%.

There were no significant differences in cumulative Cu^{64} faecal output, with copper carbonate being 62%, oxide 63% and sulphate 64% of the total administered dose. Approximately 30–35% of the dose was excreted via the faeces in the first 24 hrs. Cu^{64} from copper carbonate excreted in the urine was higher than from the sulphate or oxide. Total urinary Cu^{64} from carbonate was 3.4% of the dose compared to 1.4% for sulphate and 1.0% for oxide. Although the difference in these values was not significant, the slight increase in copper carbonate urinary excretion may be due to high initial absorption into the blood, resulting in an increase over a threshold level in the kidney.

141.3 Conclusion

There were no significant differences found in blood or plasma concentration or total faecal output of Cu^{64} . There was a greater amount of copper from copper carbonate excreted via the urine than from the oxide or sulphate. The availabilities of the three forms of inorganic copper compounds for swine were considered to be similar.

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/27

A6.2(27), Bioavailability of copper

141.4 Reliability

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

2

141.5 Deficiencies

Yes

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

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

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

[REDACTED]

Materials and Methods

• [REDACTED]

Results and discussion

• [REDACTED]

Conclusion

[REDACTED]

Reliability

[REDACTED]

Acceptability

[REDACTED]

Remarks

[REDACTED]

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/27

A6.2(27), Bioavailability of copper

Figure A6.2(27)-1. Cu^{64} in blood, plasma, urine and faeces of swine from orally administered copper sulphate, oxide and carbonate.

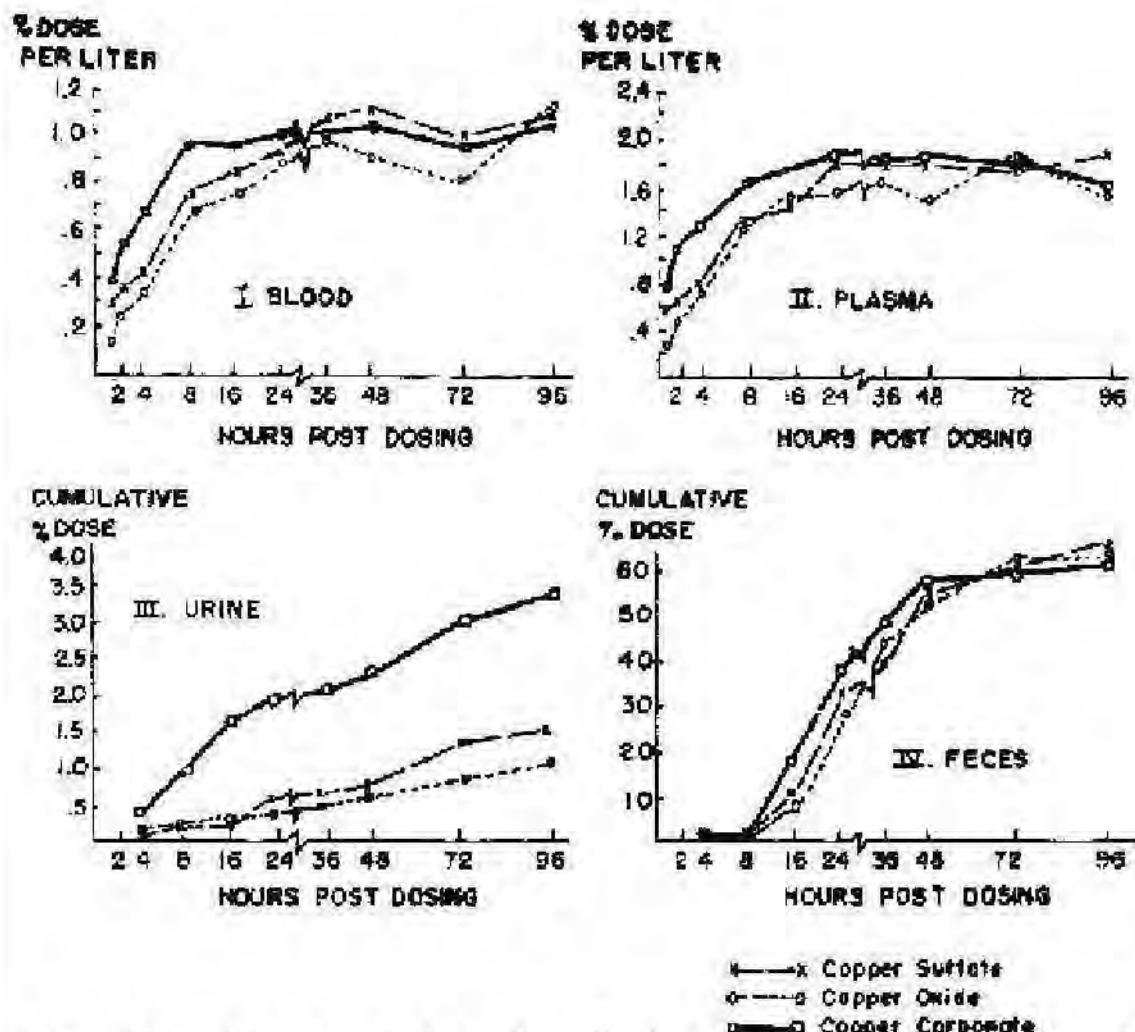


Figure 1. Cu^{64} in blood, plasma, urine and faeces of swine from orally administered sulfate, oxide and carbonate.

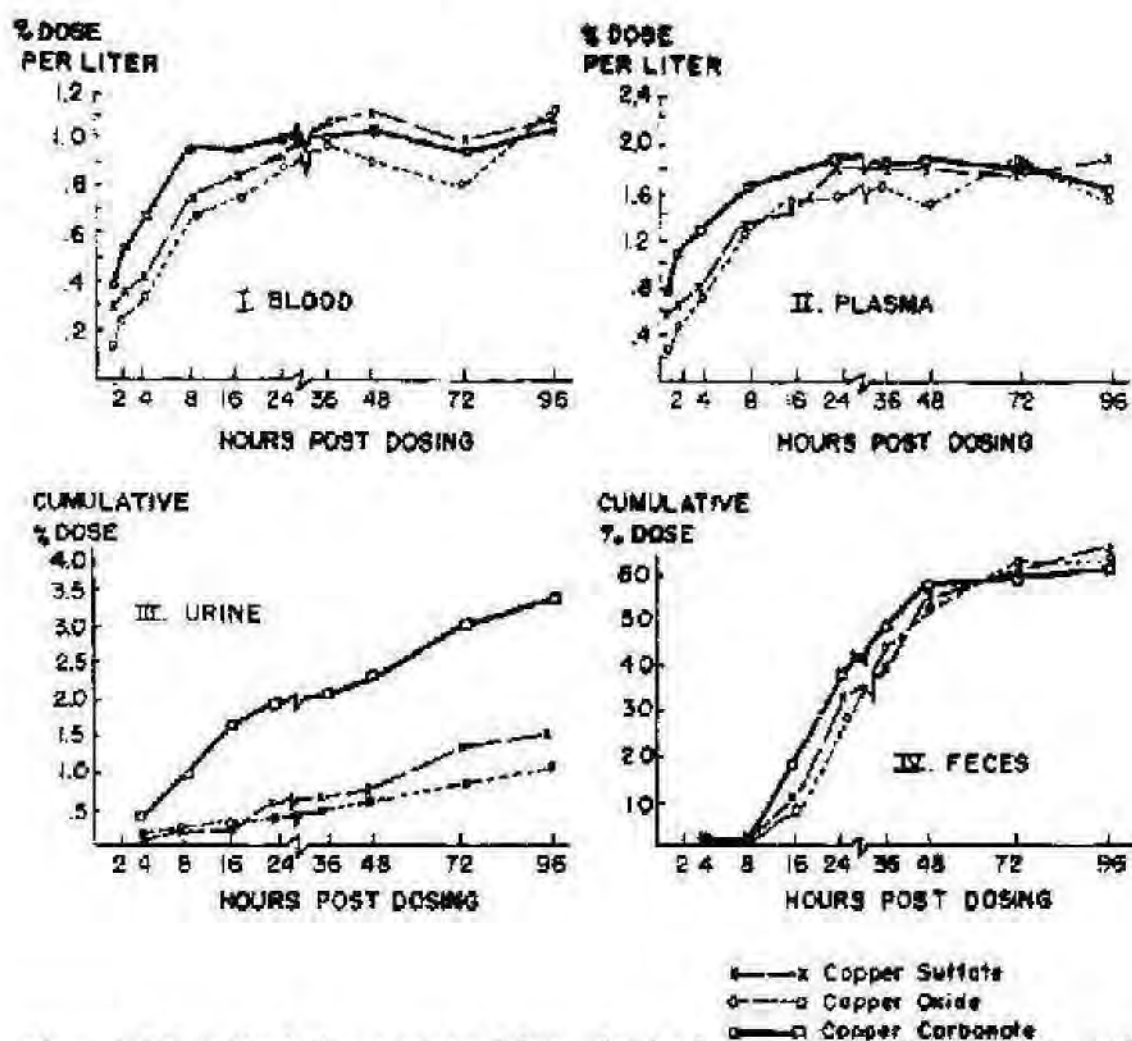
COMMENTS FROM ...

Date

Give date of comments submitted

Table A6.2(27)-1. Excretion and Retention of Cu^{64} By Swine Dosed Orally with Labelled Compounds

Treatment	Cu Carbonate	Cu Oxide	Cu Sulfate
No. of pigs	6	6	5
Feces (% of dose)	61.5	62.8	64.2
Urine (% of dose)	3.4*	1.0	1.4
Retained (% of dose)	35.1	36.2	34.4

* $P < 0.01$ Figure A6.2(27)-1. Cu^{2+} in blood, plasma, urine and faeces of swine from orally administered copper sulphate, oxide and carbonate.Figure 1. Cu^{2+} in blood, plasma, urine and feces of swine from orally administered sulfate, oxide and carbonate.

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/28

A6.2(28), Bioavailability of copper

Official
use only

142 REFERENCE

142.1 Reference

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

Bunch, R.J., Speer, V.C., Hays, V.W., Hawbaker, J.H. and Catron, D.V. (1961). Effects of copper Sulfate, Copper oxide and Chlortetracycline on Baby Pig Performance. J. Animal Sci. **20**: 723-726 (published).

142.2 Data protection

No
(indicate if data protection is claimed)

142.2.1 Data owner

Give name of company

Public domain

142.2.2 Criteria for data protection

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

143 GUIDELINES AND QUALITY ASSURANCE

143.1 Guideline study

No. This was a non-regulatory study carried out to investigate the effects of copper sulphate, copper oxide and chlortetracycline on growth performance in pigs. No guidelines are available to address this objective. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*

143.2 GLP

No. This was a non-regulatory study.

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

143.3 Deviations

No. Not applicable to non-guideline studies.

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

144 MATERIALS AND METHODS

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

144.1 Test material

Cu²⁺ as Copper sulphate.

Cu²⁺ as Copper oxide.

Chlortetracycline.

144.1.1 Lot/Batch number

Not available

144.1.2 Specification

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

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/28

A6.2(28), Bioavailability of copper

144.1.2.1 n	Description	If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution) See section 3.1
144.1.2.2	Purity	Give purity in % of active substance [REDACTED]
144.1.2.3	Stability	Describe stability of test material Not available
144.1.2.4 lling	Radiolabel	Give structural location of radio labelling, give reason if not labelled Not deemed necessary for the purposes of this study.
144.2 Test Animals		Non-entry field
144.2.1 Species		Pigs
144.2.2 Strain		Crossbred.
144.2.3 Source		Not stated.
144.2.4 Sex		50% of animals were male and 50% were female.
144.2.5 Age/weight at study initiation		Young adults recommended Age: Average ages at the start of Experiments 1, 2 and 3 were 16, 13 and 22 days, respectively. Weight: Average weights at the start of Experiments 1, 2 and 3 were 10, 9.5 and 14 lb, respectively.
144.2.6 Number of animals per group		The numbers of animals used for Experiments 1, 2 and 3 were 128, 80 and 144, respectively.
144.2.7 Controls		Yes
144.3 Administration/ Exposure		(fill in respective route in the following, delete other routes) Oral administration of the test substances in the diet.
144.3.1 Duration of treatment		The length of the experimental periods ranged from 4 weeks (Experiment 1) to 6 weeks (Experiments 2 and 3).
144.4 Procedures		Non-entry field
144.4.1 Experiment 1		Dietary treatments administered were 4 levels of copper sulphate (0, 125, 250 or 375 ppm Cu) in the presence of 2 levels of protein (16% and 20%). Four replicates of 4 pigs per pen were used in a split plot design so that litter-mate control could be maintained across copper levels (sub-plot treatments) with protein levels being the main plot treatments.
144.4.2 Experiment 2		Animals were allotted to a randomised block design involving 4 replicates of 5 ration treatments. The pigs were randomly allotted, 4 pigs per pen, to treatments from litter-mate outcome groups. Copper was fed at the levels of 0, 125, 250 and 375 ppm as copper oxide and at 250 ppm as copper sulphate for a 6-week experimental period. Faecal flora counts were made at the end of the experimental period. The liver and loin samples were assayed for copper. The tissue samples were taken from a ground mixture of the whole liver and a slice 1 inch thick from the <i>longissimus dorsi</i> region of the tenth rib. Duplicate analyses were made on each sample.
144.4.3 Experiment 3		Animals were allotted to a randomised block design involving 6

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replications of 4 ration treatments. The pigs were randomly allotted, 4 pigs per pen, to treatments from litter-mate outcome groups. The 4 treatments in this 6-week experiment were a control ration, 250 ppm of Cu as copper oxide, 250 ppm of copper as copper sulphate, and 50 mg chlortetracycline per lb of feed. Faecal flora counts were made at the end of the experimental period.

144.4.4 Statistics

The data were analysed according to analysis of variance methods. Any statements of statistical significance refer to a probability level of 5% unless otherwise noted. In all cases the pen of pigs was considered as the experimental unit in the analysis and interpretation of data.

145 RESULTS AND DISCUSSION

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

145.1 Results

Non-entry field.

145.1.1 Experiment 1

A summary of the data is presented in **Table A6.2(28)-1**. The higher (22%) level of protein improved total gain and efficiency to feed conversion. The response to copper levels was similar on both the 16 and 22% protein groups, with maximum gains and most efficient feed conversion being observed when 250 ppm of copper was included in the diet. The quadratic regressions of gains and feed per pound of gain on copper levels were statistically significant. The haemoglobin levels of the pigs were depressed by increased copper levels; however, the depression was not as great on the level of 125 or 250 ppm as on the level of 375 ppm. The quadratic response of haemoglobin level on copper levels was statistically significant. The interaction between copper levels and protein on gains, feed conversion or haemoglobin responses was not statistically significant.

145.1.2 Experiment 2

A summary of the data is presented in **Table A6.2(28)-2**. There was essentially no difference in total gain or efficiency of feed conversion by the pigs on the different copper oxide levels or between copper oxide and copper sulphate treated groups. However, there was significant improvement observed in both total gain and efficiency of feed conversion by the pigs on all copper levels as compared with the pigs on the control ration. The haemoglobin concentration of the pigs fed copper sulphate was significantly lower as compared with all other treatments. However, copper oxide had little effect on the haemoglobin concentration. A lower haemoglobin level was observed for pigs fed the highest level of copper oxide (375 ppm of Cu), but this difference was not statistically significant. The amount of Cu in loin tissue did not appear to be affected by the feeding of copper oxide. However, the Cu content of the loin tissues of copper sulphate-fed pigs was increased 3-fold over the control animals. The feeding of copper sulphate increased the Cu content of the liver 10-fold over the control. The highest level of copper oxide fed doubled the liver copper content as compared with the controls.

The faecal floral counts made at the end of the experimental period are presented as the log of the total count per gram of faeces **Table A6.2(28)- 2**.

145.1.3 Experiment 3

A summary of the data is presented in **Table A6.2(28)-3**. There was essentially no difference in total gains among the three treatments. However, all three treatments significantly ($P < 0.01$) increased the total gain per pig as compared with the pigs fed the control ration. None of the

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differences in feed conversion between the control and three treatments was statistically significant.

The haemoglobin concentration of the copper sulphate-fed pigs was significantly lower as compared with all other treatments. There were no significant differences in the haemoglobin concentration between the pigs fed the control ration and those on copper oxide and chlortetracycline-supplemented rations.

145.2 Discussion

Four levels of copper sulphate (0, 125, 250 and 375 ppm Cu) were fed with each of two protein levels (16 and 22%). The gain and efficiency of feed conversion were improved by both the higher (22%) protein level and copper sulphate. Maximum gain and feed efficiency resulted from 250 ppm of copper. Haemoglobin concentrations were depressed with increased copper levels. There was no significant interaction between protein level and added copper sulphate.

In the second experiment, four levels of Cu as copper oxide (0, 125, 250 and 375 ppm) and one level of Cu as copper sulphate (250 ppm) were compared. All levels of Cu significantly improved feed conversion and rate of gain. The improvement in gain seen with copper oxide was considered to indicate that the growth response seen with copper sulphate was attributable to the Cu radical, and not the sulphate radical. Copper sulphate significantly depressed blood haemoglobin. This was attributed to an inverse relationship between iron and copper concentrations in the liver. Changes were observed in microflora patterns on the different treatments as measured by plate count techniques. The feeding of copper oxide resulted in small increases in Cu content of the tissues, whereas the feeding of copper sulphate resulted in marked increases in tissue copper content.

Copper oxide (250 ppm Cu), copper sulphate (250 ppm Cu) and chlortetracycline (50 mg/lb) significantly improved total gain as compared to the control ration. Copper sulphate significantly depressed the haemoglobin concentration. The faecal flora patterns were also altered by these ration supplements.

145.3 Toxic effects, clinical signs

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

X

5.6 Recovery of labelled compound

state percentage

Not applicable.

146 APPLICANT'S SUMMARY AND CONCLUSION

146.1 Materials and methods

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

A study was carried out to investigate the effects of copper sulphate, copper oxide and chlortetracycline on growth performance in pigs. The study was not designed to follow an internationally accepted guideline, and was not carried out or reported in compliance with GLP.

Experiment 1: Four dietary treatments of copper sulphate were administered (0, 125, 250 or 375 ppm Cu) in the presence of 2 levels of protein (16% and 20%). Four replicates of 4 pigs/pen were used in a split plot design so that litter-mate control could be maintained across copper levels, with protein levels being the main plot treatments.

Experiment 2: Animals were allotted to a randomised block design

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involving 4 replicates of 5 ration treatments. The pigs were randomly allotted, 4 pigs/pen, to treatments from litter-mate outcome groups. Copper was fed at levels of 0, 125, 250 and 375 ppm as copper oxide and at 250 ppm as copper sulphate for a 6-week period. Faecal flora counts were made at the end of the experimental period. Liver and loin samples were assayed for copper. Tissue samples were taken from a ground mixture of whole liver and a slice from the *longissimus dorsi* region of the tenth rib. Duplicate analyses were made on each sample.

Experiment 3: Animals were allotted to a randomised block design involving 6 replications of 4 ration treatments. Pigs were randomly allotted, 4 pigs/pen, to treatments from litter-mate outcome groups. The 4 treatments in this 6-week experiment were a control ration, 250 ppm Cu as copper oxide, 250 ppm Cu as copper sulphate, and 50 mg chlortetracycline /lb feed. Faecal flora were counted at the end of the experimental period.

Statistics: Data were analysed by analysis of variance. Statements of statistical significance refer to a probability level of 5% unless otherwise noted. The pen of pigs was considered to be the experimental unit.

146.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Experiment 1: The higher (22%) level of protein improved total weight gain and efficiency of feed conversion. The response to copper levels was similar on both the 16 and 22% protein groups, with maximum gains and most efficient feed conversion being observed when 250 ppm of copper was included in the diet. Haemoglobin levels were depressed by increased copper; however, the depression was not as great at 125 or 250 ppm as at 375 ppm. The interaction between copper levels and protein on gains, feed conversion or haemoglobin responses was not statistically significant.

Experiment 2: There was no difference in total weight gain or feed conversion efficiency resulting from the different copper oxide levels. Similarly, there was no difference between results obtained using copper oxide and copper sulphate. However, there was significant improvement observed in both total gain and efficiency of feed conversion by the pigs on all copper levels as compared with the pigs on the control ration. The improvement in gain seen with copper oxide was considered to indicate that the growth response seen with copper sulphate was attributable to Cu radical, and not the sulphate radical.

Haemoglobin concentrations in animals fed copper sulphate were significantly lower than those resulting from other treatments. This was attributed to an inverse relationship between liver copper and iron. Copper oxide had little effect on haemoglobin concentration (a slightly lower level in pigs fed 375 ppm Cu from copper oxide was not statistically significant). The amount of Cu in loin tissue did not appear to be affected by feeding of copper oxide. However, the Cu content of the loin tissues of copper sulphate-fed pigs (16 ppm) was increased 3-fold over control animals (5 ppm). The feeding of copper sulphate increased the Cu content of the liver (218 ppm) 10-fold over the control (22 ppm). The highest level of copper oxide doubled liver Cu content to 44 ppm.

Changes were observed in microflora patterns on the different treatments.

Experiment 3: There was essentially no difference in total gains among the three treatments. However, all treatments significantly increased total gain per pig compared with the control. Differences in feed conversion between control and treatment pigs were not statistically significant.

Haemoglobin concentration of copper sulphate-fed pigs were significantly

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146.3 Conclusion

lower than all other treatments. There were no significant differences in haemoglobin concentration between pigs fed the control ration and those on copper oxide and chlortetracycline-supplemented rations.

Faecal flora patterns were altered by these ration supplements.

The addition of both copper oxide and copper sulphate to the diet of pigs resulted in bodyweight gains and increased feed conversion efficiency, compared with control animals. However, concentrations of copper in liver and loin resulting from the administration of copper from copper sulphate were markedly higher than those resulting from copper oxide. This suggests that copper derived from the sulphate has a higher degree of bioavailability than that of the oxide. Addition of copper sulphate (250 ppm Cu) to feed also resulted in depressed haemoglobin concentrations, indicating a competitive inhibition of iron uptake by bioavailable Cu^{2+} . This effect was not statistically significant in animals fed copper oxide.

146.4 Reliability

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

2

146.5 Deficiencies

Yes

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

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

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

Evaluation by Competent Authorities

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

EVALUATION BY RAPPORTEUR MEMBER STATE

Date

Materials and Methods

Results and discussion

Conclusion

Reliability

Acceptability

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Remarks

Table A6.2(28)-1. Effect of Protein and CuSO₄·5H₂O Levels on Performance of baby Pigs (Experiment 1)^a

Protein level	16				22			
% CuSO ₄ ·5H ₂ O level, (ppm Cu)	0	125	250	375	0	125	250	375
Total gain per pig, lb. ^b	11.8	14.6	15.3	13.2	12.1	15.3	16.0	14.8
Feed/gain, lb. ^b	2.18	2.03	1.96	2.17	2.03	1.79	1.82	1.82
Haemoglobin, gm. % ^b	10.1	10.2	10.0	8.3	11.2	10.5	10.2	8.5

^a 4 pens of 4 pigs each per ration treatment.

^b Significant quadratic regression of gains/gain and haemoglobin level on copper levels.

COMMENTS FROM ...

Date

Give date of comments submitted

Copper Oxide

Table A6.2(28)-1. Effect of Protein and CuSO₄·5H₂O Levels on Performance of baby Pigs (Experiment 1)^a

Protein level	16				22			
% CuSO ₄ ·5H ₂ O level, (ppm Cu)	0	125	250	375	0	125	250	375
Total gain per pig, lb. ^b	11.8	14.6	15.3	13.2	13.2	15.3	16.0	14.8
Feed/gain, lb. ^b	2.18	2.03	1.96	2.17	2.03	1.79	1.82	1.82
Haemoglobin, gm. % ^b	10.1	10.2	10.0	8.3	11.2	10.5	10.2	8.5

^a 4 pens of 4 pigs each per ration treatment.

^b Significant quadratic regression of gains/gain and haemoglobin level on copper levels.

Table A6.2(28)-2. Copper Sulphate and Copper Oxide for Baby Pigs (Experiment 2)

Item	Treatments				
		CuO (ppm of Cu)			Cu ₂ SO ₄ (ppm of Cu)
	0	125	250	375	250
Total gain per pig, lb	30.3	34.3	33.4	34.4	35.4
Feed/gain, lb	1.93	1.79	1.85	1.79	1.76
Haemoglobin, gm. %	10.1	10.3	10.0	9.7	8.6
Liver copper, ppm ^b	22	..	32	44	218
Loin copper, ppm ^b	5	..	4	6	16
Fecal flora counts, ^c log no. per gram					
Coliforms	6.35	5.78	5.75	5.67	5.83
Lactobacilli	9.50	9.50	9.70	9.38	8.20
Total aerobes	8.81	8.76	9.41	9.45	7.84
Total anaerobes	9.68	9.63	9.76	9.65	8.89
Streptococci	7.52	8.02	8.07	7.49	6.84
Staphylococci	5.47	5.31	6.68	5.62	6.10
Molds and yeasts	4.71	5.14	4.70	4.74	5.27

^a 4 pens of 4 pigs each per ration treatment.

Copper Oxide

- ^b On a dry weight basis (liver, 8 pigs per observation) (loin, 4 pigs per observation)
- ^c Log of total count taken from a one fram wet sample (6 pigs per observation)

Copper Oxide

Table A6.2(28)-3. Copper Sulphate, Copper Oxide and Chlortetracycline for Baby Pigs (Experiment 3)^a

Item	Control	Treatments		
		CuSO ₄ ^b	CuO ^b	CTC ^c
Total gain per pig, lb	32.6	38.8	37.9	38.7
Feed/gain, lb	1.95	1.82	1.88	1.91
Haemoglobin, gm %	10.5	10.1	10.6	10.7
Fecal flora counts, ^d log no. per gram				
Coliforms	6.16	6.44	5.58	5.91
Lactobacilli	9.20	9.03	9.36	9.30
Total aerobes	8.45	8.25	8.70	8.51
Total anaerobes	9.34	8.92	9.35	9.51
Staphylococci	5.33	5.44	5.36	5.18
Streptococci	8.26	7.95	8.25	8.40
Molds and yeasts	5.87	5.70	5.33	5.28

^a 6 pens of 6 pigs per ration treatment

^b Copper compounds fed at level of 250 ppm of copper

^c 50 mg of chlortetracycline per lb.

^d Log of total count taken from a one gm. wet sample (12 pigs per observation)

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Official
use only

147 REFERENCE

147.1 Reference

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

Bunch, R.J., Speer, V.C., Hays, V.W. and McCall, J.T. (1963). Effects of High Levels of Copper and Chlortetracycline on Performance of Pigs. J. Animal Sci. **22**: 56-60 (published).

No

147.2 Data protection

(indicate if data protection is claimed)

147.2.1 Data owner

Give name of company

Public domain

147.2.2 Criteria for data protection

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

148 GUIDELINES AND QUALITY ASSURANCE

148.1 Guideline study

No. This was a non-regulatory study carried out to compare the effects of copper oxide and copper sulphate on rate of gain of feed conversion to that of chlortetracycline and to study the influence of supplemental iron and zinc on the response to high levels of copper. No guidelines are available to address this objective.

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

148.2 GLP

No. This was a non-regulatory study.

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

148.3 Deviations

Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.

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

149 MATERIALS AND METHODS

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

149.1 Test material

Cu²⁺ as copper sulphate.

Cu²⁺ as copper oxide.

Chlortetracycline.

149.1.1 Lot/Batch number

Not available

149.1.2 Specification

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

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149.1.2.1	Description	<i>If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)</i> See section 3.1
149.1.2.2	Purity	<i>Give purity in % of active substance</i> [REDACTED]
149.1.2.3	Stability	<i>Describe stability of test material</i> Not available
149.1.2.4 ng	Radiolabelling	<i>give structural location of radio labelling, give reason if not labelled</i> Not deemed necessary for the purposes of this study.
149.2 Test Animals		Non-entry field
149.2.1 Species		Pigs
149.2.2 Strain		Crossbred.
149.2.3 Source		Not stated.
149.2.4 Sex		50% of animals were male and 50% were female.
149.2.5 Age/weight at study initiation		<i>Young adults recommended</i> Age: Three weeks old at the commencement of Experiment 1
149.2.6 Number of animals per group		The study consisted of three experiments involving 264 animals. The pigs were allotted to treatments from litter-mate outcome groups of similar initial weights. Equal numbers of males and females were distributed across treatments.
149.2.7 Controls		Yes
149.3 Administration/ Exposure		<i>(fill in respective route in the following, delete other routes)</i> Oral administration of the test substances in the diet.
149.3.1 Duration of treatment		Treatment in Experiment 1 lasted from 3 weeks of age until the pigs weighed 200 lb (market weight). Treatment in Experiments 2 and 3 lasted 56 and 55 days, respectively.
149.4 Procedures		Non-entry field
149.4.1 Experiment 1		A split-pilot design was employed to compare a control ration to rations containing copper sulphate (250 ppm Cu), copper oxide (250 ppm Cu) or chlortetracycline (50 mg/lb) and to compare the response of including the additives in the diet until the pigs weighed 125 lb to that of including the additives to market weight (200 lb). The main-plot treatment was the additives to 125 lb versus the additives to 200 lb, and the sub-plot treatments consisted of the control, copper sulphate, copper oxide and chlortetracycline. Feed and water were available to the pigs at all times. Average daily gain and feed required per lb of gain were recorded for the experimental period from 3 weeks of age to 200 lb of bodyweight. 54 pigs from the control and copper treatments were slaughtered as they individually reached 200 lb.
149.4.2 Experiment 2		Six ration treatments each replicated 5 times, with 4 pigs per pen in a randomised block design, were compared. The ration treatments included: (1) 163 ppm of supplemental zinc; (2) 141 ppm of supplemental iron, or (3) a combination of 163 ppm zinc, and 141 ppm iron and each of these 3 diets with or without 250 ppm copper from

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copper sulphate.

Total gain and feed required per lb of gain were measured for a 56 day experimental period. Haemoglobin levels were measured at 2, 4 and 6 weeks after the start of the experiment.

149.4.3 Experiment 3

Four sets of 6 litter-mates representing as nearly as possible the average treatment effects were selected from Experiments 2 and 3. Twelve of these pigs were continued on the same 6 treatments as in Experiment 2. The remaining 12 were fed diets of similar composition but containing corn with 20% moisture and the rations were subjected to heat treatment by storing the ration at 90°F for 2 weeks before treatment. This treatment of the feed was employed to observe the possible detrimental effects of excess moisture and high temperatures, particularly in the copper supplemented diets. The pigs were individually fed, twice daily. Gain and feed required per lb of gain were recorded for a 55 day experimental period.

149.4.4

References to statistical significance refer to a probability level of 5%, unless otherwise stated. In each experiment, the pen of pigs was treated as the experimental unit.

150 RESULTS AND DISCUSSION

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

150.1 Results

Non-entry field.

150.1.1 Experiment 1

Results are presented in **Table A6.2(29)-1**. Each of the additives significantly improved rate of gain and feed conversion to 125 lb body weight (bw). The rate of gain of the animals receiving Cu supplementation from copper sulphate after 125 lb bw was decreased to such an extent that the gains for the entire experimental period equalled that of the controls. When copper sulphate was removed at 125 lb and fed the control ration thereafter the pigs gained significantly faster than the controls. Those pigs fed the copper oxide from 125 lb to 200 lb gained faster than the controls, but when copper oxide was removed at 125lb, the pigs gained slower than those fed the control diet.

The pigs fed chlortetracycline to 200 lb gained significantly faster than the controls. Much of the response was prior to 125 lb bw and chlortetracycline had little effect on the rate of gain thereafter.

Except for the treatment including copper sulphate during the entire period, each of the additives significantly ($P < 0.01$) improved feed conversion efficiency for the period from 3 weeks of age to 200 lb bw.

Liver Cu was significantly increased by addition of Cu to the diet, and significantly higher levels of Cu were found in livers of pigs fed copper sulphate than in those fed copper oxide. Levels of Cu were significantly increased in livers of pigs fed Cu supplemented rations from 3 weeks of age to 200 lb compared to those receiving Cu only to 125 lb.

The concentration of Cu in the loin was significantly increased by supplementation with either copper sulphate or copper oxide. This increase apparently resulted from supplementation up to 125 lb, as removing the Cu from the diet at this point had no effect on the concentration of copper in the loin at market weight.

There was significantly less iron in the livers of the pigs fed diets supplemented with either copper sulphate or copper oxide, but the

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150.1.2 Experiment 2

addition of copper sulphate from 3 weeks to market weight had the greatest effect on suppressing the storage of iron in the liver.

Results are presented in **Table A6.2(29)-2**. The average gain of pigs fed the 3 diets containing copper sulphate was significantly greater than that of the pigs receiving the low-copper diets. There was no response to copper when the diet contained no added zinc. However, this apparent Zn x Cu interaction was not statistically significant. Pigs receiving supplemental zinc gained significantly faster than the pigs receiving no supplemental zinc. The effects of ration treatment on feed conversion were small and non-significant. There were no significant treatment differences in haemoglobin levels after 2 or 4 weeks on experiment, however, after 6 weeks on test those pigs receiving supplemental Fe had significantly higher levels of blood haemoglobin than did those receiving no supplemental Fe. Also, the interaction of Cu and Fe on haemoglobin levels was statistically significant, that is, the depressing effect of high copper was greater in the absence of supplemental iron.

150.1.3 Experiment 3

Results are presented in **Table A6.2(29)-1**. The addition of copper sulphate to the diet significantly increased the average rate of gain but had no significant effect on the feed required per lb of gain. Pigs fed the high-moisture feed which had been stored at high temperatures gained faster on less feed per lb of gain than did the pigs receiving the feed of the lower moisture levels and not stored at high temperatures.

150.2 Discussion

In the first experiment, each of the additives improved rate of gain and feed efficiency when fed to 200 lb or when fed to 125 lb and withdrawn thereafter, with the exception of copper sulphate fed to 200 lb. Copper levels in the livers of pigs fed supplemental copper were higher than pigs fed the control ration. Significantly higher levels of copper were found in livers of the pigs receiving copper sulphate than in those receiving copper oxide. There was significantly less iron in livers of pigs fed copper sulphate, compared to those fed copper oxide.

In the second trial, the effect of zinc and iron on the response to copper was studied. Pigs receiving the copper supplemented rations gained significantly faster than the pigs receiving no added copper. When the diet was not supplemented with zinc there was no response to copper supplementation. Haemoglobin levels were lowered in the presence of added copper and in the absence of supplemental iron.

In the third experiment, pigs receiving added copper gained significantly faster than the controls. Heat and water treatment of the diets did not significantly affect the response to copper.

150.3 Toxic effects,
clinical signs

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

5.7 Recovery of
labelled compound

state percentage
Not applicable.

151 APPLICANT'S SUMMARY AND CONCLUSION

151.1 Materials and
methods

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

A study was carried out to compare effects of copper oxide and copper sulphate on rate of gain of feed conversion to that of chlortetracycline. The study was not designed to follow internationally accepted

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guidelines, and was not carried out or reported in compliance with GLP. The study consisted of three experiments involving 264 crossbred pigs. Equal numbers of males and females were distributed across treatments.

Experiment 1: A split-plot design was employed to compare a control ration to rations containing copper sulphate (250 ppm Cu), copper oxide (250 ppm Cu) or chlortetracycline (50 mg/lb) and to compare the response of including the additives in the diet until the pigs weighed 125 lb to that of including the additives to market weight (200 lb). The main-plot treatment was the additives to 125 lb versus the additives to 200 lb, and the sub-plot treatments consisted of the control, copper sulphate, copper oxide and chlortetracycline. Three pens per treatment were used, each containing 6 pigs. Feed and water were available *ad libitum*. Average daily gain and feed required per lb of gain were recorded for the experimental period from 3 weeks of age to 200 lb of bodyweight. 54 pigs from the control and copper treatments were slaughtered as they individually reached 200 lb.

Experiment 2: Six ration treatments were compared, each replicated 5 times, with 4 pigs per pen in a randomised block design. The ration treatments included: (1) 163 ppm of supplemental zinc; (2) 141 ppm of supplemental iron, or (3) a combination of 163 ppm zinc, and 141 ppm iron and each of these 3 diets with or without 250 ppm copper from copper sulphate. Total gain and feed required per lb of gain were measured for a 56 day experimental period. Haemoglobin levels were measured at 2, 4 and 6 weeks after the start of the experiment.

Experiment 3: Four sets of 6 litter-mates, representing average treatment effects, were selected from experiments 2 and 3. Twelve animals continued on the same 6 treatments as in experiment 2. The remaining 12 were fed diets of similar composition, but containing corn with 20% moisture, and the rations were subjected to heat treatment by storing at 90°F for 2 weeks. This treatment was employed to investigate the effects of excess moisture and high temperature, particularly in copper-supplemented diets. Pigs were individually fed, twice daily. Gain and feed required per lb of gain were recorded for a 55 day period.

Summarize relevant results; discuss dose-response relationship.

Experiment 1: Each of the additives significantly improved rate of gain and feed conversion to a bodyweight of 125 lb. The rate of gain of animals receiving Cu from copper sulphate after 125 lb bw was decreased such that gains for the entire experiment equalled those of the controls. When copper sulphate was removed at 125 lb and the control ration fed, pigs gained significantly faster than controls. Pigs fed copper oxide from 125 lb to 200 lb gained faster than controls, but when copper oxide was removed at 125lb, pigs gained more slowly than control pigs. Except for the treatment including copper sulphate during the entire period, each of the additives significantly improved feed conversion efficiency for the period from 3 weeks of age to 200 lb bw.

Liver levels of Cu were significantly increased by addition of Cu to the diet, and significantly higher levels were found in the livers of pigs receiving copper sulphate than for copper oxide. Cu levels were significantly higher in livers of pigs fed Cu supplemented rations from 3 weeks of age to 200 lb, compared to those receiving Cu only to 125 lb.

The concentration of Cu in the loin was significantly increased by supplementation with either copper sulphate or oxide. This increase resulted from supplementation up to 125 lb, as removal of Cu from the

151.2 Results and discussion

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Specify section no., heading and species as appropriate

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diet at this point had no effect on Cu concentration in the loin at 200 lb. There was significantly less iron in the livers of pigs fed diets containing either copper sulphate or copper oxide. Addition of copper sulphate from 3 wks to 200 lb was most effective at suppressing iron storage.

Experiment 2: The effect of zinc and iron on the response to copper was studied. The average gain of pigs fed the 3 diets containing the copper sulphate was significantly greater than that of pigs receiving low-copper diets. There was no response to Cu when the diet contained no added zinc. However, this apparent Zn x Cu interaction was not statistically significant. Pigs receiving supplemental zinc gained significantly faster than pigs receiving no supplemental zinc. The effects of ration treatment on feed conversion were small and non-significant. There were no significant differences in haemoglobin levels after 2 or 4 weeks. However, after 6 weeks, pigs receiving supplemental Fe had significantly higher levels of blood haemoglobin than did those receiving no supplemental Fe. Also, the interaction of Cu and Fe on haemoglobin levels was statistically significant (i.e. the depressing effect of high copper was greater in the absence of supplemental iron).

Experiment 3: Addition of copper sulphate to the diet significantly increased average rate of gain, but had no significant effect on the feed required per lb of gain. Pigs fed high-moisture feed which had been stored at high temperatures gained faster on less feed per lb of gain than those given feed of lower moisture levels not stored at high temperature.

151.3

Conclusion In the first experiment, each of the additives improved rate of gain and feed efficiency when fed to 200 lb or when fed to 125 lb and withdrawn thereafter, with the exception of copper sulphate fed to 200 lb. Copper levels in the livers of pigs fed supplemental copper were higher than pigs fed the control ration. Significantly higher levels of copper were found in livers of the pigs receiving copper sulphate than in those receiving copper oxide. There was significantly less iron in livers of pigs fed copper sulphate, compared to those fed copper oxide.

In the second experiment, pigs receiving added copper gained significantly faster than pigs receiving no added copper. When the diet was not supplemented with zinc there was no response to added copper. Haemoglobin levels were lowered in the presence of added copper and in the absence of supplemental iron.

In the third experiment, pigs receiving added copper gained significantly faster than the controls. Heat and water treatment of the diets did not significantly affect the response to copper.

151.4 Reliability

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

2

151.5 Deficiencies

Yes

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

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

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

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

COMMENTS FROM ...

Date

Give date of comments submitted

Copper Oxide

Table A6.2(29)-1. Effect of Copper Sulfate, Copper Oxide and Chlortetracycline on Pig Performance and Levels of Iron and Copper in Liver and Loin Tissue (Experiment 1)

	Basal	CuSO ₄	CuO	CTC	Basal	CuSO ₄	CuO	CTC ^c
Additive fed 14 to 125lb	0	250 ^a	250 ^a	50 ^b	0	250 ^a	250 ^a	50 ^b
Additive fed 125 to 200lb	0	0	0	0	0	250	250	50
No. of pigs per pen	6	6	6	6	6	6	6	6
No. pens per treatment	3	3	3	3	3	3	3	3
<i>Av. daily gain, lb</i>								
14 to 125 lb	1.14	1.29	1.25	1.33	1.08	1.22	1.27	1.27
125 to 200 lb	1.79	1.90	1.65	1.83	1.73	1.67	1.84	1.92
14 to 200 lb	1.38	1.47	1.40	1.49	1.38	1.38	1.46	1.47
<i>Feed/gain, lb</i>								
14 to 125 lb	2.59	2.48	2.51	2.64	2.73	2.53	2.46	2.51
125 to 200 lb	3.88	3.63	3.78	3.63	4.18	4.11	3.62	3.82
14 to 200 lb	3.18	2.91	2.99	2.99	3.30	3.18	2.89	3.02
No. pigs sampled for tissue analysis	9	9	9	• • •	9	9	9	• •
<i>Tissue level of Cu, ppm of dry matter</i>								
Liver ^c	19	51	54	• • •	26	28	28	
Loin ^c	6.3	9.0	8.2		5.7	7.3	10.3	
<i>Tissue level of Fe, ppm of dry matter</i>								
Liver ^c	867	819	836	• • •	835	285	665	
Loin ^c	54	67	76		57	58	107	

Copper Oxide

^a ppm of elemental copper.

^b Chlortetracycline, mg/lb

^c Coefficients of variation are as follows: gain to 125 lb., 5.4; gain 125 to 200 lb., 8.7; gain 14 to 200 lb., 4.0; feed/gain to 125 lb, 4.0; feed/gain 125 to 200 lb., 8.6 feed/gain 14 to 200 lb., 3.4 liver Cu, 91; loin Cu, 66 liver iron, 32 and loin copper, 47.

Copper Oxide

Table A6.2(29)-2. Effect of Copper, Iron and Zinc on Baby Pig Performance (Experiment 2)

	Supplemental Levels					
	0	0	0	250	250	250
Cu (ppm)	0	0	0	250	250	250
Fe (ppm)	141	0	141	141	0	141
Zn (ppm)	163	163	0	163	163	0
No. pigs per pen	4	4	4	4	4	4
No. pens per treatment	5	5	5	5	5	5
Total gain lb, ^{a,b}	52.8	52.1	52.2	57.4	56.4	52.3
Feed/gain, lb	2.24	2.14	2.13	2.20	2.12	2.17
14 day Hb, gm. %	11.1	11.3	11.0	10.5	11.2	11.0
28 day Hb, gm %	9.7	9.2	9.8	9.5	8.8	9.4
42 day Hb, gm.%	10.2	10.1	10.2	10.6	9.0	10.6

^a Coefficients of variation are as follows: gain 6.4; feed/gain, 4.7 and 42 day Hb, 5.8.

^b 0 vs 250 ppm copper significant (P<.05)

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IUCLID: 5.0/30	A6.2(30), Bioavailability of copper

Official
use only**152 REFERENCE**

- 152.1 Reference** *Author(s), year, title, laboratory name, laboratory report number, report date (if published, list journal name, volume: pages)
If necessary, copy field and enter other reference(s).*
Cromwell, G.L., Stahly, T.S. and Monegue, H.J. (1989). Effects of Source and Level of Copper on Performance and Liver Copper Stores in Weanling Pigs. J. Animal Sci. **67**: 2996-3002 (published).
- 152.2 Data protection** No
(indicate if data protection is claimed)
- 152.2.1 Data owner *Give name of company*
Public domain
- 152.2.2 Criteria for data protection Choose one of the following criteria (see also TNsG on Product Evaluation) and
No data protection claimed
delete the others:

153 GUIDELINES AND QUALITY ASSURANCE

- 153.1 Guideline study** No. This was a non-regulatory study carried out to determine the efficacy of various levels of dietary Cu as a growth promotant for weanling pigs and to determine the relative effectiveness of the sulphate (CuSO₄) and oxide (CuO) forms of Cu as growth promotants. No guidelines are available to address this objective. *(If yes, give guidelines; if no, give justification, e.g. "no guidelines available" or "methods used comparable to guidelines xy")*
- 153.2 GLP** No. This was a non-regulatory study.
(If no, give justification, e.g. state that GLP was not compulsory at the time the study was performed)
- 153.3 Deviations** Yes. Refer to section 5.3.2 for a general discussion of deviations and deficiencies.
(If yes, describe deviations from test guidelines or refer to respective field numbers where these are described, e.g. "see 3.x.y")

154 MATERIALS AND METHODS

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

- 154.1 Test material** Cu²⁺ as Copper sulphate (CuSO₄.5H₂O).
Cu²⁺ as Copper oxide (CuO).
- 154.1.1 Lot/Batch number Not available
- 154.1.2 Specification Deviating from specification given in section 2 as follows
(describe specification under separate subheadings, such as the following; additional subheadings may be appropriate):

Section A6.2**Metabolism in mammals****Annex Point IIA6.2***Specify section no., heading and species as appropriate***IUCLID: 5.0/30****A6.2(30), Bioavailability of copper**

154.1.2.1 Descrip *If appropriate, give e.g. colour, physical form (e.g. powder, grain size, particle size/distribution)*
tion
See section 3.1

154.1.2.2 Purity *Give purity in % of active substance*


154.1.2.3 Stabilit *Describe stability of test material*
y
Not available

154.1.2.4 Radiola *give structural location of radio labelling, give reason if not labelled*
belling
Not deemed necessary for the purposes of this study.

154.2 Test Animals Non-entry field

154.2.1 Species Pigs

154.2.2 Strain Hampshire-Yorkshire crossbred.

154.2.3 Source Not stated.

154.2.4 Sex 50% of animals were male and 50% were female.

154.2.5 Age/weight/heig *Young adults recommended*
ht at study
initiation
Age: 28 days at the start of the study.
Weight: Average initial weights ranged from 6.7 to 8.1 kg.

154.2.6 Controls Yes

154.3 Administration/ *(fill in respective route in the following, delete other routes)*

Exposure Oral administration of the test substances in the diet.

154.3.1 Duration of The length of the experimental periods ranged from 28 days (Experiments 1 to 4) to
treatment 33 days (Experiment 5).

154.4 Procedures Non-entry field

154.4.1 Experiment 1 Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal diet plus 125 or 250 ppm Cu from CuSO₄ and basal plus 125 or 250 ppm Cu from CuO. These diets were fed for 28 days to 3 replicates of 5-6 pigs/pen. The average bodyweight of the pigs increased over the course of the study from 6.9 kg to 15.6 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

154.4.2 Experiment 2 Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal diet plus 125 or 250 ppm Cu from CuSO₄ and basal plus 125 or 250 ppm Cu from CuO. These diets were fed for 28 days to 5 replicates of 5-6 pigs/pen. The average bodyweight of the pigs increased over the course of the study from 8.1 kg to 17.8 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

154.4.3 Experiment 3 Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal diet plus 125, 250 or 500 ppm Cu from CuSO₄ and basal plus 125, 250 or 500 ppm Cu from CuO. These diets were fed for 28 days to 2 replicates of 5-6 pigs/pen. Average bodyweight of the pigs increased over the course of the study from 7.1 kg to 12.2 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

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- 154.4.4 Experiment 4 Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal diet plus 125, 250 or 500 ppm Cu from CuSO₄ and basal plus 125, 250 or 500 ppm Cu from CuO. These diets were fed for 28 days to 2 replicates of 5-6 pigs/pen. Average bodyweight of the pigs increased over the course of the study from 6.9 kg to 13.2 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.
- 154.4.5 Experiment 5 Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal with 0, 125, 250, 375 or 500 ppm Cu from CuSO₄. These diets were fed for 33 days to 4 replicates of 5-6 pigs/pen. The average bodyweight of the pigs increased over the course of the study from 6.7 kg to 18.0 kg.
- At the end of the test period, eight pigs that had been fed the 500 ppm diet (2 pigs that were closest to the pen average from each replication) were switched to the basal diet. Four of these pigs (one randomly selected pig/replication) were killed 14 days after withdrawal of Cu and the remaining 4 pigs were killed 28 days after withdrawal of dietary Cu to determine the depletion rate of Cu from the liver.
- The single pig taken from each pen at the end of the experiment to determine liver Cu (*cf.* 3.4.1 – 3.4.4) was chosen before pigs were selected for the depletion study.
- 154.4.6 Cu analysis Following removal of the liver, the gall bladder and extraneous fat were removed, the liver was homogenised in a blender, and the samples were freeze-dried. After wet ashing, the liver was assayed for Cu by atomic absorption spectrophotometry.
- 154.4.7 Statistics Data were subjected to analysis of variance. There was no evidence of experiment x treatment interaction ($P > 0.03$) for any of the criteria in the combined analysis of data from Experiments 1 and 2, so these were pooled. The same was true of data from Experiments 3 and 4. Also, the lack of an experiment x treatment interaction allowed pooling of data from 0, 125 and 250 ppm treatments of all 5 experiments. Orthogonal contrasts of basal vs. added Cu, level of added Cu and source of Cu were made in Experiments 1 to 4. In nearly all cases, the Cu source x Cu level interaction was significant ($P < 0.01$); therefore, a second set of nonorthogonal contrasts of the basal vs. certain treatment groups were made. In Experiment 5, orthogonal polynomials were used to make linear and nonlinear contrasts among treatment means. In all cases, the pen was considered the experimental unit.

155 RESULTS AND DISCUSSION

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

155.1 Results

Non-entry field.

- 155.1.1 Experiments 1 & 2 Pigs fed 125 or 250 ppm Cu from CuSO₄ gained faster ($P < 0.01$) and required less feed per unit of gain ($P < 0.01$) than those fed the control diet (**Table A6.2(30)-1**). Daily feed intake tended to be greater for pigs fed 125 or 250 ppm Cu from CuSO₄, but this difference was not significant ($P > 0.20$). Liver Cu was elevated approximately 10-fold in pigs fed 250 ppm Cu from CuSO₄, but no increase occurred when the lower (125 ppm) level was fed. Rate and efficiency of gain and liver Cu levels in pigs fed CuO were not influenced at either level of inclusion.
- 155.1.2 Experiments 3 & 4 Daily feed intake and growth rate were improved ($P < 0.01$) in pigs fed 125 or 250 ppm as CuSO₄ compared with those fed basal diet (**Table A6.2(30)-2**). The 500 ppm Cu level did not result in improved gains over controls and resulted in higher ($P < 0.05$) feed:gain ratios. As in Experiments 1 and 2, CuO at any level did not improve performance. Liver Cu increased ($P < 0.01$) in pigs fed 250 and 500 ppm Cu as CuSO₄, but was not affected by any level of Cu when added as the oxide.
- 155.1.3 Experiment 5 Rate and efficiency of gain were influenced quadratically ($P < 0.01$), as was daily feed intake ($P < 0.10$), by level of added Cu (**Table A6.2(30)-3**). Rate and efficiency

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Metabolism in mammals

Specify section no., heading and species as appropriate

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of gain improved in a curvilinear fashion with the addition of 125 and 250 ppm Cu, after which further addition of Cu resulted in a decline in performance to equal that of the controls. Based on the inflection point (calculated from the second derivative) of the quadratic equation, growth rate was maximised when the level of added Cu was 242 ppm (**Figure A6.2(30)-1**).

As in previous trials, liver Cu was not affected by the 125 ppm dietary inclusion rate, but marked increases in liver Cu occurred when the higher dietary levels of Cu were fed, resulting in a quadratic ($P < 0.05$) response pattern. The 250, 375 and 500 ppm dietary levels resulted in 17-, 41- and 72-fold increases in liver Cu, respectively. When the 500 ppm level of Cu was withdrawn from the diet, liver Cu concentrations decreased from 1513 ppm to 508 ppm within 14 d (**Figure A6.2(30)-2**). A further decrease to 274 ppm occurred after 28 d of Cu withdrawal.

155.2 Discussion

The dietary inclusion of 250 ppm Cu (as CuSO_4) improved growth rate 24 to 39% and improved efficiency of feed utilisation 11 to 21%. Feed intake was also increased by 8 to 17% in pigs fed this dietary level and source of Cu.

The 125 ppm level of Cu (as CuSO_4) was as effective as the 250 ppm level in Experiments 3 and 4, but was less effective in Experiments 1, 2 and 5. Based on analysis of the pooled data from 0, 125 and 250 ppm Cu from CuSO_4 for these five experiments, the 250 ppm dietary level of Cu was more efficacious than the 125 ppm in stimulating growth rate (263, 323 and 342 g/d for pigs fed 0, 125 and 250 ppm Cu, respectively; $P < 0.05$). Based on these means, the 125 ppm dietary level of Cu was about 75% as effective in improving rate of gain and about 60% as effective in improving feed:gain as the 250 ppm dietary level of Cu.

The magnitude of the improvement in growth rate and feed:gain ratio was diminished when Cu (as CuSO_4) was increased to 375 ppm and was completely lost when the diet was increased to 500 ppm Cu. This dietary level does not, however, depress growth rate compared with pigs fed a low-Cu control diet, although it did reduce efficiency of feed utilisation in some instances (**Table A6.2(30)-2**).

Liver tissue of control pigs averaged 22 ppm Cu on a dry mass (DM) basis. Liver Cu was not increased by feeding 125 ppm Cu as CuSO_4 , but liver Cu concentration was elevated 6- to 17-fold when 250 ppm Cu was provided. A further marked accumulation of Cu in liver tissue occurred when 375 and 500 ppm Cu as CuSO_4 was fed. The accumulation in liver Cu in pigs fed 500 ppm dietary Cu was greater in Experiment 5 than in Experiments 3 and 4, perhaps because pigs were on test slightly longer (33 versus 28 days) and because pigs in experiment 5 consumed more feed (590 vs. 434 g/d; 19.47 vs. 12.15 kg total feed) and hence consumed more total Cu than did pigs in Experiments 3 and 4. According to the results of Experiment 5, this Cu is rapidly depleted from the liver after pigs are returned to a low-Cu diet.

The results of this study indicate that CuO was ineffective as a growth promotant at the levels tested. Even a 500 ppm level of Cu as CuO did not increase liver Cu above control levels (**Table A6.2(30)-3**), suggesting that Cu as CuO is very poorly available.

155.3 Toxic effects, clinical signs

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

5.8 Recovery of labelled compound

state percentage
Not applicable.

156 APPLICANT'S SUMMARY AND CONCLUSION

156.1 Materials and

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

Section A6.2

Metabolism in mammals

Annex Point IIA6.2

Specify section no., heading and species as appropriate

IUCLID: 5.0/30

A6.2(30), Bioavailability of copper

methods

deviations from test guidelines

A study was carried out to determine the efficacy of various levels of dietary Cu as a growth promotant for weanling pigs and to determine the relative effectiveness of the sulphate (CuSO_4) and oxide (CuO) forms of Cu as growth promotants. Five experiments involving 460 Hampshire-Yorkshire crossbred pigs were conducted. The pigs were weaned at 28 ± 2 days of age and allotted at random to treatment from weight outcome groups. Sex was balanced in each treatment and in all cases the pen was considered to be the experimental unit. Data were subjected to analysis of variance procedures. The study was not designed to follow internationally accepted guidelines, and was not carried out or reported in compliance with GLP.

Experiment 1: Dietary treatments administered were the basal diet (containing 30 ppm Cu); basal diet plus 125 or 250 ppm Cu from CuSO_4 and basal plus 125 or 250 ppm Cu from CuO . Diets were fed for 28 days to 3 replicates of 5-6 pigs/pen. The average bodyweight of the pigs increased over the course of the study from 6.9 kg to 15.6 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

Experiment 2: Dietary treatments administered were the basal diet; basal diet plus 125 or 250 ppm Cu from CuSO_4 and basal plus 125 or 250 ppm Cu from CuO . Diets were fed for 28 days to 5 replicates of 5-6 pigs/pen. The average bodyweight of the pigs increased over the course of the study from 8.1 kg to 17.8 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

Experiment 3: Dietary treatments administered were the basal diet; basal diet plus 125, 250 or 500 ppm Cu from CuSO_4 and basal plus 125, 250 or 500 ppm Cu from CuO . Diets were fed for 28 days to 2 replicates of 5-6 pigs/pen. Average bodyweight increased over the course of the study from 7.1 kg to 12.2 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

Experiment 4: Dietary treatments administered were the basal diet; basal diet plus 125, 250 or 500 ppm Cu from CuSO_4 and basal plus 125, 250 or 500 ppm Cu from CuO . Diets were fed for 28 days to 2 replicates of 5-6 pigs/pen. Average bodyweight increased over the course of the study from 6.9 kg to 13.2 kg. At the end of the experiment, one pig from each pen (the pig closest to the pen average weight) was slaughtered and the liver removed for copper analysis.

Experiment 5: Dietary treatments administered were the basal diet; basal with 0, 125, 250, 375 or 500 ppm Cu from CuSO_4 . Diets were fed for 33 days to 4 replicates of 5-6 pigs/pen. Average bodyweight increased during the study from 6.7 kg to 18.0 kg. At the end of the test period, eight pigs that had been fed the 500 ppm diet (2 pigs that were closest to the pen average from each replication) were switched to the basal diet. Four of these pigs (one randomly selected pig/replication) were killed 14 days after withdrawal of Cu and the remaining 4 pigs were killed 28 days after withdrawal of dietary Cu to determine the depletion rate of Cu from the liver. A single pig taken from each pen at the end of the experiment to determine liver Cu was chosen before pigs were selected for the depletion study.

Cu Analysis: Following removal of the liver, the gall bladder and extraneous fat were removed, the liver was homogenised in a blender, and the samples were freeze-dried. After wet ashing, the liver was assayed for Cu by AAS.

156.2 Results and discussion

Summarize relevant results; discuss dose-response relationship.

Experiments 1 & 2: Pigs fed 125 or 250 ppm Cu from CuSO_4 gained faster and required less feed per unit of gain than those fed the control diet. Daily feed intake was greater for pigs fed 125 or 250 ppm Cu from CuSO_4 , but the difference was not significant. Liver Cu was elevated up to 10-fold in pigs fed 250 ppm Cu from CuSO_4 , but no increase occurred when 125 ppm was fed. Rate and efficiency of

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Specify section no., heading and species as appropriate

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gain and liver Cu levels in pigs fed CuO were not influenced at either level of Cu. *Experiments 3 & 4:* Feed intake and growth rate were improved in pigs fed 125 or 250 ppm Cu as CuSO₄ compared with those fed basal diet. 500 ppm Cu did not result in improved gains and resulted in higher feed:gain ratios. CuO at any level did not improve performance. Liver Cu increased in pigs fed 250 and 500 ppm Cu as CuSO₄, but was not affected by any level when added as the oxide.

Experiment 5: Rate and efficiency of gain were influenced quadratically, as was daily feed intake, by level of added Cu. Rate and efficiency of gain improved in a curvilinear fashion with the addition of 125 and 250 ppm Cu, after which further addition of Cu resulted in a decline in performance to equal that of the controls. Based on the inflection point of the quadratic equation, growth rate was maximised when the level of added Cu was 242 ppm.

Liver Cu was not affected by the 125 ppm dietary inclusion rate, but increases in liver Cu occurred when higher dietary levels of Cu were fed, resulting in a quadratic response pattern. The 250, 375 and 500 ppm dietary levels resulted in 17-, 41- and 72-fold increases in liver Cu, respectively. When the 500 ppm level of Cu was withdrawn, liver Cu concentrations decreased from 1513 ppm to 508 ppm within 14 days. A further decrease to 274 ppm occurred after 28 days of Cu withdrawal.

156.3 Conclusion

The results of this study indicate that feeding 250 ppm Cu as CuSO₄ markedly improves rate and efficiency of gain in weanling pigs. Exposure of young pigs to 500 ppm Cu for 4 to 5 weeks appears not to be detrimental, but does not stimulate growth. Although CuO is a more concentrated form of Cu (75% Cu) than CuSO₄ (25% Cu), Cu in the oxide form is ineffective as a growth promotant. Based on liver Cu stores, the bioavailability of Cu from CuO is low relative to that of CuSO₄.

156.3.1 Reliability

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

2

156.3.2 Deficiencies

Yes

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

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

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